IN VIVO STUDIES ON THE CHEMISTRY OF PAIN: THE ROLE OF NEUROPEPTIDES.

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ABSTRACT

Although a number of peptides have recently been implicated in analgesia, rigorous evidence on the characterisation and quantitation of these substances in pain processes has been notably absent. This is partly due to the enormous complexity of peptidergic systems present in the central nervous system. However, many of the major factors contributing to our limited knowledge of brain chemistry arise from the technical problems related to purification, characterisation and quantitation of very complex biological extracts.

To overcome some of these technical problems, a useful model has been developed based upon the push-pull cannula perfusion technique which allows the analysis of peptides (and other substances) released into the third ventricular perfusate following periaqueductal gray (PAG) stimulation in the conscious unrestrained rat. With this model, reproducible generation of analgesic states has been achieved in a quantifiable manner.

Specific high sensitivity quantitation of the peptides was performed by radioimmunoassay of relevant fractions after purification of the perfusate on a high yield, high resolution reverse phase HPLC system. Simultaneous monitoring of a number of neuropeptides was therefore achieved with each perfusate, and samples were taken before, during and post stimulation, with the animal thus serving as its own control.

Using this model, the role of exogenous and endogenous methionine enkephalin and CCK_8 in analgesia has been investigated. It has been definitively shown for the first time that CCK_8 in the third ventricle does not increase upon PAG stimulation, and methionine enkephalin, rather surprisingly, is

not detectable in the same samples. A direct correlation of the animal results with human data has been made for the first time, validating the animal studies.

It has been demonstrated that the dose of naloxone employed in stimulation-induced analgesia is critical, thus resolving naloxone's role in such studies.

Novel experiments on the in situ metabolism of CCK_8 and methionine enkephalin have demonstrated unambiguously their rapid metabolism.

The model developed can be used for precise chemical mapping of intrinsic peptide systems that are activated by stimulation of the PAG. This will be of considerable importance to our understanding of the physiological events associated with pain and analgesia.

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
APUD	Amine precursor uptake and decarboxylation
CCK8	Cholecystokinin octapeptide
CNS	Central nervous system
CSF	Cerebrospinal fluid
DAMME	D-Ala methionine enkephalinamide
5HIAA	5hydroxyindole acetic acid
HPLC	High pressure liquid chromatography
5HT	5Hydroxytryptamine
HVA	Homovanillic acid
%MPE	Percentage maximum possible effect
PAG	Periaqueductal gray
PE	Polyethylene
SIA	Stimulation induced analgesia
TRH	Thyrotropin-releasing hormone
YGGFM	Tyrosine glycine glycine phenylalanine methionine

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FOR MY FAMILY

CHAPTER 1

INTRODUCTION

The brain is immensely complex, both structurally and functionally, and has often been compared to a computer, a comparison that does not do justice to the brain. One of the major differences between brains and computers is the reliance of the latter on but one kind of signal - electrical impulses - for communicating among their components. Nerve cells in the brain and elsewhere also use electrical signals, but in addition they communicate chemically with neurotransmitters. It is not known how many neurotransmitters there are, but extensive efforts to identify the chemicals and map the neurons producing them are currently under way. The goal is a better understanding of how the brain receives information from the body and the environment, integrates it into a picture of what is happening and then tells the body how to react.

The explosion of research into neuroactive and other peptides has been from a realisation of their fundamental role in the control of major body functions. Furthermore the action and interrelationship of many neuropeptides has led to a belief that an imbalance of these substances may give rise to many mental and physical illnesses including senile dementia, schizophrenia, Parkinsonism and other disorders (Mass 1975, Meltzer et al. 1976, Morris et al. 1980). One of the most striking examples of this upsurge of interest has been in the area of pain research.

It is well known that pain perception and responsiveness can be modulated by a large number of factors including pharmacological and psychological variables. One of the most

important aspects of this modulation is pain inhibition or analgesia, a reduction of responsiveness to normally noxious stimuli. Yet the mechanisms underlying analgesia remain largely obscure.

The opioids (any natural or synthetic drug that has morphine-like pharmacological action) are employed primarily for the relief of pain but their use entails the risk of producing physical and sometimes psychological dependence. Morphine, the alkaloid that gives opium its analgesic properties, remains the standard against which new analgesics are measured. Although newer analgesics possess special properties, none is clinically superior in relieving pain.

HISTORICAL

The psychological effects of opium may have been known to the ancient Sumerians (4000 B.C.) whose ideograph for the poppy was hul ('joy') plus gil ('plants'), but the first undisputed reference to poppy juice is found in the writings of Theophrastus in the third century B.C. Arabian traders introduced the drug to the Orient, where it was employed mainly for the control of dysenteries.

In 1680 the English physician Thomas Sydenham wrote: "Among the remedies which it has pleased Almighty God to give man to relieve his sufferings, none is so universal and so efficacious as opium". In the eighteenth century opium smoking became popular in the Orient.

Opium has been used as a drug at least since classical Greek times, not only of course because it deadens pain but because it gives rise to euphoria. The word comes from the Greek - opion - poppy juice; the drug is present in the milky

exudate obtained by incising the unripe seed pod of the poppy Papaver Somniferum (fig. 1 and 2). In 1803, a young German pharmacist, Friedrich Serturner, isolated an opium alkaloid that he named morphine after Morpheus, the Greek god of dreams. The discovery of other alkaloids in opium quickly followed that of morphine (codeine by Robiquet in 1832, papaverine by Merck in 1848) and by the middle of the nineteenth century the use of pure alkaloids rather than crude opium preparations began to spread throughout the medical world.

The invention of the hypodermic needle and the parenteral use of morphine tended to produce a more severe variety of compulsive drug use. The extent of the opiate use problem in the U.S.A. was accentuated by the influx of opium-smoking Chinese labourers, the widespread use of morphine among wounded Civil War soldiers, and the unrestricted availability of opium that prevailed until the early years of this century. The recognition of this serious liability of morphine stimulated search for potent analgesics that would not lead to compulsive drug use.

The recently discovered natural morphine-like substances in the brain (Hughes et al. 1975) promises to open new avenues to an understanding of precisely where in the body opiates act, how they do so, and why they are addictive.

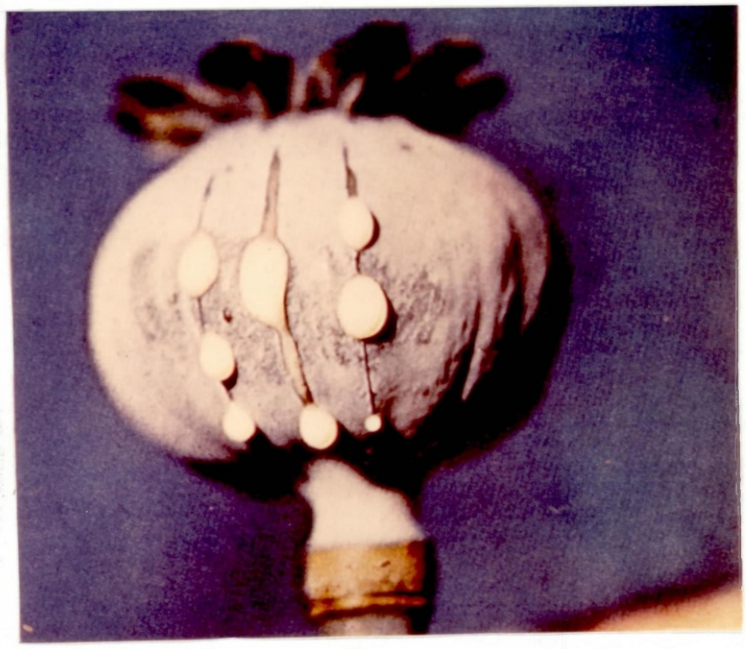
EARLY METHODS USED FOR PAIN RELIEF

Chronic or prolonged pain in man has proved remarkably resistant to treatment in spite of the immense effort that has been devoted to the solution of this problem. The two treatment procedures that were most commonly employed, administration of

Fig. 1 - THE OPIUM POPPY PLANT (PAPAVER SOMNIFERUM)



Fig. 2 - THE MILKY EXUDATE OF THE UNRIPE CAPSULE OF PAPAVER



narcotic analgesics and placement of discrete lesions within the central nervous system (CNS), have been disappointing for different reasons. Narcotic drugs, while capable of producing powerful analgesia, unfortunately produce rapid tolerance and dependence contra-indicating their use over long periods of time. Surgical attempts to excise pain by the placement of discrete lesions within the "pain pathways" or elsewhere are inherently undesirable since destroyed neural tissue is irretrievably lost. In addition these procedures have not been consistently successful and often produce undesirable side effects.

The possibility of an alternative approach to the treatment of chronic pain has been suggested by a body of evidence indicating that within the CNS there exists a neural substrate capable of a powerful active suppression of pain perception. First, a number of observations show that psychological factors can result in the total suppression of pain appreciation even in the presence of highly noxious stimuli. The group of soldiers studied by Beecher (1959) reported little or no distress from wounds comparable to those requiring administration of large doses of analgesics in a civilian population. The relief felt by these soldiers upon being removed from the dangers of the battlefield was so great that they failed to recognise their pain or ignored it. Pavlov's dog (Pavlov 1927) gave no overt pain response to electric shock, burning or cutting, after these stimuli came to be associated with the presentation of food.

ANATOMY AND PHYSIOLOGY OF CEREBROSPINAL FLUID

The brain and spinal cord are delicate semi-solid structures requiring protection and support. The brain is covered by three membranes. A clear fluid, cerebrospinal fluid (CSF), surrounds it and it is encased in a bony vault, the skull. The most external membrane is a dense connective tissue envelope known as the dura mater. The innermost connective tissue membrane is the pia mater, a thin translucent membrane adherent to the surface of the brain and spinal cord which accurately follows every contour. Between these membranes is a delicate layer of reticular fibres forming a web-like membrane, the arachnoid. The pia mater and the arachnoid have a similar structure and collectively are called the lepto-meninges.

The cerebrospinal fluid (CSF) is a clear colourless liquid containing small amounts of protein, glucose and potassium and relatively large amounts of sodium chloride. The CSF serves to support and cushion the CNS against trauma. It has been suggested that the CSF has nutritive functions and that it serves to remove the waste products of neuronal metabolism. Because the brain is nearly incompressible within the cranium, the combined volumes of brain, CSF and intracranial blood must be maintained at a constant level. The volume of any one of these components can be increased only at the expense of one or both of the others.

The CSF is in intimate contact with all structures of the central nervous system - CNS and CSF are separated from the blood by the so-called blood-brain barrier and blood-CSF barrier respectively; this separation assists in the maintenance of homeostasis and relative independence from changes in composition

of the blood, since the functioning of all neurons of the CNS are dependent upon the maintenance of a physical and chemical milieu within certain narrow limits. Because of these anatomical and physiological relationships, biochemical events in the CNS may be reflected in the composition of the CSF or in a perfusate of the CSF space; thus, analysis of the CSF or perfusate of the spaces can often provide information on the chemistry of the brain and spinal cord without sampling the nervous tissue itself.

CSF fills the brain ventricles and the central canal of the spinal cord (internal fluid) as well as the subarachnoid spaces of the brain and spinal cord (external fluid). The choroid plexuses are considered to be the main source of CSF. They are highly vascular structures protruding into brain ventricles and covered by modified ependymal cells. These cells transport solutes from choroidal stroma into the ventricular CSF and vice versa. CSF is formed continuously and has to be drained away at the same rate. The bulk absorption or drainage of CSF takes place through the arachnoid villi, which permits flow of the fluid in one direction only (from CSF to blood) so that the passage of the blood into subarachnoid fluid is prevented. CSF flows into the venous blood through arachnoid villi when its pressure is greater than that of the blood.

CSF circulates from the place of its formation to the absorption sites but this circulation is slow (Davson 1967). The CSF in spinal subarachnoid space (spinal or lumbar fluid) seems to be a backwater of the main stream of CSF and its circulation is practically nonexistent.

PHYSIOLOGY OF PAIN

During the last few years, there have been numerous studies in understanding the neural mechanisms underlying the perception of pain. From what we now know, it is easy to see why the mechanism of pain perception has remained enigmatic for so long. However, it is now firmly established that small diameter fibres, whose endings are distributed throughout the body, including skin, muscles and viscera, respond specifically to painful stimuli. These fibres, on entering the spinal cord, branch profusely and form synapses in the superficial layers of the dorsal horn, mainly in the marginal zone and the substantia gelatinosa. Here the fine terminals of axons and dendrites, together with collaterals of ascending and descending fibre tracts, form a dense jungled neuropil, the precise wiring diagram of which has yet to be elucidated. There are two distinct groups of spinal neurons which respond to painful stimuli, one located in the superficial layer (lamina 1) and involved mainly in transmitting sharp pain, and the other in the deeper layers of the dorsal horn (laminae IV and V) responding to intense pain (Besson 1978). Through a dense meshwork of short and long axons these cells transmit messages further centrally. Some of these axons terminate within the spinal cord (Cervero et al. 1979) whereas others ascend to the brainstem reticular nuclei (Zemlan et al. 1978). The longest bundle proceeds to the thalamus (Chung et al. 1979) accompanied by other axons arising from the brainstem. In contrast to the other senses, the neural organisation of the pain system is characterised by elaborate feedback networks. Terminals of at least three descending fibre tracts, which originate from the brainstem nuclei, form synapses in the dorsal horn (Basbaum and

Fields 1978). In addition numerous short axons of spinal neurons traverse a few segments, their terminals interlacing with the dendrites of pain cells located further rostrally (Kerr et al. 1978).

The interplay between the incoming pain messages and the descending influence from the brainstem has recently been the focus of intensive investigation. In 1964 Taub first discovered that spinal neurons responding to painful stimuli could be silenced when certain areas of the brainstem were stimulated electrically. This initial finding of descending inhibitory pathways, confirmed subsequently by others, has recently been extended. The physiological effect on spinal neurons of electrical stimulation of the brainstem is now known to be confined to cells responding to pain, especially those in laminae IV and V (Fields et al. 1977, Hayes et al. 1979). Other neurons nearby which convey, for example, information concerning touch or temperature are uninfluenced by this manoeuvre. Careful mapping has revealed that there are discrete nuclei in the brainstem, stimulation of which produces potent neuronal inhibition and behavioural analgesic effects. Among these are the nucleus raphe magnus and the periaqueductal gray (PAG) (Fields 1977, Guilbaud et al. 1977, Willis et al. 1977, Oliveras et al. 1979).

The raphe magnus is known to be the origin of certain descending fibre tract (Basbaum and Fields 1979, Zemlan et al. 1979), but the periaqueductal gray, which is located along the length of the aqueduct, seems to exert its influence indirectly on the dorsal horn through the nucleus raphe magnus.

The analgesic effect of morphine and related compounds can now be understood in terms of their inhibitory influence on the

transmission of pain. For reasons which are not entirely clear morphine reduces the efficiency of synaptic transmission between pain fibres entering the spinal cord and their target cells in the dorsal horn (Le Bars 1975, Jurna and Heinz 1979, Sastry 1979).

A small quantity of morphine injected into the substantia gelatinosa reduces sensitivity to pain when tested either behaviourally (Yaksh and Rudy 1976) or electro-physiologically (Duggan et al. 1977). The action of morphine, however, is also mediated by the descending inhibitory system of the brain stem. From numerous studies involving gross lesions, microinjection of morphine and biochemical mapping of the distribution of opiate receptors, the same conclusion emerges. The cells in the PAG and the nucleus raphe magnus, richly endowed with opiate receptors, detect the presence of morphine and transmit this message down to the dorsal horn of the spinal cord (Dickenson et al. 1979, Yaksh and Rudy 1979). Here the terminals of the descending fibre release serotonin (5 HT) (Yaksh and Tyce 1979) which, through mechanisms yet to be discovered, interferes with excitatory synaptic processes between primary pain fibres and spinal neurons (Jordan et al. 1978). This inhibitory modulation on the transmission of pain is the essence of Melzack and Wall's Gate Theory.

Melzack and Wall (1965) Gate control theory of Pain has led to attempts to control pain by activation of inhibitory mechanisms. The theory suggests that pain control may be activated by the enhancement of normal physiological activities rather than their disruption by destructive irreversible lesions. Basically, the theory proposes that neural mechanisms in the dorsal horns of the spinal cord act like a gate that can

increase or decrease the flow of nerve impulses from peripheral fibres to the CNS. Somatic input, therefore, is subjected to the modulatory influence of the gate before it evokes pain perception and response. Melzack and Wall proposed that large fibre inputs tend to close the gate whereby the small fibre inputs generally open it, and the gate is also profoundly influenced by descending inhibition from the brain.

One of the major results of the Gate Control Theory was the discovery by Wall and Sweet (1967) that selective activation of large fibres in peripheral nerves by direct or transcutaneous electrical stimulation produces striking relief of several forms of chronic pain associated with nerve lesions. Many studies have since then demonstrated the effectiveness of transcutaneous electrical nerve stimulation for a variety of pain states, including low back pain, pain related to reflex sympathetic dystrophy and myafascial pains. The procedure generally is to allow the patient to wear the stimulating device so that electrical stimulation can be applied at low levels for long periods of time. The pain is rarely abolished but is often diminished to bearable levels so that patients who were previously immobilised are now able to work and lead more normal lives. To be sure, not all patients are helped; and some forms of chronic pain are relieved more than others. Furthermore, some patients who are helped at first report decreasing effectiveness after several months. Nevertheless the procedure is sufficiently effective and simple that it has become a major form of treatment of chronic pain.

The technique of acupuncture, which the Chinese have traditionally used for combating pain, seems also to be based on the Melzack and Wall theory. Thus it is now becoming clear

that the analgesic effect of acupuncture is the result of interaction between impulses from the acupuncture point and that this interaction takes place at different levels of the CNS. There are several lines of evidence to suggest that endogenous opiate systems are operating during this process. Pomeranz and his colleagues (1979) have shown that the analgesic effect produced by acupuncture in animals is abolished if naloxone is injected. Also several clinical studies note that strong acupuncture is most effective for alleviating chronic pain in humans (Andersson et al. 1973, Melzack 1975 and Sjohind and Eriksson 1979), and the analgesia so produced is antagonised by injection of naloxone. In retrospect, it is clear that the Chinese have been at the forefront of research into pain.

This thesis deals with research into pain mechanisms activated by electrical stimulation of the periaqueductal gray (PAG).

ELECTRICAL STIMULATION OF THE BRAIN

Electrical stimulation of the brain has become an important tool in the study of complex phenomena in the central nervous system (CNS), and was the earliest of the three classic techniques of ablation, stimulation and recording. After the introduction of experimental stimulation of the brain with electricity by Fritsch and Hitzig, clinicians were able to exploit seizure symptomatology in a systematic way tc examine brain structure.

In general, the reason for experimental stimulation of the brain is obviously to reproduce the function of the region stimulated, so that the complicated circuitry of the central

nervous system can be functionally dissected. It is tempting therefore, when one finds that stimulation has elicited some observable function, to conclude that function is controlled by, represented in, or caused by the region of stimulation. This tempting logic is of course by no means true. Even if one considers only the complexity of tissues stimulated by any given electrical current, there are fibres, inhibitory and excitatory cells, dendrites, cell bodies and so on, all synchronously influenced in a way which must never occur in response to a naturally occurring incoming volley of nerve impulses with their precise temporal-spatial patterning.

An alternative approach used to study brain mechanisms is that of chemical stimulation. The classic work of Sechenov (1965) in demonstrating the role of the midbrain reticular formation, utilized crystals of salt as the stimulating element, and in that tradition, investigators since have turned increasingly to the use of ions such as potassium or sodium, or transmitters, to try more precisely to replicate the natural processes of the brain. Thus chemical stimulation can be viewed perhaps as an extension or refinement of the method of electrical stimulation. We know, for instance, that an electrical current, in an artificial way, depolarises (or hyperpolarises) a large population of neurons indiscriminately, and the firing or discharge rate of all the neurons in the area of stimulation is altered. In order to "dissect" out the function of a given region of the brain, the local application of a chemical transmitter, for example, parallels quite closely what is actually occurring biologically at the local site.

It is felt that, aside from its theoretical and clinical implications, the technique of stimulation induced analgesia

can have considerable value as a method for studying pain inhibition in general.

SIMILARITY OF STIMULATION-INDUCED ANALGESIA AND MORPHINE ANALGESIA

A fascinating aspect of stimulation-induced analgesia is that it seems to share common features with narcotic analgesia, such as site of action in the brain. Electrical stimulation of periventricular and periaqueductal sites appears to be the most effective in producing analgesia (Mayer et al. 1971). Similarly microinjections of morphine into these same regions of the brain have also been shown to yield analgesia (Tsou and Jang 1964, Lotti, Lomax and George 1965, Herz et al. 1970, Yeung Yaksh and Rudy 1977). Furthermore, morphine is capable of inhibiting some spinal flexor reflexes to noxious stimuli, reflexes which are not vulnerable to other analgesics. The best studied of these is the tail flick reflex in the mouse or rat. Stimulation-induced analgesia also inhibits this reflex, and can be as potent as large doses of morphine (Mayer et al. 1971). Electrophysiological and lesion studies (Irwin et al. 1951, Satoh and Takagi 1971) have pointed out that the morphine inhibition of spinal reflexes is mediated through the activation of descending inhibitory system arising from the brainstem.

REGIONS OF THE BRAIN INVOLVED IN STIMULATION-INDUCED ANALGESIA

It is of considerable importance that analgesia produced by electrical stimulation of the brain can be extremely potent. The magnitude of the effect varies with the intensity, frequency and duration of the stimulation pulses, but has been reported to eliminate totally any overt response to tissue destructive pinch (Liebeskind et al. 1973, Mayer et al. 1974, Soper 1976),

heat (Mayer et al. 1974, Ruda et al. 1976) and pinprick (Balagura et al. 1973, Soper 1976). Extremely high doses of systemically administered morphine (50 mg/kg.) can produce effects of comparable magnitude, but severe side effects such as catatonia, ataxia, sedation and muscular rigidity are present at these doses (Mayer et al. 1974).

The anatomical substrate for stimulation induced analgesia (SIA) appears concentrated in medial brain stem structures extending from the medial diencephalon caudal to the medullary raphe nuclei. The region of the periaqueductal gray matter (PAG) in the midbrain has been most extensively studied and has been shown to be an active site in the rat, (Reynolds 1969, Balagura et al. 1973, Mayer et al. 1971, Mayer and Liebeskind 1974, Rhodes 1975, Soper 1976), cat, (Liebeskind et al. 1973, Melzack et al. 1974, Oliveras et al. 1974, Sewell et al. 1974), rhesus monkey (Goodman et al. 1975, Ruda 1976), and man (Richardson et al. 1973, Adams 1976). Additional active sites have been reported in more rostral periaqueductal regions and sites adjacent to the third ventricle in the rat, (Mayer et al. 1971, Mayer and Liebeskind 1974, Rhodes 1975, Soper 1976) and man (Richardson and Akil 1973, Adams 1976). The periaqueductal gray matter has been shown to be high in opiate binding sites (Kuhar et al. 1973). A number of other brain regions (e.g. amygdala, corpus striatum) have also been shown to have high affinities for opiates (Kuhar et al. 1973), yet morphine microinjection into these areas does not yield analgesia, indicating that the opiate binding is not a significant condition for involvement in analgesic mechanisms.

Although this study is concerned with analgesia resulting from stimulation of the PAG, it should be noted that there are

several other sites which when electrically stimulated will produce analgesia both in experimental animal studies and in the clinical situation with humans.

Recent examination of more caudal brain structures has revealed that electrical stimulation of the medullary raphe nuclei results in potent analgesia in both the rat (Oleson et al. 1975, Prodfit et al. 1975) and cat (Oliveras et al. 1974). Two other areas in the medulla producing SIA include the nucleus reticularis magnocellularis (Nmc) and the nucleus reticularis gigantocelluris (Ngc). Many studies have shown that stimulation of these regions inhibits the responses of sensory neurons in the medullary (Hu et al. 1979, Lovick et al. 1979, Dostrovsky 1980, Dostrovsky and Shah 1980) and spinal (Fields et al. 1977, Willis et al. 1977, McCreery et al. 1979, Pearson et al. 1979, Gebhart et al. 1980, Gray et al. 1980, Haber et al. 1980) dorsal horns neurons. This region of the medullary reticular formation receives efferents from the PAG and also has efferent connections with the spinal cord (Basbaum et al. 1976).

Evidence is accumulating that a neural system originating in nucleus raphe magnus, descending in the dorsal lateral funiculus, and terminating in the dorsal horn of the spinal cord is at least one inhibitory system that is activated during stimulation induced analgesia (Oliveras et al. 1974). Interference with this pathway by lesions reduced the analgesic effectiveness of brain stimulation.

Perhaps the most studied region from the medial brain stem has been the medial forebrain bundle - lateral hypothalamic region. Stimulation of this region in the rat has been reported to result in analgesia utilizing various measures including pinprick, hot plate, jump to electric shock, and escape from

electric shock (Cox et al. 1965, Balagura et al. 1973, Yunger et al. 1973, Rose 1974). Several aspects of analgesia produced by stimulation of these regions suggest that mechanisms other than those described for stimulation of medial brain stem regions are involved. For instance,

1. Stimulation of medial forebrain bundle-lateral hypothalamic areas does not produce analgesia which outlasts the period of stimulation.
2. Stimulation of these regions does not inhibit spinal cord nociceptive reflexes (Mayer et al. 1974).

Different neurohumoral mechanisms appear to underlie the two phenomena (Akil et al. 1972, Yunger et al. 1973). Some evidence is available that stimulation of this area can reduce clinical pain in man (Ervin et al. 1968).

Another region for which there is some evidence of analgesic activity is the septal region (Kasper 1964, Breglio et al. 1970). This area has not been studied extensively but since the analgesia produced by stimulation there does not affect spinal cord nociceptive reflexes (Mayer et al. 1974), it seems most likely that other mechanisms are involved. Stimulation of the central septal area has been reported to be effective in relieving clinical pain syndromes in man (Heath et al. 1960, Gol 1967). Other regions which changes in pain-related behaviour result from stimulation include the dorsomedial thalamus (Mayer et al. 1971) and the ventral tegmentum (Mayer et al. 1974). Further study of these regions is required to determine the neural mechanisms of analgesia involved.

EARLY REPORTS ON ANALGESIA PRODUCED BY STIMULATION OF THE PAG

In 1969, Reynolds first showed that electrical stimulation of the PAG area produced analgesia in the rat so profound as to permit abdominal surgery without anaesthesia (Reynolds 1969). This new method of inducing powerful analgesia, which involved focal stimulation of certain brain loci, has been shown by Mayer (Mayer et al. 1971) not to be attributed to either electrographic seizures, or to general sensory or motor deficits. Also its action cannot be viewed as resulting from a functional lesion caused by stimulation since permanent lesions of these same sites has not led to a clear reduction in pain responsiveness (Liebman et al. 1970). Stimulation-induced analgesia have also been reported in human clinical work (Heath and Mickle 1960, Gol 1967, Ervin Mark and Stevens 1968, Richardson and Akil 1977, Akil, Richardson, Barchas and Li 1978, Akil, Li, Richardson, Hughes and Barchas 1978, Bloom et al. 1978, Amano et al. 1980) and in a few other studies with laboratory animals, cat (Liebeskind, Guilbaud, Besson and Oliveras 1973, Melzack and Melinkoff 1974), monkey, (Oliveras, Besson, Guilbaud and Liebeskind 1974) and rat (Reynolds 1969, Mayer, Wolfle, Akil, Carder and Liebeskind 1971, Akil and Mayer 1972, Mayer and Liebeskind 1974, Akil and Liebeskind 1975, Yeung, Yaksh and Rudy 1977). However, in all these studies, no chemical identifications were made of the neurochemicals involved in analgesia.

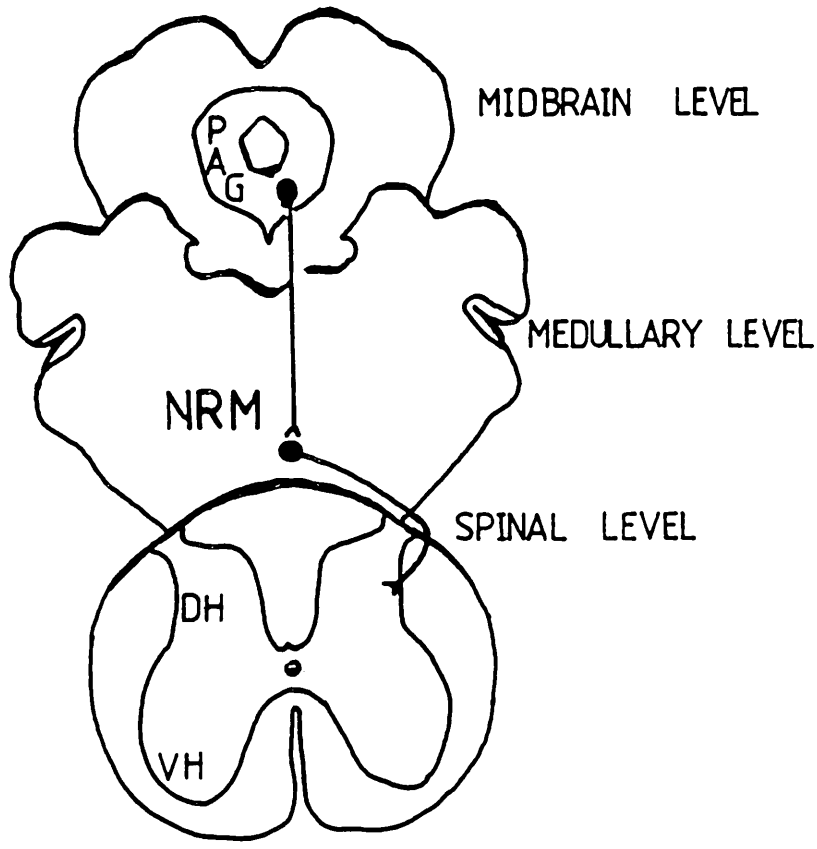
MECHANISMS: ANATOMY AND PHYSIOLOGY OF DESCENDING PAIN CONTROL SYSTEMS

PAG INVOLVEMENT IN ANALGESIA

The mechanism by which manipulations of the PAG produces analgesia is uncertain. This area is rich in both endorphins and opiate receptors (as mentioned before). Local injection of opiates into the PAG produces analgesia (Pert et al. 1974, Yaksh et al. 1976) and injection of naloxone (a specific opiate antagonist) into the same region blocks the analgesia resulting from systemic opiate administration (Vigourel et al. 1973). Thus the PAG is crucial for both stimulation produced and opiate analgesia, but, if the PAG were a crucial relay station for pain messages, opiates could simply act there to block pain by a direct inhibitory effect on relay neurons or the terminals of incoming axons. However, since lesions of the PAG or injection of local anaesthetic into the PAG do not produce analgesia this explanation is unlikely. Furthermore, in the PAG, electrical stimulation and glutamate injections, (Mayer et al.1976, Behbehani et al. 1978) both of which are generally excitatory to neurons, produce analgesia.

The evidence thus points to an indirect action of PAG neurons in the production of analgesia. The observation that stimulation of the PAG inhibits both spinal nociceptive reflexes (Mayer et al. 1971) and dorsal horn neurons sensitive to noxious stimulation (Oliveras et al. 1974) led to the hypothesis that midbrain stimulation induced analgesia depends on descending pathways from the PAG to the spinal cord. These studies were consistent with those indicating that morphine also acts primarily at the brainstem to produce analgesia indirectly by inhibition of spinal pain transmission neurons (Satoh et al.

Fig. 3 - DESCENDING PROJECTIONS TO THE DORSAL HORN OF THE SPINAL CORD INVOLVED IN PAIN PROCESSING.



PAG = periaqueductal grey; NRM = nucleus raphe magnus; DH = dorsal horn; VH = ventral horn.

1971).

The pathways involved in the descending control of pain transmission is shown in fig. 3. Three anatomical entities are critically involved, the midbrain PAG, the medullary NRM and the superficial layers of the spinal dorsal horn. Electrical stimulation of either the PAG or the NRM inhibits pain transmission neurons and produces profound analgesia.

1. PAG - at the level of the PAG, the descending inhibitory system can be activated by a variety of methods, including opiate microinjection, glutamate infusion and electrical stimulation. The PAG has moderate concentrations of enkephalin. Electrical stimulation may work partially by enkephalin release and partially by direct activation of the postsynaptic efferent cell. How endorphins act in the PAG is unclear. Opiate iontophoresis excites some PAG neurons and inhibits others but only the inhibition is naloxone reversible (Gent et al. 1976).

2. Nucleus Raphe Magnus - Whatever the effect of opiates is on PAG neurons, there is a large direct projection from the PAG to the NRM, and excitation of projecting PAG neurons excites NRM neurons, including raphe spinal efferents. The synaptic transmitter linking the PAG to the NRM is unknown, however, the presence of enkephalin containing cell bodies in or near the NRM is intriguing (Hökfelt et al. 1977). The nearby gigantocellular reticular formation also projects to the NRM. Although the NRM does not receive direct input from the spinal cord, physiological studies demonstrate significant spinal influences on NRM neurons.

Raphe Spinal Connections - Through the dorsolateral funiculus, the NRM inhibits spinal dorsal horn neurons implicated in pain

transmission. There is some evidence that 5HT is involved in this action, presumably because NRM efferents are serotonergic. Thus 5HT antagonists or depleters interfere with both opiate analgesia and SIA (Tenen 1968, Akil et al. 1975).

Spinal Dorsal Horn - At the level of the spinal dorsal horn, it is clear the NRM input is inhibitory. What is not clear is the mechanism of this inhibition. There are enkephalinergic neurons and a dense concentration of opiate receptor in the substantia gelatinosa but there is little evidence to show that the enkephalinergic neurons mediate descending inhibition of spinal nociceptive neurons.

When peripheral nociceptors are activated, they excite dorsal horn pain transmission neurons, which ultimately leads to the perception of pain and a behavioural response. Some "pain-transmision neurons" or their collaterals indirectly provide excitatory input to raphe-spinal neurons, which in turn descend to inhibit the very pain-transmission cells that activate them, thus completing a negative feedback loop (fig.3).

The hypothesis that peptides may act as transmitters has already generated new and fruitful approaches to the study of pain, so before describing the experiments on pain, it is worth mentioning a little about the general aspects of peptides, since they are emerging as compounds of immense interest and importance.

NEUROPEPTIDES (GENERAL ASPECTS)

HISTORICAL

The realisation that mammalian neurons produced and released peptides followed du Vigneaud's characterisation of oxytocin and vasopressin as octapeptides (du Vigneaud 1954).

These two hormones are released into the blood in the posterior pituitary from the end of neurons which originate in the supra-optic and paraventricular nuclei of the hypothalamus. These neurons, previously described by the Scharrers, Bargmann and others (Knowles 1974), were the first peptide neurons identified in the mammalian brain. The next peptide neurons to be discovered were those containing the three hypothalamic hormones, thyrotropin releasing hormone (TRH), lutenising hormone releasing hormone (LHRH) and growth hormone release inhibiting hormone (somatostatin) (Schally et al. 1973, Guillemin 1978). These neurons are also hypothalamic in origin, they also secrete their content into the blood vessels and are therefore also neuro-secretory. Neuroanatomically, these systems have been assumed to correspond to the parvocellular tuberoinfundibular neuron described by Szentagothai et al. (1962). The major impetus for the study of neurons containing either the hypothalamic peptide hormones or the host of other chemically identified peptides came when the peptides became available in amounts sufficient for the production of antibodies. This made it possible to measure peptides in tissues and body fluids by radioimmunoassay (Yalow 1978) and also to localise peptides at the cellular and ultrastructural level using immunocytochemistry.

THE IMMUNOHISTOCHEMICAL TECHNIQUE

Immunohistochemistry (Coons 1941) has evolved as an extremely useful technique for visualising different types of compounds in nervous and other tissues (Livet 1978, Jones et al. 1978). In principle, any substance can be traced provided that 1. it is available in pure form,

2. it is immunogenic (or can be rendered immunogenic by conjugation to carrier protein) and
3. it can be retained in tissue sections during processing for immunohistochemistry.

In addition to this universal applicability, this method has the advantage that it can be combined with other types of histochemical techniques. Furthermore, "double staining" techniques allow visualisation of more than one substance in a given section.

With the immunohistochemical approach antisera raised against peptides coupled to bovine serum albumin are applied to tissue sections and visualised by fluorescent or other types of markers. Various modifications can be used, the classical indirect immunofluorescene technique of Coons and collaborators (Coons et al. 1978), the peroxidase - antiperoxidase (PAP) technique (Sternberger et al. 1970). Apart from limitations of sensitivity and the ability of the antibodies to penetrate to intracellular storage sites, the major problem is the specificity of the immunological reaction. Thus an antiserum may not only react with the proper antigen but also cross-react with structurally similar peptides. Hence the cautionary use of terms such as "Substance P immunoreactive" and "Substance P-like immunoreactivity". Further research may show that, when multiple systems are described with a particular antibody, they are not homogenous with regard to their peptide content. Thus, for example, of the more than thirty groups of substance P immunoreactive cells found in the rat central nervous system (Ljungdahl et al. 1978), perhaps only a fraction of them contain the genuine undecapeptide (Chang et al. 1971).

The complexity of this problem can be well illustrated by

the opioid peptides (Li 1964, Hughes et al. 1975, Elde 1976, Watson 1977, Rossier 1977, Bloom 1978). The rapid progress in this area of neuroscience can be largely attributed to its successful structure elucidation (Hughes et al. 1975) using mass spectrometric techniques, and the subsequent synthesis of these peptides. Now several other larger opioid peptides have been shown to contain one or other of these peptide sequences (Lewis et al. 1978, Yang et al. 1978, Kanagawa et al. 1979). Using antisera to leucine and/or methionine enkephalin, many groups have described the central cellular localisation of enkephalin neurons (Elde et al. 1976, Hökfelt et al. 1977, Simantov et al. 1977, Watson et al. 1977, Bloom et al. 1978, Sar et al. 1978, Uhl et al. 1979 and Larssom et al. 1979). Because of the problem with crossreactivity, it was not possible to decide whether the two pentapeptides were present in separate or in the same neurons, and only recently have Larsson et al. (1979) provided evidence that leucine and methionine enkephalin neurons constitute separate systems. It remains to be established to what extent the enkephalin neurons contain the pentapeptides, as opposed to the large opioid peptides from which, in some cases, they are likely to be derived. It is clear, however, that a separate β- endorphin system exists in the rat brain (Rossier et al. 1977, Watson et al. 1977).

A further problem with the immunocytochemical mapping of peptide neurons comes in visualising peptides in the cell soma and axon. This is probably because they contain peptides either in low concentrations or in precursor form. In the latter case an antibody cross reacting with a precursor is an asset rather than a hindrance. Another approach for visualising

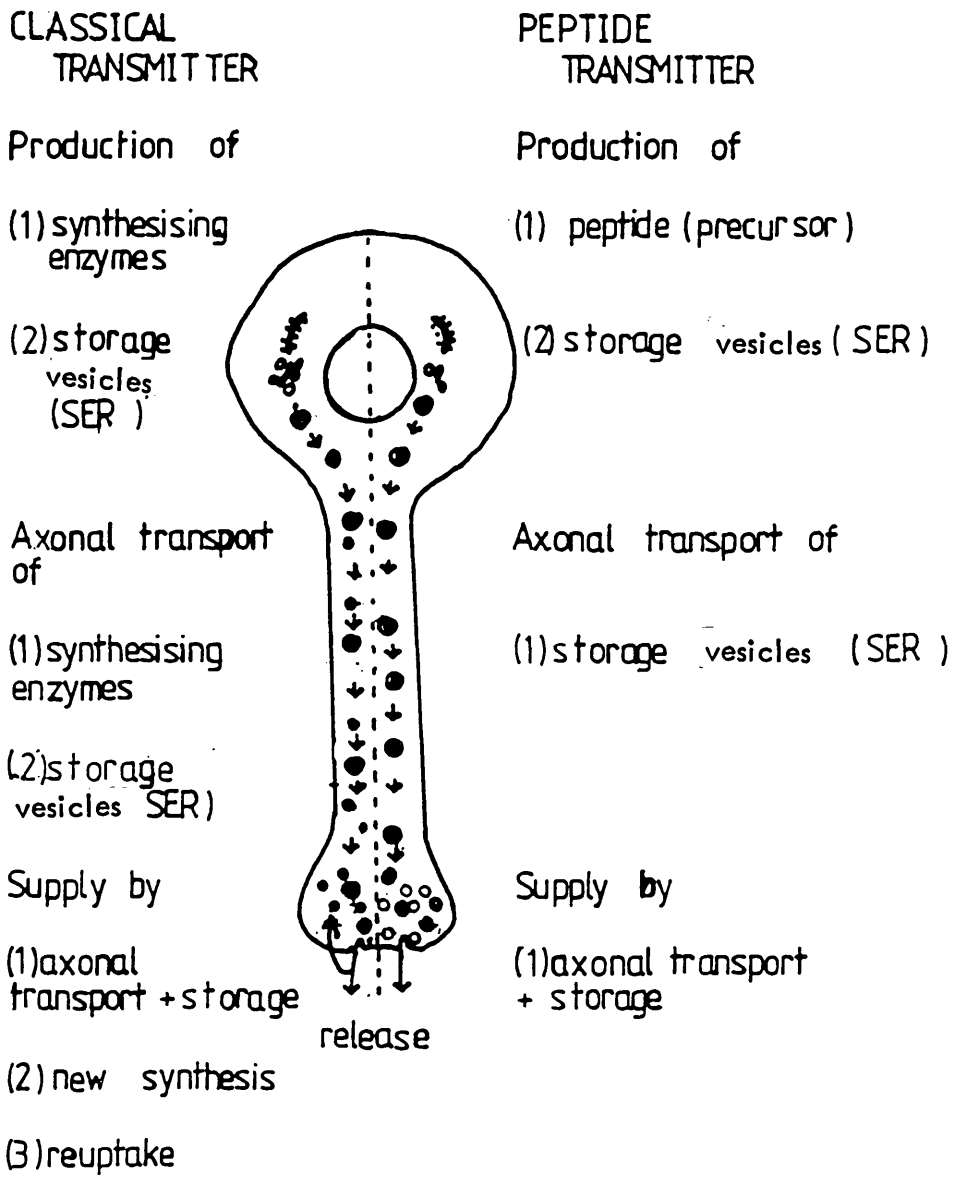
peptides in cell somata is the use of colchicine, a drug which inhibits axonal flow (Dahlstrom 1968), and in this way increases peptide levels. Peptides in axons may be detected by ligation of nerves (Lubinska et al. 1963) with the resulting accumulation of the peptide.

SOME CHARACTERISTICS OF PEPTIDE NEURONS

Only limited information is available on the structural, biochemical and physiological properties of peptide neurons. Nevertheless, it seems that they do not have unique characteristics which would allow their identification on purely morphological grounds. Peptide neurons may be small or large, multipolar or pseudo-unipolar, their nerve endings may make morphologically defined synapses or they may act as receptors over long distance and they may have long or short projections. Nor does ultrastructural analysis reveal any fine structural feature exclusively characteristic of peptide neurons. One feature that is common to several classes of peptide neurons is the large granular vesicle (Goldsmith 1977), a type originally described in peripheral adrenergic neurons (Grillo et al. 1962).

Although functional evidence suggests that at least some peptides act as neurotransmitters (Nicoll 1976, Otsuka et al. 1977, Cuello et al. 1978, Emson 1979), the method of replenishment of peptide neurotransmitters to nerve endings seems to be different from that of classical neurotransmitters (e.g. noradrenaline) (the difference is shown schematically in fig. 4). For example, intraneural noradrenaline levels are kept fairly constant by the efficient replacement of released transmitter by 1. enzymatic synthesis in the nerve endings,

Fig. 4 - SCHEMATIC DRAWING OF A NEURON DEMONSTRATING SOME DIFFERENCES BETWEEN A NEURON USING A 'CLASSICAL' TRANSMITTER AND A PEPTIDE TRANSMITTER.



SER = smooth endoplasmic reticulum

2. re-uptake from the extraneuronal (synaptic) space through an active membrane mechanism, and
3. supply of amine in storage vesicles (or their precursors) from the cell body via axonal transport (Iversen 1967).

Peptides, on the other hand, are probably in the form of a larger precursor molecule (Gainer et al. 1977) without local synthesis in nerve endings. As no re-uptake mechanisms seem to operate for peptides in nerve endings, every single peptide molecule released from a nerve ending must be replaced by axonal transport. This comparatively inefficient and slow mechanism should be reflected in the dynamics of synaptic events at peptide synapses (Bloom 1978). Perhaps peptides are released intermittently rather than tonically. Such an intermittent release may, however, be compensated for by a long duration of action. The amounts of peptide released may also be much smaller than those of classical transmitters. This would be in line with the rather low concentration of peptides found in the CNS (approximately 1,000 times less than monoamines and 100,000 times less than amino acids). Furthermore, peptides may activate receptors at much lower concentrations than the classical transmitters, a situation which may compensate for an inefficient replacement of released transmitter.

DISTRIBUTION OF PEPTIDE NEURONS

Early studies both with radioimmunological and immunohistochemical techniques indicated that the three hypothalamic hormones TRH, LHRH and somatostatin, were present outside the hypothalamus. The finding of these neurosecretory products, previously assumed to be exclusively involved in pituitary control, also in for example, the spinal cord was followed by

reports of the existence of so-called gut hormones such as vasoactive intestinal polypeptide (VIP) (Said et al. 1975, Larsson et al. 1976, Fuxe et al. 1977) and cholecystokinin (CCK) (Vanderhaeghen 1976) in many areas of the brain. A hypothesis has since emerged according to which the central and peripheral nervous system and the endocrine system have a number of peptides, or perhaps families of peptides, in common (Gotto et al. 1979). Although differences can be observed between various species, the occurrence of peptide-containing neurons is widespread.

CENTRAL PEPTIDE NEURONS

More or less extensive maps of the many central peptide systems have been published for immunoreactive substance P (Ljungdahl et al. 1978, Cuello et al. 1978), enkephalin (Elde et al. 1976, Hökfelt et al. 1977, Simon et al. 1977, Watson et al. 1977, Bloom et al. 1978, Sar et al. 1978, Uhl et al. 1979, Larsson et al. 1979), neurotensin (Uhl et al. 1979) CCK (Innis et al. 1979, Larsson et al. 1979, Loren et al. 1979) and bradykinin (Corren et al. 1979). Although they overlap in many areas, each central peptide system has a unique distribution pattern of cell bodies. For example, although both substance P and enkephalin immunoreactive cell bodies have been described in more than thirty areas of the CNS, they overlap only slightly and, when present in the same nuclei, the cells do not seem to be identical. Coexistence of two peptides has, however, been described. Immunoreactive ACTH and β-endorphin are present in the same neurons in the hypothalamus (Bloc et al. 1978, Watson et al. 1978, and Nilaver et al. 1979) and TRH-like immunoreactivity has been observed in substance P

(and 5HT) immunoreactive neurons of the medulla oblongata (Hökfelt et al. 1980).

It is clear from the data collected so far that some areas of the brain are rich and others are poor in peptide-containing neurons. The hypothalamus, the amygaloid complex, certain nuclei of the medulla oblongata and the dorsal horn of the spinal cord are examples of areas of the rat brain where most peptide fibres are observed. The cerebral cortex seems to lack many peptides but is rich in somatostatin, VIP and gastrin (CCK-like peptides, all present in interneuron (Emson et al. 1979)). Of particular interest are the median eminence of the hypothalamus and the dorsal horn of the spinal cord.

The first immunohistochemical identification of a small peptide in neuron was of LHRH immunoreactive fibres observed in the external layer of the median eminence of the hypothalamus (Calas at al. 1973). TRH and somatostatin fibres were also later found in this brain region. Lesion studies suggest that these three peptides do not originate in the basal hypothalamus as originally assumed, but deeper within the hypothalamus (Hökfelt 1980). Instead, the arcuate neurons with short projections to the median eminence seem to contain small classical transmitters such as dopamine (Fuxe 1964), GABA (Walaas et al. 1978) or acetylcholine (Carson et al. 1977) which may exert part of their action at the median eminence level rather than being released into portal vessels. It may be, therefore, that a set of short tuberoinfundibular dopamine, gamma aminobutyric acid (GABA), and acetylcholine neurons act as a type of "gating system", controlling the release of hypothalamic hormones into the portal vessels via an axo-axonic influence (Hökfelt 1980).

The dorsal horn of the spinal cord receives a complex input of peptide-containing neurons. Immunoreactive substance P, somatostatin, VIP and gastrin/CCK are present in primary sensory neurons (Lundberg et al. 1978, Hökfelt et al. 1979, Larsson et al. 1979) substance P (and perhaps somatostatin) is also observed in propriospinal neurons or spinal interneurons (Ljungdahl et al. 1978, Barber 1979). Neurotensin (Innis et al. 1979) and enkephalins (Hökfelt et al. 1977) are present in interneurons (or possibly proprispinal neurons). These findings suggest that peptides play an important part in the processing of sensory information at the spinal level.

PERIPHERAL AND SENSORY PEPTIDE NEURONS

The widespread distribution of peptides in peripheral neurons has now been firmly established. Initial observations concerned substance P in primary sensory neurons (Lembeck 1975, Takahashi et al. 1975, and Hökfelt 1975) but this and other peptides are also present in virtually all parts of the autonomic nervous system, particularly the gut and certain autonomic ganglia. There are well documented examples of primary sensory neurons containing immunoreactive substance P, somatostatin (Hökfelt et al. 1976), VIP (Lundberg et al. 1978) and gastrin/CCK (Larsson et al. 1979, Lundberg et al. 1978). It is not yet absolutely clear whether these peptides can ever occur in the same neurons, but substance P and somatostatin at least do not (Hökfelt et al. 1976). Substance P-like immunoreactivity has also been observed in the vagus nerve (Pernow 1953, Euler 1963, Lundberg et al. 1978, Gamsce et al. 1979) and in taste neurons of the cat (Lundberg et al. 1979). Thus substance P may be involved in sensory processes of various

types including pain (Henry 1977).

If substance P is a transmitter substance in primary sensory neurons one might expect its main action to be exerted at the central branches in the spinal cord or brainstem. A role for substance P in the periphery has, however, long been suspected (Hinsey et al. 1930, Henry 1977, Lembeck et al. 1977). Olgarth et al. (1977) have demonstrated that substance P can be released from peripheral, presumably, sensory branches in the tooth pulp.

GASTROINTESTINAL TRACT

Peptide neurons containing immunoreactive substance P (Pearse et al. 1975, Nilsson et al. 1975), VIP (Bryant 1976), somatostatin (Hökfelt et al. 1975, Costa et al. 1977), enkephalin (Elde et al. 1976, Alumets et al. 1978, Linnoila et al. 1978), neurotensin (Schultzberg et al. 1979), gastrin/CCK and bombesin (Dockray 1979) have been identified in the gastrointestinal tract. Analysis of neurons stained consecutively with different antibodies has revealed that substance P, VIP and enkephalin are present in separate systems (Schultzberg et al. 1979). In the proximal colon the somatostatin and gastrin/CCK-like peptides coexist in some neurons, although separate somatostatin and gastrin/CCK neuron seem to exist. Most of these peptides are intrinsic to the gut but are complicated by the fact that the peptides are also present in extrinsic neurons of, for example, the vagus nerve.

TRACING PEPTIDE PATHWAYS

Although the localisation of peptide-containing cell bodies and nerve terminals is now well documented, comparatively little is known about the connecting axons because their peptide

content is usually too low to detect. Similar problems with catecholamine axons were solved by transection experiments, in which the pile-up of transmitter on the cell body side of the lesion allowed mapping of the axon pathway (Ungerstedt 1971). Even so, a very large number of consecutive sections must be processed in order to follow long pathways, for example, from the lower brainstem to the forebrain. Anterograde and retrograde tracing techniques (Cowan et al. 1975), in particular, retrograde peroxidase tracing (Kristensson et al. 1971 and La Vail 1972) combined with immunochemical staining with antiserum to tyrosine hydroxylase (Ljungdahl et al. 1975) has enabled the projection of dopaminergic neurons to be mapped. Retrograde fluorescent dye tracing has been combined with peptide immunohistochemistry (Hökfelt et al. 1979) to map, for example, an enkephalin immunoreactive pathway from the lower medulla oblongata to the spinal cord (Hokfelt et al. 1979).

COEXISTENCE OF PEPTIDES AND CLASSICAL TRANSMITTERS

The occurrence of more than one transmitter substance in one neuron has been observed in the invertebrate nervous system by several groups (Kerhu et al. 1967, Hanley and Cottrell 1974, Hanley et al. 1974, Cottrell 1976, Osborne 1977). Brownstein et al. (1974) used the term "coexistence" in describing this phenomenon. However, as was discussed by Osborne (1979) it has in no case been established that the coexisting substance fulfil transmitter roles in the particular neuron. Another type of coexistence has been found in developing mammalian autonomic neurons. Such neurons may, at least in in-vitro conditions, switch from a cholinergic to an adrenergic transmitter, and may at some stage contain both acetylcholine

(ACh) and noradrenaline. It remains, however, to be shown whether or not such coexistence also occurs in adults but Burns and Rand (1965) discussed the idea that ACh occurs in noradrenergic (NA) neurons and controls NA release. (For a review in this field see Patterson (1978)).

The coexistence of peptides and amines is well known in endocrine cells, particularly in the gastrointestinal tract (Pearse 1969). According to Pearse, these cells belong to the APUD system, the cells which are "neuroendocrine-programmed cells originating from the ectoblast"(Pearse 1979). As neurons have the same embryonic origin it was postulated that they also may contain both a peptide and an amine. The fact that several examples have been observed in a relatively short time may indicate the generality of this situation (Schultberg et al. 1979, Hökfelt et al. 1977, Lundberg et al. 1979).

The possible occurrence of two transmitters in the same nerve terminal raises the question of their subcellular storage sites. In most types of nerve endings, small (diameter about 500 A) and large (diameter about 1000 A) granular vesicles can be distinguished. Ultrastructural immunocytochemical studies indicate that peptides are usually present in the large vesicles (Goldsmith 1977). No ultrastructural immunocytochemical studies have been performed on nerve endings identified and characterised as containing both a peptide and an amine. It may, however, be speculated that, as monoamines seem to be present both in small and large vesicles (Tranzer et al. 1967), peptides and amines may coexist in the large vesicles, whereas the small ones may contain only the amine (Pickel et al. 1977). A differential subcellular storage compartment is, however, suggested by the fact that even very

high doses of reserpine, which almost completely deplete the monoamine stores, do not seem to affect substance P levels (Paasonen 1956, Hökfelt 1978).

It has generally been assumed that one neuron produces and releases only one transmitter, a concept often referred to as Dale's principle (Eccles 1957, Burnstock 1976). It seems, therefore, important to understand the possible functional significance of two putative transmitters in one neuron. Note that coexistence does not follow a simple pattern. For example, many more substance P immunoreactive cell groups have been observed (Ljungdahl et al. 1978) than those containing both substance P and 5HT. In no case do all of the neurons that contain a particular classical transmitter also contain the same peptide and vice versa. Several explanations seem possible, but an attractive hypothesis is that each type of neuron defined by the classical neurotransmitter that it contains, is subdivided into groups characterised by the peptide they contain.

The occurrence of a peptide in a population of dopamine neurons is interesting in view of our extensive knowledge of the functional role of these dopamine systems. In particular, the mesolimbic dopamine systems have been associated with higher mental functions and according to the so-called dopamine hypothesis, disturbances in this system may represent one component in the pathogenesis of schizophrenia (Matthyse and Kety 1975). If a CCK-like peptide is released together with dopamine, the peptide could also be involved in the aetiology and symptomatology of schizophrenia. It has been speculated (Hökfelt 1980) that an imbalance between peptide and amine in fact, could be an aetiology factor for schizophrenia, whereby

a loss or decrease in peptide would lead to an overactive dopamine system (Matthysse and Kety 1975).

Finally, the coexistence of putative peptides and biogenic amine transmitters raises questions about substitution therapy. The belief that some mental and neurological disorders are due to degeneration of transmitter-characterised systems has formed the basis of therapy by administration of the lost transmitter (or its precursor). The best example is Parkinson's disease which is now, due to the pioneering work of Carlsson, Cotzias and Homyiewicz and others (Yahr 1977), known to be due to the degeneration of dopamine systems in the basal ganglia and which can be treated by peripheral administration of L-dopa, the precursor of dopamine. It is obvious that if a neuron releases two transmitters, substitution should, at least in certain conditions, consequently occur with both substances. It may therefore be that future substitution therapy will have to consider administration of two or more components. This argument is, however, not valid if the peptide acts on a presynaptic receptor. For this particular system other aspects must be considered in relation to drug therapy.

Summarising then, it would seem that although our understanding of the functional role of peptides is still incomplete, interesting discoveries have been made. For example, the posterior pituitary hormones seem to be involved in many processes (De Wied and Versteeg 1975), central endorphins are important in pain mechanisms (Terenius 1978), substance P in primary sensory neurons may be involved in processing of pain and in axon reflexes causing vasodilation in skin (Henry 1977), CCK octapeptide causes saiety (Gibbs et al. 1973), angiotensin II injected intracerebrally stimulates drinking and increases

blood pressure (Fitzsimons 1972) and LHRH increases sexual behaviour (Moss 1971). The effects mentioned above represent a narrow selection of the work going on in the field of neuropeptide physiology but illustrate the wide variety of functions in which peptides may be involved. Finally, peptides have been identified in the brain, spinal cord and periphery. Several of these peptides were already known as peripheral hormones. Thus, one and the same peptide can be present in intestinal endocrine cells, gut neurons, sensory neurons of various types and in a large number of central systems. This may be a way of economising on genetic material. At present the identification of the intraneural peptides rests mainly on immunological techniques.

There is experimental evidence that at least some of these peptides, for example substance P, act as neurotransmitters. However, comparatively little is known about their exact physiological role. This is partly due to a lack of drugs which can influence peptide-induced events at synapses. The wealth of such drugs discovered and developed for studies of monoaminergic mechanisms has been an important reason for the rapid advancement of our understanding of the roles of dopamine, noradrenaline and 5HT neurons in the brain in normal and pathological conditions (Usdin et al. 1979). Whilst awaiting the development of equivalent drugs for peptides, some results can be expected from the use of antisera which block peptide induced effects (Cheramy 1978).

CHAPTER 2

DEVELOPMENT OF AN ANIMAL MODEL TO STUDY
1. ANALGESIA
2. THE RELEASE OF PEPTIDES

During the course of this work, many peptides have been implicated in analgesia. [Table 1].

TABLE 1. PEPTIDES IMPLICATED IN ANALGESIA

β- Endorphin	Tseng et al. 1976
Enkephalins	Bradley et al. 1976
Substance P	Malick et al. 1978
ACTH	Walker et al. 1981
TRH	Boschi et al. 1983
Dynorphin	Goldstein et al. 1980
β- Casomorphins	Grecksch et al. 1981
Caerulein	Jurna and Zetler 1981
CCK_8	" " " "
Neurotensin	Martin et al. 1981
Kyotorphin	Takagi et al. 1981
Vasopressin	Berkowitz et al. 1982

The evidence for this involvement in analgesia is not based on identification of the released peptide but purely on micro-injection studies.

In a study on the role of central nervous system (CNS) neuropeptides in pain, it is first necessary to define certain aspects of the proposed work. The following questions need to be answered:

A. How will analgesia be induced and measured?

B. At which point of the CNS will samples be removed, and how can one concomitantly collect samples and induce analgesia?

C. Can neuropeptides be reliably identified and quantified, and can more than one neuropeptide in the same sample be detected?

The above questions can all be resolved by the development of a suitable animal model. This model should have the following properties:

1. It should be used to induce analgesia reproducibly, relatively easily and conveniently. The analgesia so produced should be potent enough to be able to quantify it, using a reliable behavioural test.
2. It should allow sampling from the CNS system concomitant with the production of analgesia. It should also have the added advantage of being able to provide continuous sampling from, for example, the CSF system, i.e., before analgesia has been induced, during the analgesic state and post stimulation. This would thus allow a comparison of the peptides present at these times. Such a system would thus allow the study of peptides (and other substances) released into the ventricular system before, during and post stimulation.
3. Of importance is that the model should be such that each animal would be its own control. Thus avoiding the need to use large numbers of animals and the handling of complex statistics.
4. It should allow such a study as described above to be made while the animal is freely moving and, most importantly, the animal should at no time exhibit signs of stress or any abnormal behaviour.

A. The method used to induce analgesia in this study is that of electrical stimulation of the periaqueductal gray (PAG). It has been proven that analgesia produced by electrical stimulation of the PAG can be extremely potent (Reynolds 1969, Mayer et al. 1974).

It is appropriate also to mention here a little about the technical details involved when using brain stimulation techniques. There are several types of electrodes one can use for brain stimulation studies. The physical configuration of any electrode, its size and length, the amount of exposed tip and so on, will depend upon its intended use. Except for the dimensions, the basic decision to be made in constructing electrodes has to do with their spatial configuration: monopolar, bipolar (or multipolar) or concentric.

The monopolar electrode, with a reference lead elsewhere on the animal, stimulates the largest area. For practical purposes in a small brain, the electrode can be assumed to stimulate the brain as a whole with the point of most intense stimulation, of course, being the tip of the electrode. The intensity falls off as the square of the distance away from the electrode. The intervening tissue between the brain and the reference point (which may include muscle, heart retina or other tissues) is also stimulated. For this reason the monopolar electrode is in general unsatisfactory for precise work. The large stimulus field, however, does increase the chance of finding an excitable area and may sometimes be useful in a preliminary exploration of the cortex or some subcortical point.

The bipolar electrode is composed of two monopolar electrodes placed close together but with tip separations of

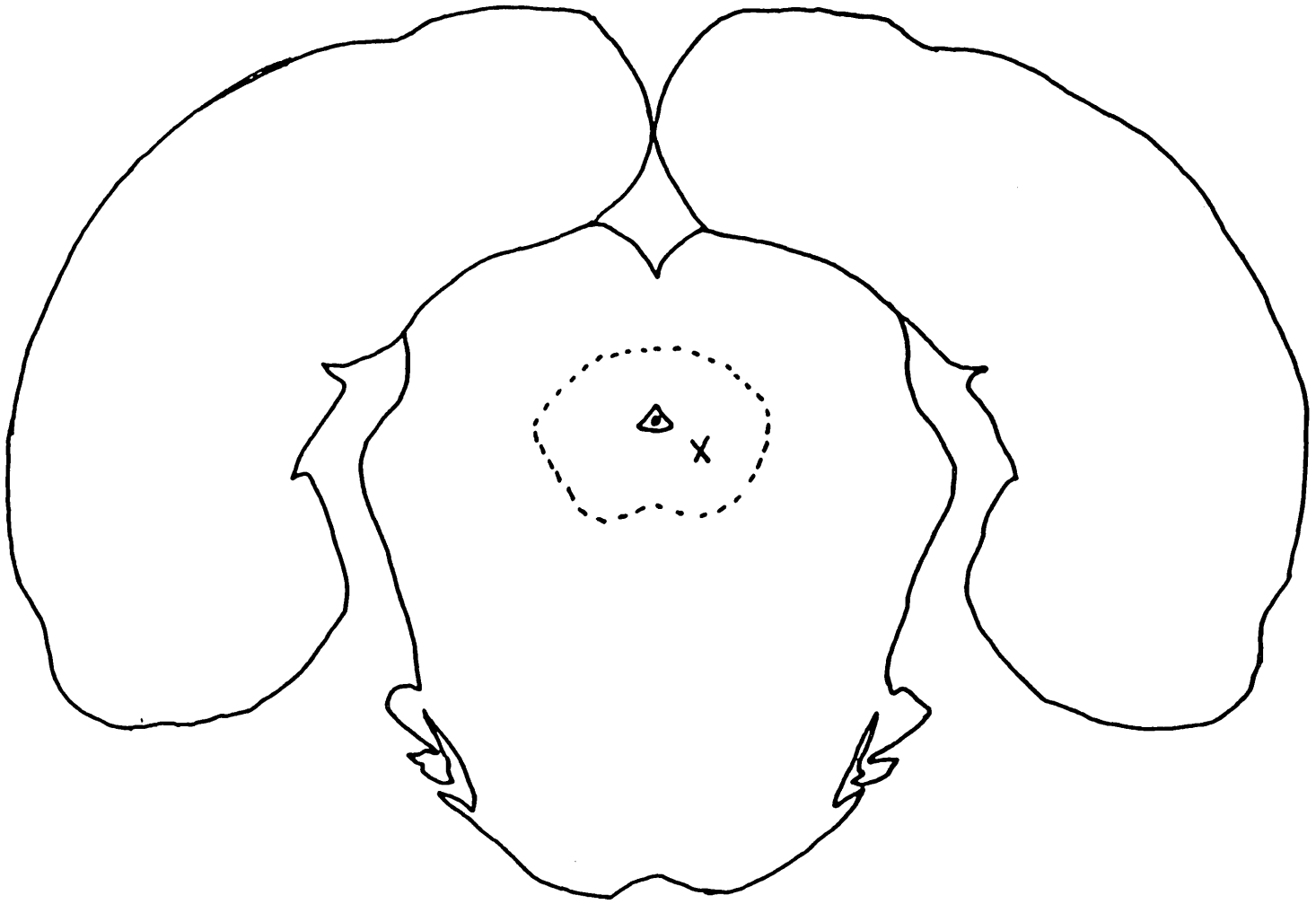
one-half to one millimeter. The most intensely stimulated region is that lying between the two electrode tips where the lines of force are concentrated and a symmetrical field surrounding them. The bipolar electrode provides a much more discrete location of the stimulus than does the monopolar and is generally preferred.

The multipolar electrode is ordinarily used only in bipolar configuration, the larger array serving as a convenience to the experimenter to permit exploration of a series of brain sites without the trauma of moving the electrode. This has been particularly valuable in human studies with chronically implanted electrodes.

The concentric electrode, usually made with the exposed tip of a cannula or hypodermic needle within which is an insulated wire bare only at the cross tip, has an even "tighter" field pattern than does the bipolar. Its field can be thought of as sharply focussed between the protruding tip and the surrounding circle so that it stimulates predominantly in the direction in which it is aimed and thus allows for some accurate positioning for exciting structures which are not mechanically penetrated by the electrode.

Bipolar electrodes have been used throughout this project. A typical electrode placement in the PAG of the rat is shown in fig. 5. There are some potential problems arising from brain stimulation studies. The most critical aspect of the technique is simply that of any good surgical technique. Sterilisation of the electrodes (with 70% ethanol) is quite important since a microabscess at the tip of an electrode can substantially alter stimulation results. Delgado (1961) reported that as many as fifty electrodes could be left for long periods of time

Fig. 5 - LOCATION OF ELECTRODE TIP IN MIDBRAIN



X = histological verification of electrode tip location. (from the atlas of Pellegrino and Cushman)

without significantly altering the normal functioning of the monkey brain, suggesting that the brain is fairly resistant to the trauma of implanting the electrodes. Tissue trauma may also result from chemical irritation due to the composition of the metal electrode or of the insulating material. A great variety of metals have been used for chronic implantation. These include copper, silver, stainless steel, platinum, gold, tungsten, molybdenum and platinum chloride and silver. Fischer et al. (1961) implanted insulated and uninsulated copper, silver chloride and stainless steel wires in the cat brain for periods of two to four months to test for toxicity - silver chloride and copper electrodes were poorly tolerated even when insulated, and caused extensive tissue degeneration in the area surrounding the uninsulated wire. There was no noticeable tissue reaction to stainless steel, even around the bare wire.

Electricity has certain advantages over other methods of stimulation. It can be delivered to a precise point within the brain by a relatively small mechanical unit, a wire. The dimensions of the stimulus delivered, that is, its amplitude, duration, shape and frequency of repetition, can be precisely controlled and measured. The stimulus may be applied conveniently by remote control and the onset and termination of its effect may be quite prompt as compared to similar changes following chemical stimulation. For all these reasons, it remains a powerful and useful experimental tool.

In any study involving analgesia in animals, it is first essential to decide:

a. on a method to inflict pain, e.g., burn, shock or squeeze;
b. on a behavioural response, such as a twitch or flinch.

Pain can be prcduced by four main methods, thermal,

electrical, mechanical and chemical. A pain stimulus must be chosen which can be controlled and measured, and which permits the establishment of a clearly defined end point. These pain tests in animals have traditionally been used for predicting analgesic activity in man.

The thermal method involves applying heat to a sharply defined area of the animal's body and then observing the response. The response time (latency) is taken as the time between exposure to the stimulus and the response of the animal. There are three common methods using thermal stimuli. Firstly, the tail flick test (D'Amour and Smith 1943): in this test the heat source is focussed on the tail of rats or mice, and the behavioural response is the flicking of the tail away from the heat source. Secondly, the skin twitch (Winder 1950): in this test radiant heat is focussed, usually on the skin of rodents, and the behavioural response is a twitch of the skin. The time to produce the twitch is recorded. Thirdly, the hot plate method (Woodfe and McDonald 1944): this consists usually of a copper plate maintained at a constant temperature (55^{0}C). The test animal, usually a rodent, is placed on the hot plate and the first signs of discomfort are shown by the rodent sitting up on its hind paws and licking and blowing its front paws. This licking of the front paws is generally taken as the end point.

With all three methods a cut-off time is used in order to prevent any tissue damage. Analgesia is assessed by comparing response times obtained before drug administration to that obtained post drug administration. The use of thermal methods to induce pain has certain advantages over other methods. It is reliable and reproducible, i.e., good determinations of

relative potencies of narcotic analgesics are obtained. It does, however, lack the ability to detect all narcotic antagonist analgesics.

Mechanical methods employ the use of pressure on the skin of the test animal. Various mechanical devices are used to squeeze or distend the limbs of the test animal, and the end point is generally taken as the pressure necessary to induce vocalization in the animal. With this test it is difficult to standardise the stimulus, and in addition there is a risk of producing tissue damage which makes repeated determinations difficult. For these reasons, this method is not often used.

Intraperitoneal injections (ip) of certain noxious chemicals into rats or mice produces a wave of constriction and elongation, passing caudally along the abdominal wall. This response is known as "writhing" or "abdominal constriction". Chemicals producing abdominal constriction (e.g., benzoquinone, acetic acid, bradykinin, hydrochloric acid, acetylcholine and oxytocin) are injected i.p., the mice or rats are then placed in translucent boxes, and their response counted during a continuous observation for two minutes. Drugs to be tested for their ability to supress abdominal constriction responses are injected twenty minutes before injection of the noxious chemicals (constrictors). Responses are counted for two minutes afterwards. A disadvantage resulting from the use of this method is that false positives are sometimes obtained.

There are various electrical methods used, for example electrical stimulation from implanted electrodes in the tooth pulp or tail of rats. A pain reaction is assumed to be produced when the animal responds to the applied electric shock by squeaking. In each animal the individual pain threshold is

determined before the administration of the test drug and graded in volts. This was done by increasing the voltage until the animal responds with a squeak.

Two important aspects of any potential analgesic are potency and efficacy. The tail flick test, although lacking the ability to detect some narcotic antagonist analgesics, does give a good correlation of the relative potency of morphine, mepridine and pentazocine in animals with clinical data. The hot plate method, although highly predictive of clinical analgesic activity, fails to select the narcotic antagonists which are capable of relieving pain in man. Mechanical methods, however, do not seem to have this drawback.

In this research, the tail flick test was used to monitor the analgesia produced by electrical brain stimulation. The other methods were not used for obvious reasons; for example:

1. the use of electric shocks to inflict pain would involve a complex response involving both the central and peripheral nervous systems;
2. mechanical methods run the risk of producing tissue damage, and an inflammatory response may thus result. This response may mask the events occurring from electrical brain stimulation;
3. it would be unreasonable to use chemicals to inflict pain in an investigation involving the detection of chemicals causing pain relief.

In summary, the behavioural method used to monitor the analgesia produced by electrical brain stimulation is that of the tail flick test. This test was chosen for the following reasons:

firstly, the response, a spinal reflex, is relatively simple;

secondly, it is easily quantifiable. The dependent variable (response latency) is a continuous measure which allows careful examination of the time course of stimulation (or drug) effects; thirdly, it is the most widely used behavioural screen for the narcotic analgesics; this has been justified largely on the basis of the relatively good correlation between behavioural results in animal testing and clinical potency in humans.

Important experiments have been performed to validate the use of the tail flick test and electrical stimulation of the PAG in this research. These experiments are reported in later sections.

B. To understand the functions of neuropeptides in the central nervous system (CNS) it is important to know not only that these substances are present in neurons and nerve terminals, but also whether they can be released from such neuronal sites to exert influences on other neurons. The release mechanisms involved resemble the stimulus secretion coupling phenomena known to exist in neurons using the conventional neurotransmitters, or in other secretory cells in showing an absolute requirement for calcium ions in the external medium (Iversen et al. 1976, Bayon et al. 1978, Henderson et al. 1978, Pinget et al. 1979).

In the CNS, a direct demonstration of the release of a suspected neurotransmitter presumes

1. that the proper nerve pathway or set of axons has been identified and can be selectively stimulated;
2. that production and release of the "transmitter" occurs in physiological excess and can be detected in amounts secreted following excitation of nerve endings; and

3. that activation (diffusion, re-uptake, metabolism, etc.) of the transmitter must be blocked (Phillis 1970, Cooper et al. 1978).

The primary difficulty in applying these criteria in the CNS has been the inability of currently available techniques to detect release of small amounts of the peptide from a defined peptidergic pathway without destroying the functional and morphological integrity of the region being examined (Cooper et al. 1978). Thus only the resultant activity of a large population of heterogenous neurons is reflected in these studies. Therefore, because of these complexities and the heterogeneity of the brain and spinal cord, and the inaccessibility of the neurons, it is difficult to devise experiments to measure neuropeptide release from the CNS in vivo. Most studies have involved the use of in vitro model systems (Iversen et al. 1978) in which the release of substances from the nerve terminals can be studied using synaptosome preparations or small slices of brain tissue, by means of chemical depolarizing stimuli (such as high external potassium) as triggers. These preparations can be obtained from local regions of the CNS containing a particularly high concentration of the substance in question. This helps to overcome a second major difficulty, namely that the neuropeptides occur in exceedingly small amounts in the CNS tissue, and only minute quantities are released in response to stimulation.

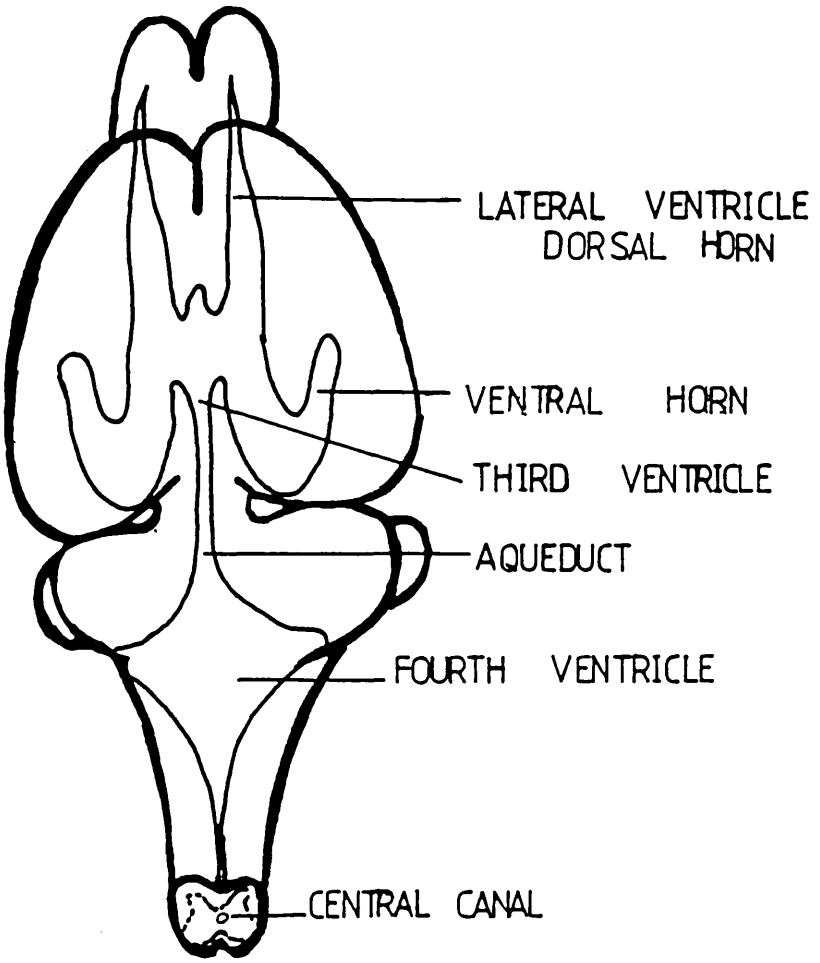
In this research, however, an in vivo system has been used. Two approaches allow the in vivo investigation of the chronic rather than the acute preparation. These are firstly, samples of cerebrospinal fluid can be collected repeatedly at given intervals. Alternatively, samples of perfusates can be obtained

from circumscribed regions of the brain. It was decided to use the latter method in this thesis, and more specifically to perfuse the ventricles.

There are several strategic points in the cerebroventricular system, (fig. 6), whereby one could conveniently sample CSF or perfusate. The arrangements of the ventricular system are essentially the same in all mammals, and consist of four communicating ventricles. The brain has two lateral ventricles, one in each of the cerebral hemispheres, the lateral ventricles communicate with the third ventricle through the foramina of Monro and the third with the fourth through the aqueduct of Sylvius (fig. 6). CSF can be taken from different parts of the system and is designated as lumbar or spinal fluid (from the spinal subarachnoid space in the lumbar region), cisternal fluid (from the cisterna magna), cortical fluid (from the cortical subarachnoid space) or ventricular fluid (from the brain ventricles).

Spinal and cisternal fluid are more readily accessible than the others, and the former is almost exclusively studied in man and the latter in animals. The reasons for the two approaches are technical. Lumbar puncture in man is a routine and relatively safe procedure, while cisternal puncture is avoided because of the possibility of injury to vital centres in the medulla. On the other hand, percutaneous puncture of the cisterna magna in animals can be easily performed, while for safe and successful sampling of lumbar fluid surgery is required. Conclusions reached from the study of cisternal fluid in animals are often compared with data in human lumbar fluid. However, cisternal and lumbar fluids must not be considered equivalent with regard either to composition or to

Fig. 6 - DIAGRAM OF THE VENTRICULAR SYSTEM OF THE RAT.



the origin of substances in them.

By perfusing the cerebral ventricles, a method is provided whereby substances which are released from the structures forming the ventricular walls can be collected, separated and then detected by various assay procedures. Following the initial studies of Leusen (1948), the method of perfusing the ventricles has become more widely used in the last two decades. In England, the pioneering work in the late 1950's and early 1960's of Feldberg and his colleagues, has provided valuable knowledge about the release of acetylcholine, serotonin and other substances.

In the United States, Pappenheimer and his colleagues have carried out extensive studies which have led to the methods for perfusing the conscious animal, which in itself has certain advantages over the anaesthesised preparation (e.g. Pappenheimer et al. 1962) for example by avoiding general chemical interference of the normal physiological state.

A method that provides for the in vivo determination of neurochemical changes in specific regions of the brain during concomitant behavioural changes, is the technique of push-pull perfusion. The concept of push-pull perfusion was introduced by Fox and Hilton (1958) and this was developed later by Gaddum (1961) who suggested its usage for brain physiology.

PRESENT WORK

In this thesis, the method of push-pull perfusion has been used to investigate the in vivo release of peptides during concomitant electrical stimulation of the peri aqueductal gray (PAG). This method was used after initial attempts using other procedures. These included:

1. perfusing from the left lateral ventricle through to the right lateral ventricle and
2. perfusing from the lateral ventricle through to the fourth ventricle.

Although these methods had successfully been used by others using larger species, i.e., the cat (Feldberg and Fleischhauer 1962), they were found not to be satisfactory using the rat for the following reasons:

a. the perfusions could not be reliably reproduced, possibly due to the thinness of the ventricular lumen in the rat;
b. the rat had to be anaesthetised during the perfusion procedures;
c. any attempts to perfuse the rat unanaesthetized could only be achieved by physically restraining the animal. Thus fear or other defence reactions were apt to overshadow the brain stimulation effect.

Later experiments were then extended to using the push-pull cannula, and confining the perfusion site to the third ventricle. Under these conditions, successful perfusions were achieved in the conscious unrestrained rat.

The decision to perfuse the ventricle in this research was taken on the assumption that neural activity is associated with the release of neurochemicals and that some of the neurochemicals might "overflow" into the extracellular space and thus be available for collection. Changes in the level of the overflow are therefore indicative of the activity in the neural system. The third ventricle was perfused in preference to the more accessible lateral ventricle since the surrounding regions have been shown to exert modulatory control over the physiological response to pain (Hökfelt et al. 1977, Oliveras et al.

1979) and also the area around the third ventricle has been shown by the local action of exogenously administered opiates to be effective in producing analgesia (Herz et al. 1970, Yeung et al. 1977). In addition, most of the human studies that have been made on stimulation induced analgesia have also sampled CSF from the third ventricle. Thus a direct comparison between animal studies and clinical studies could be made.

A most important aspect from using such a model is that the animal can be used as its own control. This is particularly advantageous when difficult techniques are used; surgery and histology are time-consuming and costly, and the use of animals should always be minimised. To compare values of a given substance under both control and experimental conditions in the same brain may provide unequivocal results. If the test animal is killed for the purpose of brain assay, the opportunity for such a comparison is lost. Thus to generate a chemical basal level under any other conditions would need large numbers of animals and complex statistics.

The X-ray localization of the cannula in a typical experiment is shown in fig. 7. The localization of the cannula and electrode in situ is shown in fig. 8. A photo micrograph of a representative cannula placement is shown in fig. 9.

There are several inherent problems using push-pull perfusion in the rat to study peptide release. As mentioned previously, peptides occur in exceedingly small quantities (approximately 1,000 times less than monoamines and 100,000 times less than amino acids). It was soon realised that in order to successfully detect peptides in the third ventricular perfusates, it was necessary to repeatedly perfuse the same animal and then pool the perfusates. It was also necessary to

Fig. 7 - THE X-RAY LOCALISATION OF A CANNULA IN A TYPICAL EXPERIMENT

GUIDE CANNULA IN SITU

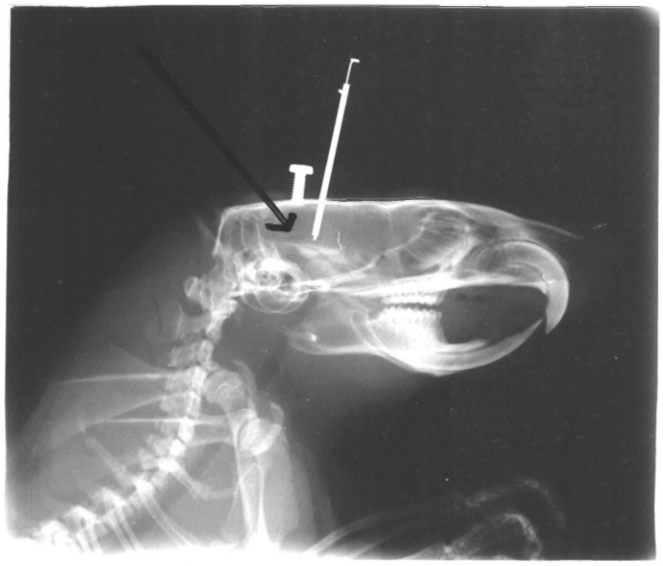
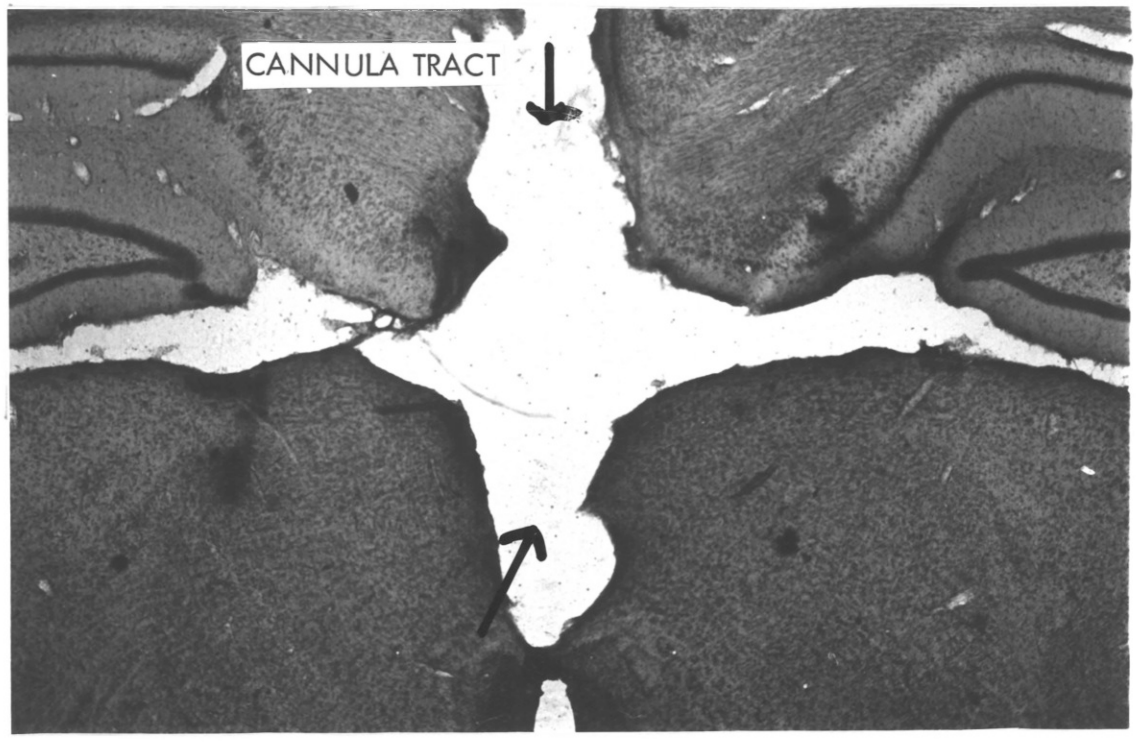


Fig. 8 - THE X-RAY LOCALISATION OF A CANNULA AND ELECTRODE IN SITU



Fig. 9 - A PHOTOMICROGRAPH OF A REPRESENTATIVE CANNULA PLACEMENT

CORONAL SECTION TAKEN THROUGH THE RAT BRAIN INDICATING THE LOCUS OF CANNULA TRACT INTO THE THIRD VENTRICLE .



THIRD VENTRICLE

have long collection periods for each sample collected. Thus, due to the nature of the work, only a limited number of experiments could be performed, particularly since the duration of an individual experiment was of the order of six to eight weeks. Despite these precautions, it is still imperative to use very sensitive assay methods to detect the peptides.

There are other precautions that are necessary when an animal is maintained for extensive investigation. Health is of prime importance. In this study, neither surgery nor experimentation was undertaken if signs of disease or ill health were manifest. In the rat, a consistent pattern of food and water intake is a major index of general physical condition. Sterile precautions were taken during the repeated perfusions. For example, after the stylet was removed from the guide cannula, special care was taken that the tips of the push-pull cannulae were neither touched by the fingers nor touched against a surface that was not sterilised. At the end of the perfusion, the stylet was first cleaned with 70% ethanol before replacement back into the guide cannula.

The maintenance of viability of the site of perfusion is a further precaution that is necessary for successful repeated perfusions. Under no circumstances should the site be left untouched for more than four days. If this happens, an encapsulating wall of fibrous material or glial tissue is deposited within the small channel that is produced by the push-pull cannula. Such a mass of "scar" tissue quickly inactivates the physiological responsivity of the locus of perfusion.

C. The multiplicity of endogenous peptides is becoming increasingly apparent. This multiplicity renders it difficult to carry out meaningful physiological/biochemical studies using only bioassays, since several of these substances occur in the same structures and interact with the same receptors. It is equally difficult to exclusively rely on radioimmunoassay (RIA), since cross reactivity, even if minimal, can become a significant factor if behavioural manipulations affect one or the other cross reacting peptide differentially. It is therefore critical to couple such procedures as radio-receptor assays (RRA) or RIA with reliable chromatographic steps which separate the various peptides.

A high resolution, reverse phase high pressure liquid chromatography (HPLC) procedure using an n-propanol:acetic acid elution system had been developed in this laboratory for the separation and characterization of biologically important peptides (Morris et al. 1980). This method at the time had been shown to be viable down to the five picomole level using radio-labelled vasoactive intestinal peptide (Morris et al. 1980). The work in this thesis demonstrated, for the first time, the applicability of the HPLC method at the femtomole level when used in conjunction with sensitive RIA techniques.

As a direct result of some of the work in this thesis, it became apparent that when a highly sensitive assay method is employed, the danger of carry-over between runs became apparent. Thus despite the excellent recoveries obtained (given in the general methods section), the small amount of unrecovered peptides can produce a significant artifact if not completely eluted. For example, if one employs 1 nmole of authentic unlabelled peptide as a standard, and recovers 99% of it, the adsorbed

1% still represents ten picomoles of peptide remaining on the column. This can then be eluted upon later runs and easily leads to artifactual quantitation in the biological samples. In some circumstances, particularly when working on individual samples of interest, it was essential to gradient elute the column many times in order to give blanks at the femtomole level. Consequently, when standard peptides were chromatographed for calibration, great care was taken to follow these standard runs with numerous blank runs (buffer with no sample added). Additionally, the blank run prior to a sample run was always collected and assayed in an identical manner to the sample run.

A high yield, high resolution, high pressure liquid chromatography (HPLC) system has been used to give specificity coupled with radioimmunoassay (RIA) to give the sensitivity required. This system allows the simultaneous monitoring of a number of neuropeptides in one experiment involving samples taken before stimulation, during stimulation and post stimulation, the animal thus serving as its own control.

The model described here thus enabled

a. reproducible generation of analgesic states in a quantifiable manner in the conscious, unrestrained, and therefore non-stressed rat, and
b. the collection of suitable biochemical samples for the study of endogenous peptide released upon electrical stimulation
c. the study of peptide metabolism in the third ventricle, and
d. the microinjection of peptides into the third ventricle.

This thesis is the first report of such a model.

In summary, the model developed involves following:

1. A method to collect third ventricular perfusate in the

conscious unrestrained rat using push-pull cannula techniques;

2. a method to induce analgesia by electrical stimulation of the periaqueductal gray;
3. a method to quantify the analgesia produced;
4. a method of separating several neuropeptides present in the same perfusate sample using reverse phase, high pressure liquid chromatography procedures;
5. a method of detecting neuropeptides present in the perfusate samples using radioimmunoassay techniques.

Individual experiments utilizing the model developed will now be described.

CHAPTER 3

EXPERIMENTS TO VERIFY THE USE OF THE TAIL FLICK TEST

Having chosen the tail flick latency test as the possible basis for screening the production of analgesia in the rat, it was firstly important to demonstrate its viability under defined sets of experimental conditions.

In particular it was necessary to define the following:

1. Was it possible to test the rat using the tail flick procedure in a reproducible and reliable manner? That is, would there be any tendency for the tail flick baseline latencies to alter with time?
2. The effect of peripherally administered morphine on the tail flick latency.
3. The effect of periaqueductal gray (PAG) stimulation on the tail flick latency.

I. THE CHANGE IN BASELINE LATENCY WITH TIME.

METHODS

A group of rats (280-320 g.) were placed in perspex restrainers one hour prior to tail flick testing. Tail flick latencies (TFL) were then determined continuously for four hours (intertrial interval = two minutes). One test session was approximately five hours in duration.

RESULTS

The individual baseline latencies obtained are shown in figs. 10 to 13. Importantly, there was no tendency for the TFL to either increase or decrease with time. In fact the latencies were fairly constant around a baseline level of three seconds throughout the four hours of testing in different animals. Thus repetitive heating of the tail or even adapta-

Fig. 10 - THE CHANGE IN BASELINE LATENCY WITH TIME

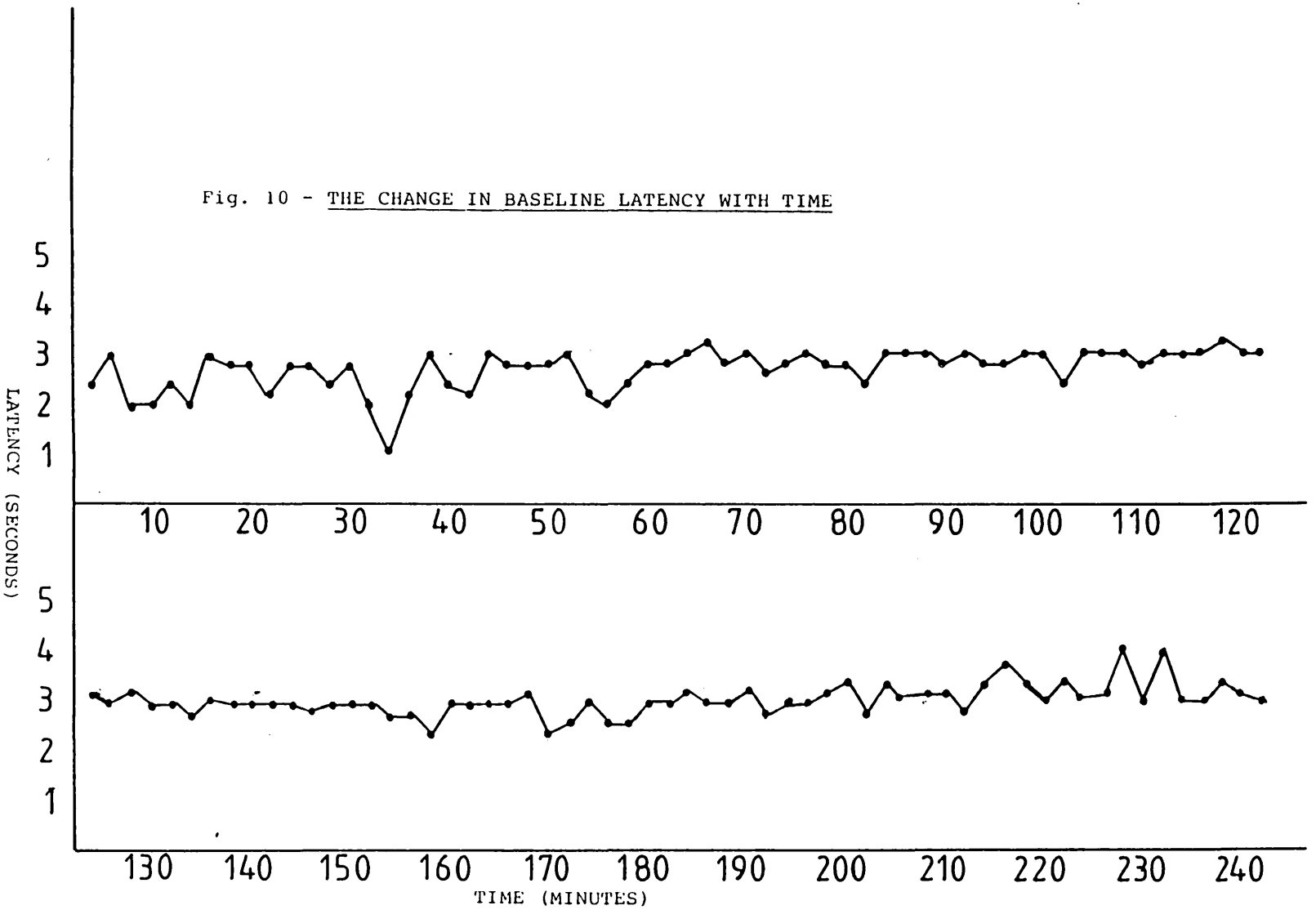


Fig. 11 - THE CHANGE IN BASELINE LATENCY WITH TIME

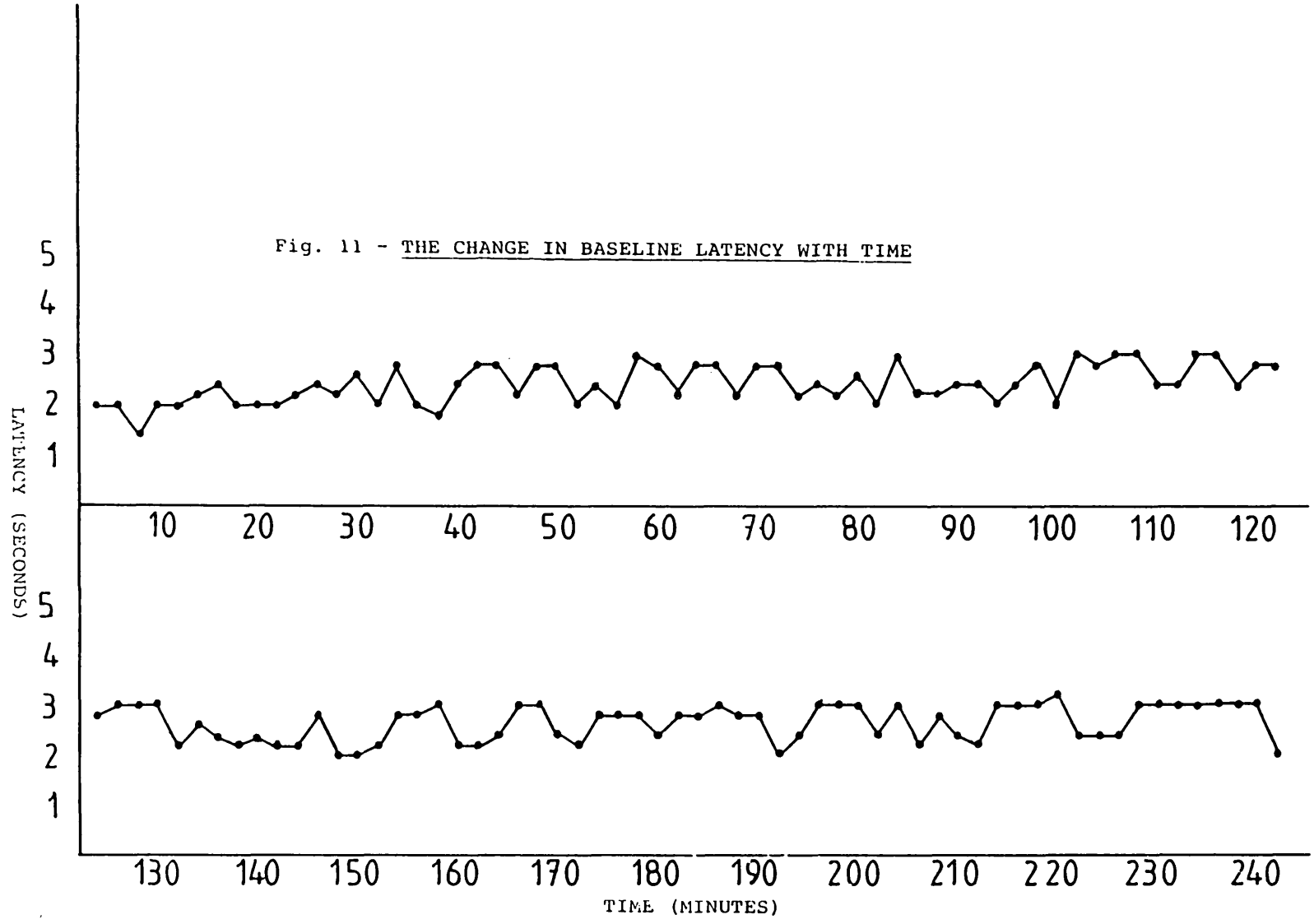


Fig. 12 - THE CHANGE IN BASELINE LATENCY WITH TIME

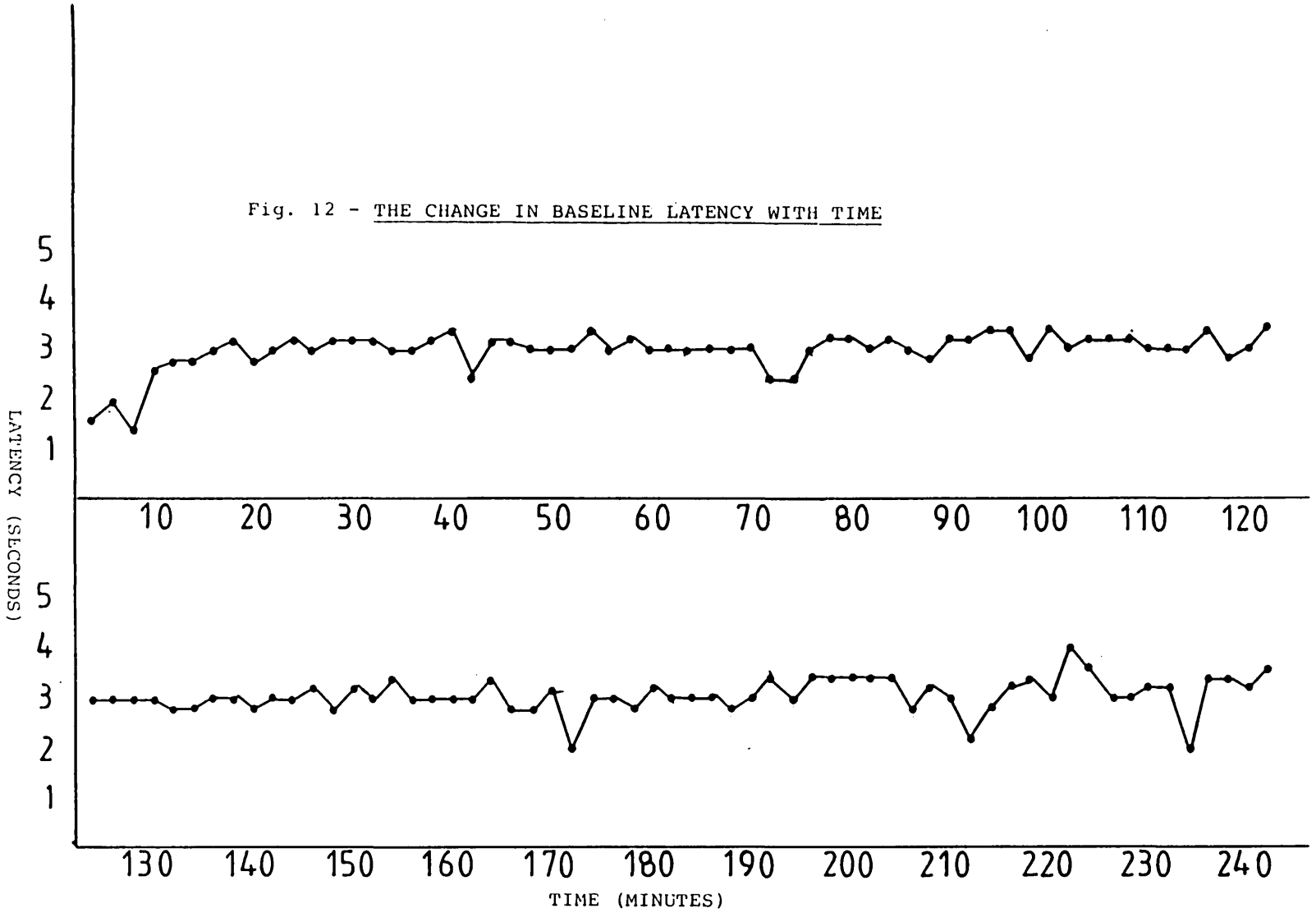
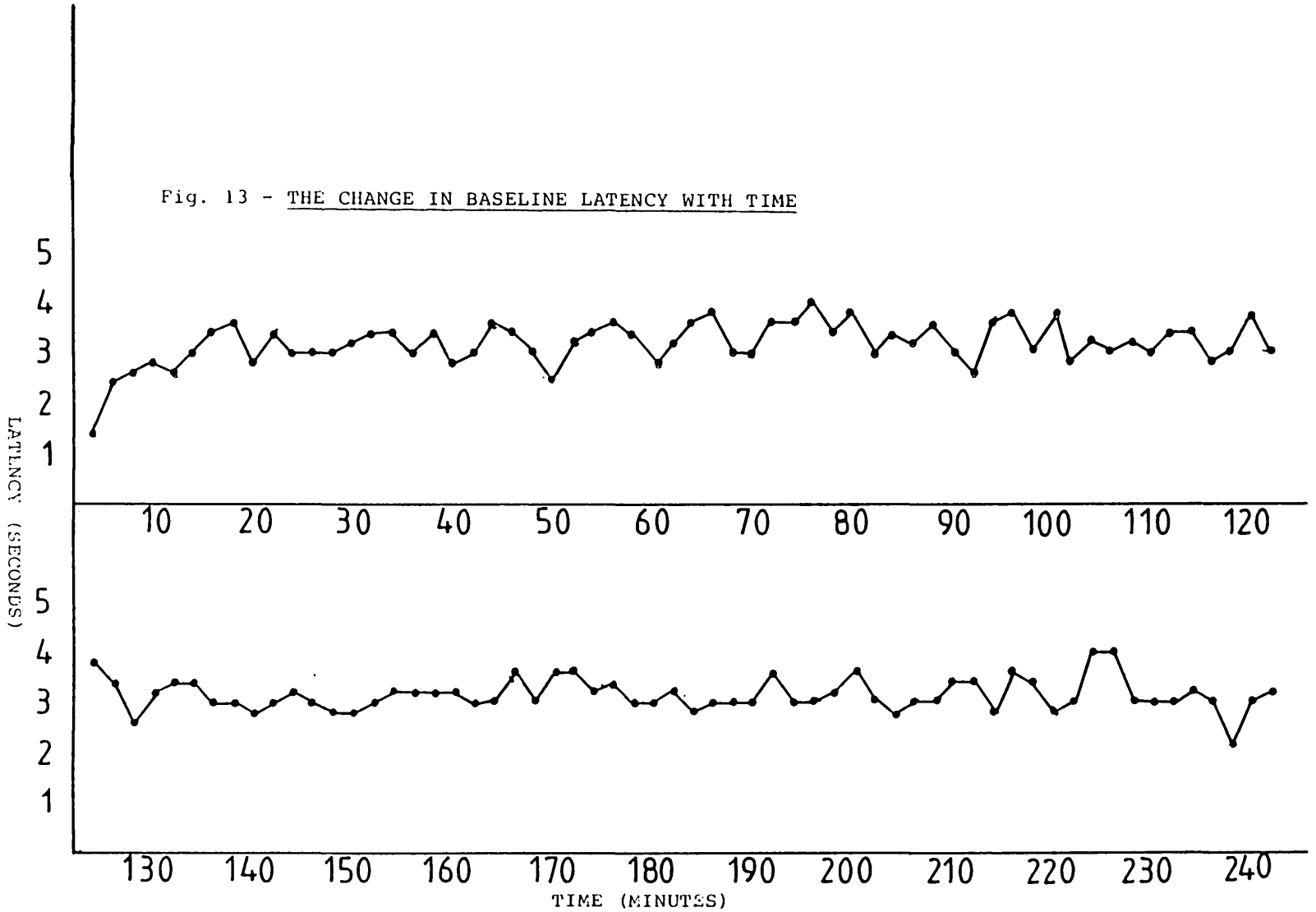


Fig. 13 - THE CHANGE IN BASELINE LATENCY WITH TIME



tion to the test situation, did not cause an alteration in the baseline level.

II. THE EFFECT OF MORPHINE ON THE TAIL FLICK LATENCY

METHODS

Experiments were carried out using male Sprague Dawley rats, weighing between 280-320 g. Groups of rats were placed in their restrainers one hour prior to TFL testing. Fifteen trials were then obtained (intertrial interval = 2 minutes) prior to morphine administration. Morphine sulphate (dissolved in saline) was injected intramuscularly (hind limb) in doses of 10 mg./kg. (n=4), 5 mg./kg. (n=3), 3 mg./kg. (n=4), 2 mg./kg. (n=4) and 0.5 mg./kg. (n=4). A control group of rats were similarly injected with saline (n=5). Each animal received one dose only. Tail flick testing was then continued for a further one and a half hours. Thus one test session, approximately three hours in duration (including a one hour adaptation period) was conducted for each animal.

RESULTS

Morphine was effective in increasing TFL (see figs. 14 and 15) in all but the lowest dose used. At a dose of 2 mg./kg. a small be reliable increase in TFL was produced. Higher doses resulted in successively greater increases in tail flick latency, the highest dose employed producing a 100% increase over basal control levels.

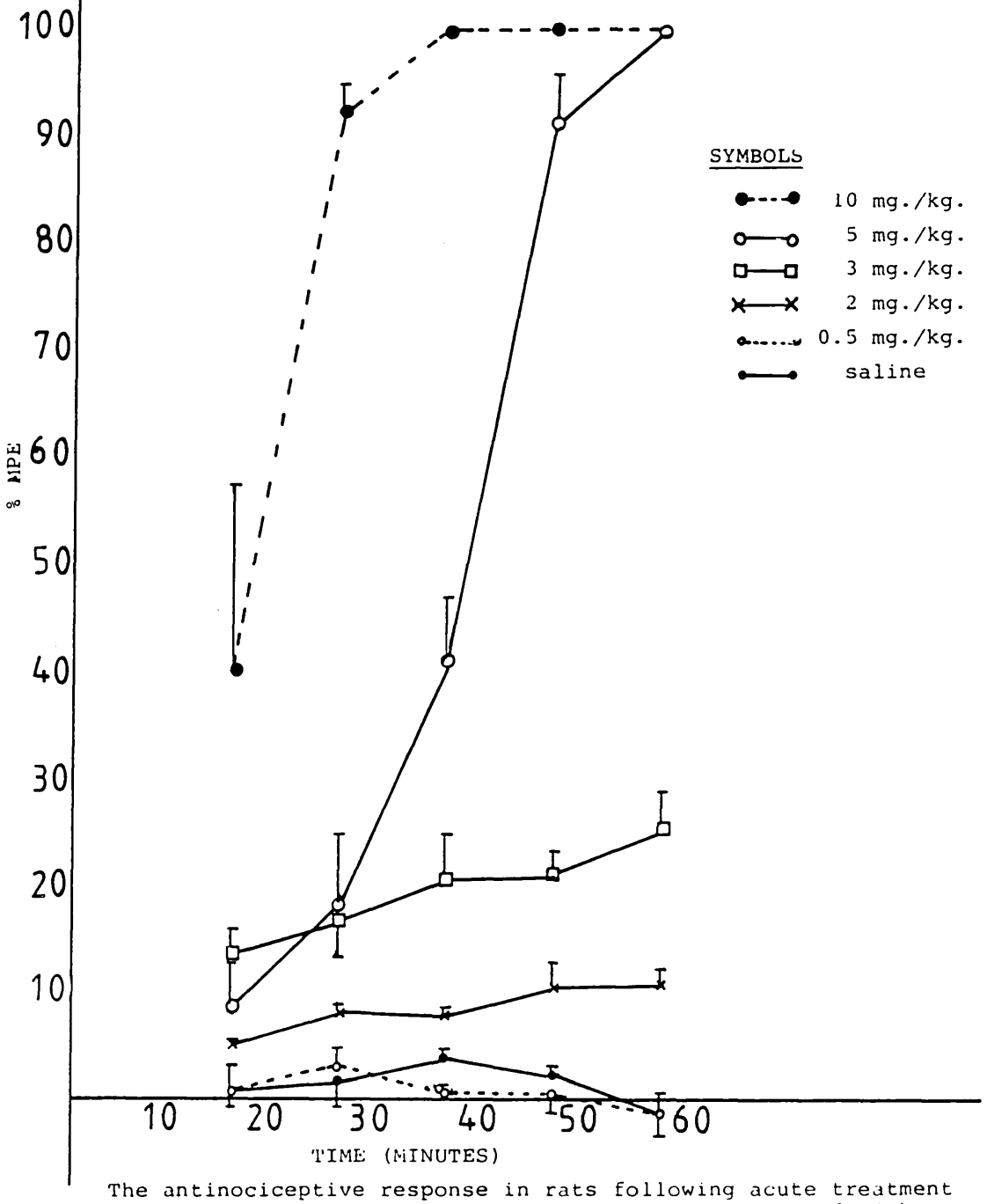
CALCULATION OF RESULTS

The results are expressed as the percentage of the maximum possible effect (% MPE). This was calculated as follows:

Baseline latencies

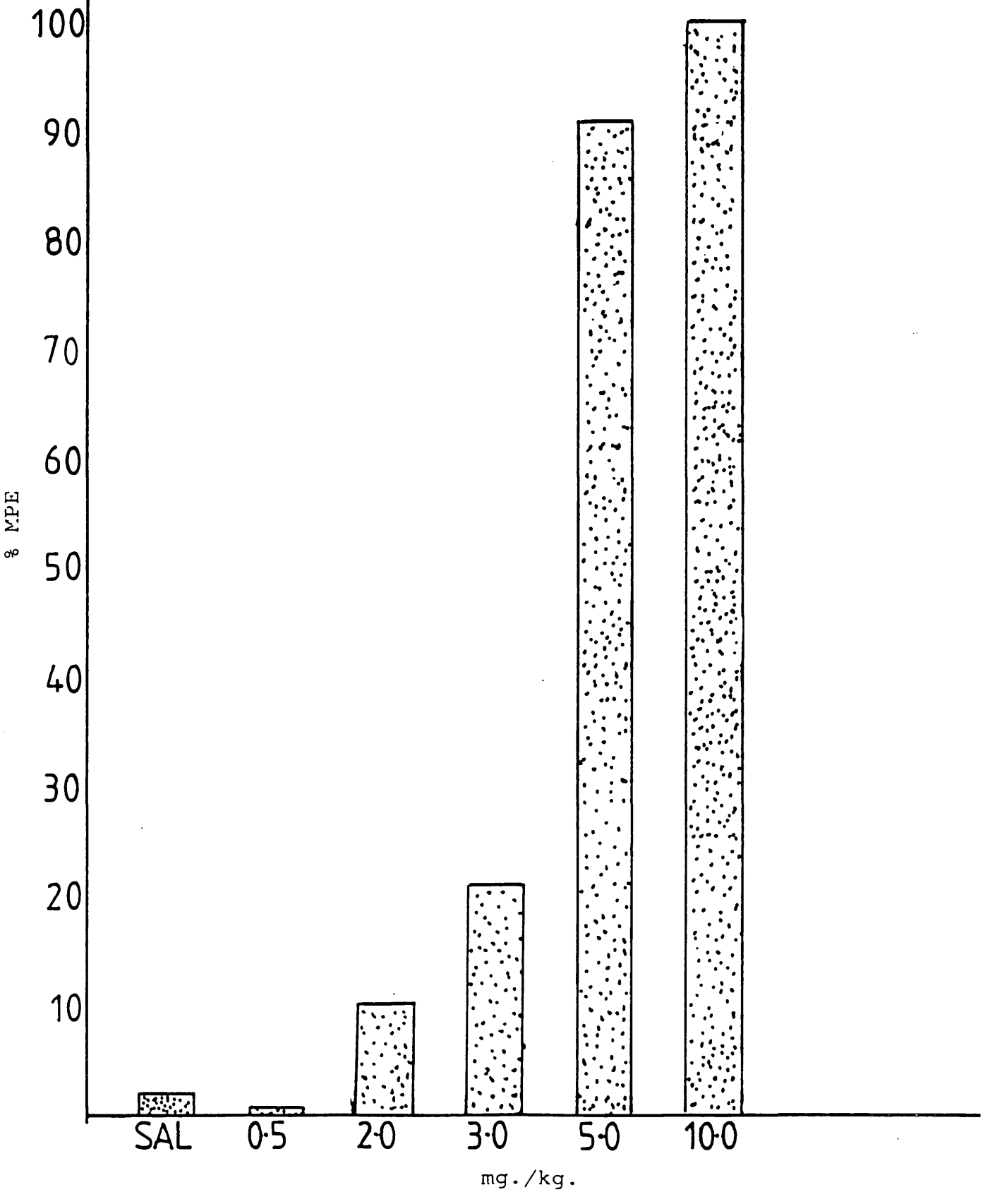
Fifteen trials were taken (intertrial interval = 2 minutes)

Fig. 14 - TIME EFFECT CURVES FOR MORPHINE



The antinociceptive response in rats following acute treatment with morphine or saline. Groups or rats were injected with a single dose of saline (control) or morphine (see text for details). Each point represents the mean (±SEM) response of 3-5 animals.

Fig. 15 - DOSE RESPONSE OF MORPHINE (40 - 50 MINUTES POST INJECTION). This plot is derived from the data presented in fig. 14.



and the response time of the last five trials were used to calculate the mean baseline latency (BL) in seconds.

Degree of Analgesia

Mean latencies were noted at the following times post injection:

10 - 20 minutes

20 - 30 minutes

30 - 40 minutes

40 - 50 minutes

50 - 60 minutes

The results were then expressed as the percentage of the maximum possible effect (MPE) using these mean values. The effect of morphine on the tail flick test was measured in terms of a change from baseline scores to the post injection scores. In addition, the fact that there was a cut-off point needed to be taken into account. Therefore, the actual change was compared to the maximum possible effect (% MPE) and computed according to the formula:

$$\% \text{ MPE} = \frac{\text{Latencies post injection - BL latency X 100}}{\text{cut-off latency - BL latency}}$$

where cut-off latency = 10 seconds

latency post injection = mean of the 5 trials immediately prior to injection.

III. THE EFFECT OF PERIAQUEDUCTAL GRAY STIMULATION ON THE TAIL FLICK LATENCY

METHODS

Rats were implanted with bipolar electrodes and allowed one week to recover from surgery. The rats were then placed in perspex restrainers one hour prior to tail flick testing. Intensities of 2, 4, 6, 8 and 10 v. (peak to peak) were used

Fig. 16 - THE EFFECT OF PAG STIMULATION ON TAIL FLICK LATENCY

Intertrial interval
= 2.0 minutes

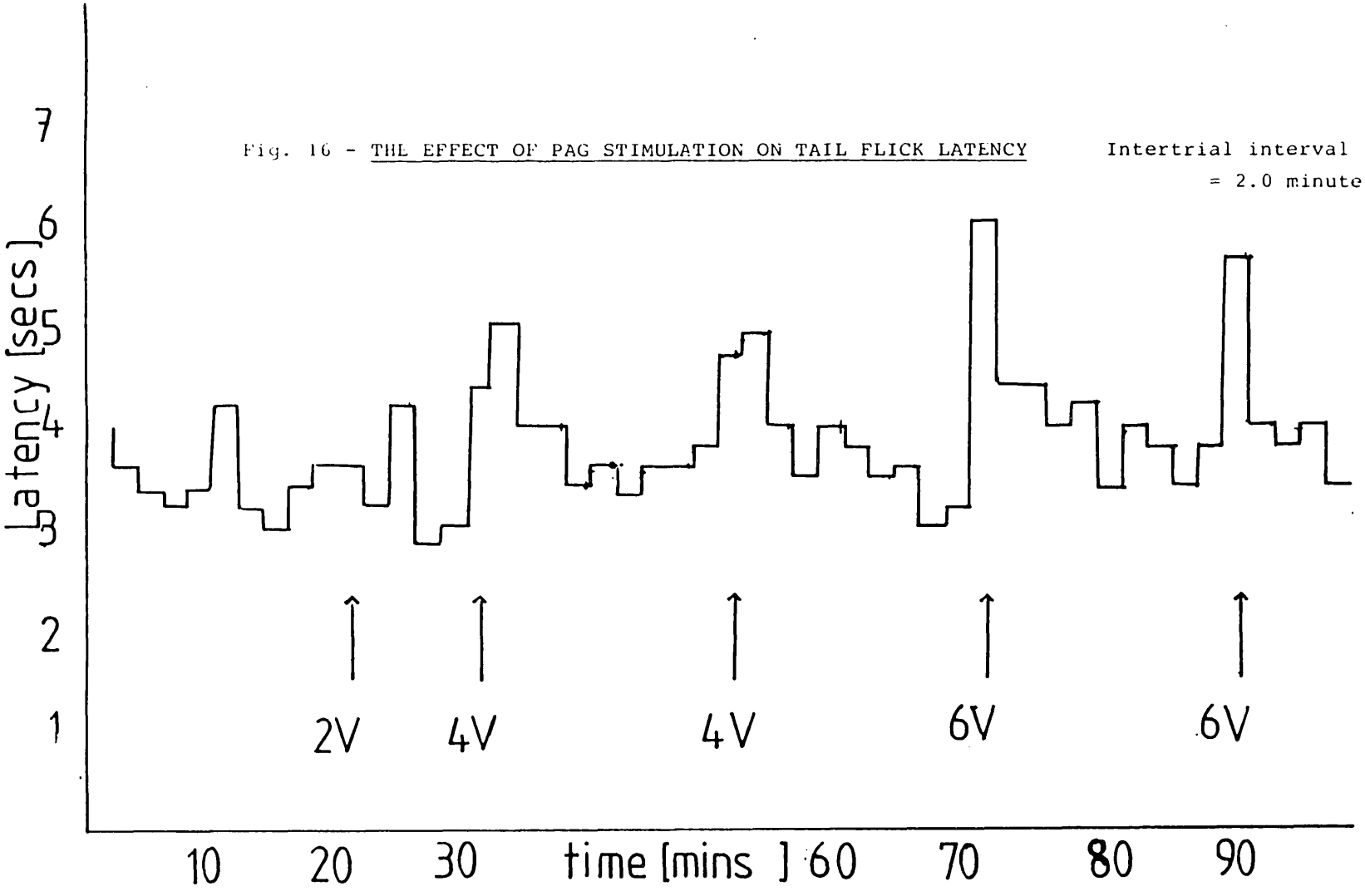
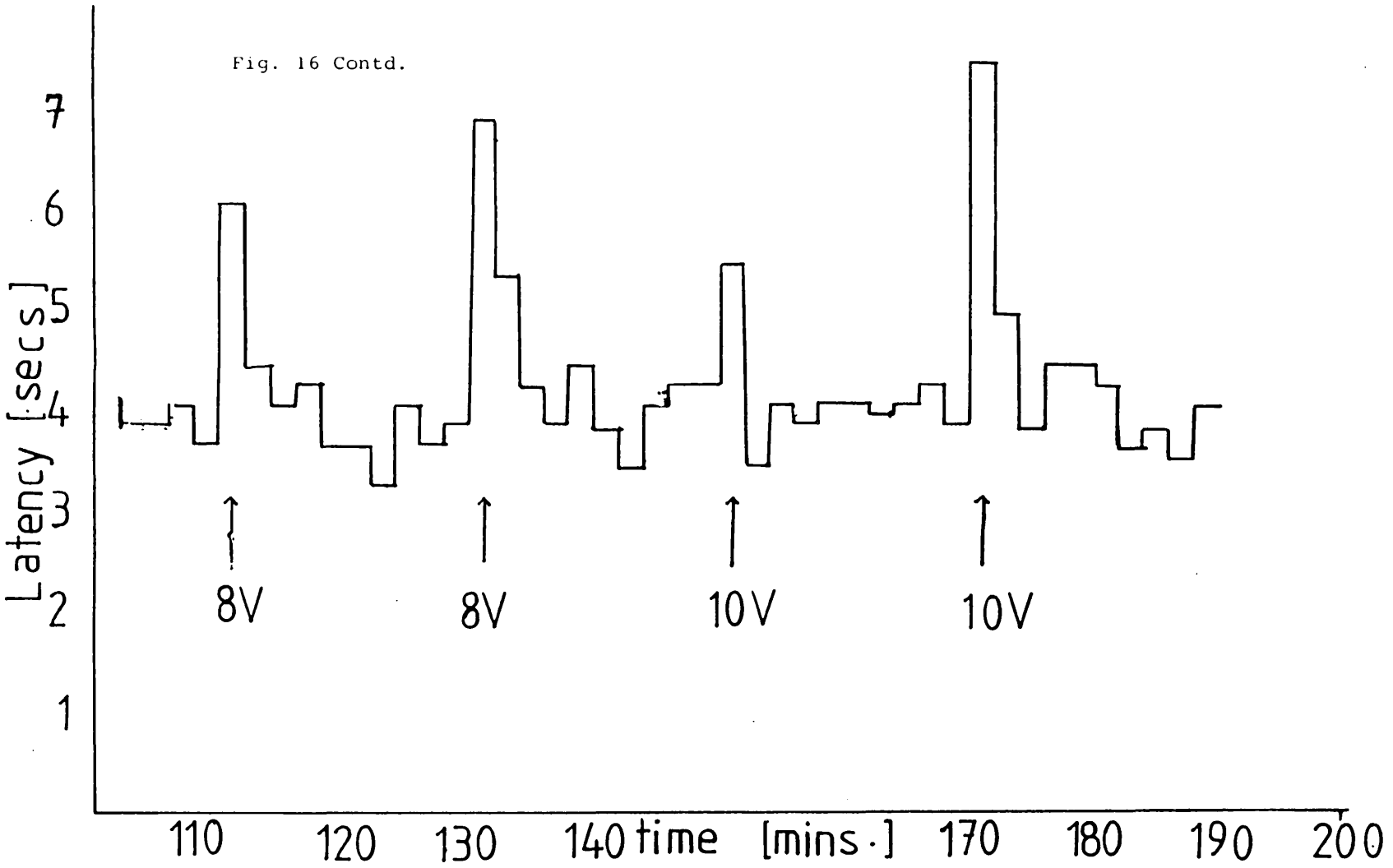


Fig. 16 Contd.


Latency [secs]
1
2
3
4
5
6
7
8V
8V
10V
10V
110
120
130
140
time [mins.]
170
180
190
200

to stimulate the PAG. The details of stimulation parameters etc. are given in the general methods section.

RESULTS

Fig. 16 shows the increase in latency observed after PAG stimulation at the different intensities for a representative rat. A stimulus-dependent increase in latency can be observed. Repeated stimulation at the same intensity did not consistently produce the same degree of analgesia. The reasons for this occurring may be due to the fact that the design of this experiment did not allow adequate time between stimulation periods. Thus it could be possible that a depletion effect could have occurred. That is, the endogenous stores of the neurochemicals mediating the response may have been exhausted from the first stimulation period. For this reason, future experimental design for screening rats utilised three stimulation intensities only, with each stimulation period being separated by at least thirty minutes.

DISCUSSION

The present experiments show that one can repeatedly test the rat using the tail flick procedure for a period of up to four hours. No generalised change in baseline latency was observed. The latency remained relatively constant (at about three seconds) throughout the testing period. Also the rat remained relatively comfortable in the restrainer, despite the long periods involved. Morphine produced a dose-dependent increase in TFL at the doses used, except with 0.5 mg./kg. of morphine. Reference to figs. 14 and 15 allows comparison of morphine's analgesic action with that produced by brain stimulation (fig. 16).

Brain stimulation also caused a stimulus-dependent increase in the TFL which was fast in onset but short in duration (not greater than six minutes). Thus peripheral administration of morphine and central stimulation of the brain both inhibit the spinally-mediated tail flick reflex.

Since future experiments were to be performed with the assumption that tail flick latencies do not change with time, it was important to demonstrate just how long one could repeatedly test the rat using this procedure. Also, the results on the effect of morphine confirm other reports of similar findings, and therefore validate the use of this procedure for the experiments conducted during this research.

The tail flick test is the most widely used behavioural screen for the narcotic analgesics. This has been justified largely on the basis of the relatively good correlation between behavioural results in animal testing and clinical potency in humans. Since the tail flick reaction is a spinal reflex and considering that morphine shows no clear effect upon peripheral nerve activity, two hypotheses have been proposed to explain the effect of morphine upon the spinal transmission of nociceptive messages: a direct spinal depressive effect and an indirect effect via supraspinal structures which modulate the activity of descending systems controlling spinal activity. Levine et al. (1982) have shown that the maximally effective subcutaneous dose of morphine was 10 mg./kg; this is in agreement also with the current findings on the effect of morphine on the tail flick latency (see fig. 15). In addition, Levine has shown that morphine (15 µg.) injected supraspinally (fourth ventricle) was consistently more effective than spinal injection (subarachnoid space at the lumbar level). However,

neither produced analgesia so potent as that following maximal systemic morphine administration (10 mg./kg.). In contrast, it was demonstrated that injection of morphine at both sites at once was as effective as systemic administration. These findings suggest that systemic opioid agonists produce analgesia by an action at both spinal and supraspinal sites. In addition, the analgesic effect of supraspinal morphine was completely prevented by pretreatment with spinal naloxone.

This finding that spinal naloxone completely antagonises supraspinal morphine-induced analgesia indicates that supraspinally-administered morphine activates a descending system which has an opioid link at the level of the spinal cord.

Thus the results from this experiment have established that:

1. the tail flick test can be used to monitor a change in pain sensitivity over several hours;
2. morphine, an efficacious analgesic, produces dose-dependent changes in latency of the tail flick reflex, confirming this well established phenomenon;
3. central PAG stimulation also alters the level of the tail flick latency.

Thus both administration of morphine (peripheral) and electrical stimulation of the PAG (central) cause inhibition of the spinally mediated tail flick reflex.

Since opioids are implicated in analgesia, it was of interest now to demonstrate the effect of a narcotic antagonist, naloxone, on the response produced by PAG stimulation. It was envisaged that the findings from such a study would contribute to a better understanding of later experiments, where the role of peptide(s) in analgesia was investigated.

CHAPTER 4

THE EFFECT OF NALOXONE ON STIMULATION INDUCED ANALGESIA

METHODS

Rats were implanted with bipolar electrodes into the periaqueductal gray (PAG). Details of the implantation procedure are as in the general methods section. After a one week recovery period, rats were screened to determine the appropriate parameters of brain stimulation. Rats were put in restrainers one hour prior to tail flick testing, then fifteen control trials were obtained. To determine the voltage intensity producing maximal analgesia, three stimulation intensities were used, 4v., 8v., and 16v. Fifteen trials followed each stimulation period. At the end of this screening procedure, five trials were obtained prior to injection of naloxone or saline. Naloxone (dissolved in saline) was then administered i.m. either 10 mg./kg. or 1.0 mg./kg. Tail flick testing was continued for ten minutes post naloxone, then the brain was stimulated at the predetermined intensity. Further trials were then conducted for one hour post naloxone, and the brain stimulated once again at the same intensity used previously.

CALCULATION OF RESULTS

The degree of analgesia produced by electrical brain stimulation was expressed as a percentage and derived from the ratio of actual change in response time (T) from baseline (BL) to the maximum possible change according to the formula:

$$\% \text{ MPE} = \frac{T - BL}{\text{cut-off latency} - BL} \times 100$$

where baseline value was taken as the mean latency of the first

Fig. 17 - STIMULUS-EFFECT PLOT FOR PAG STIMULATION

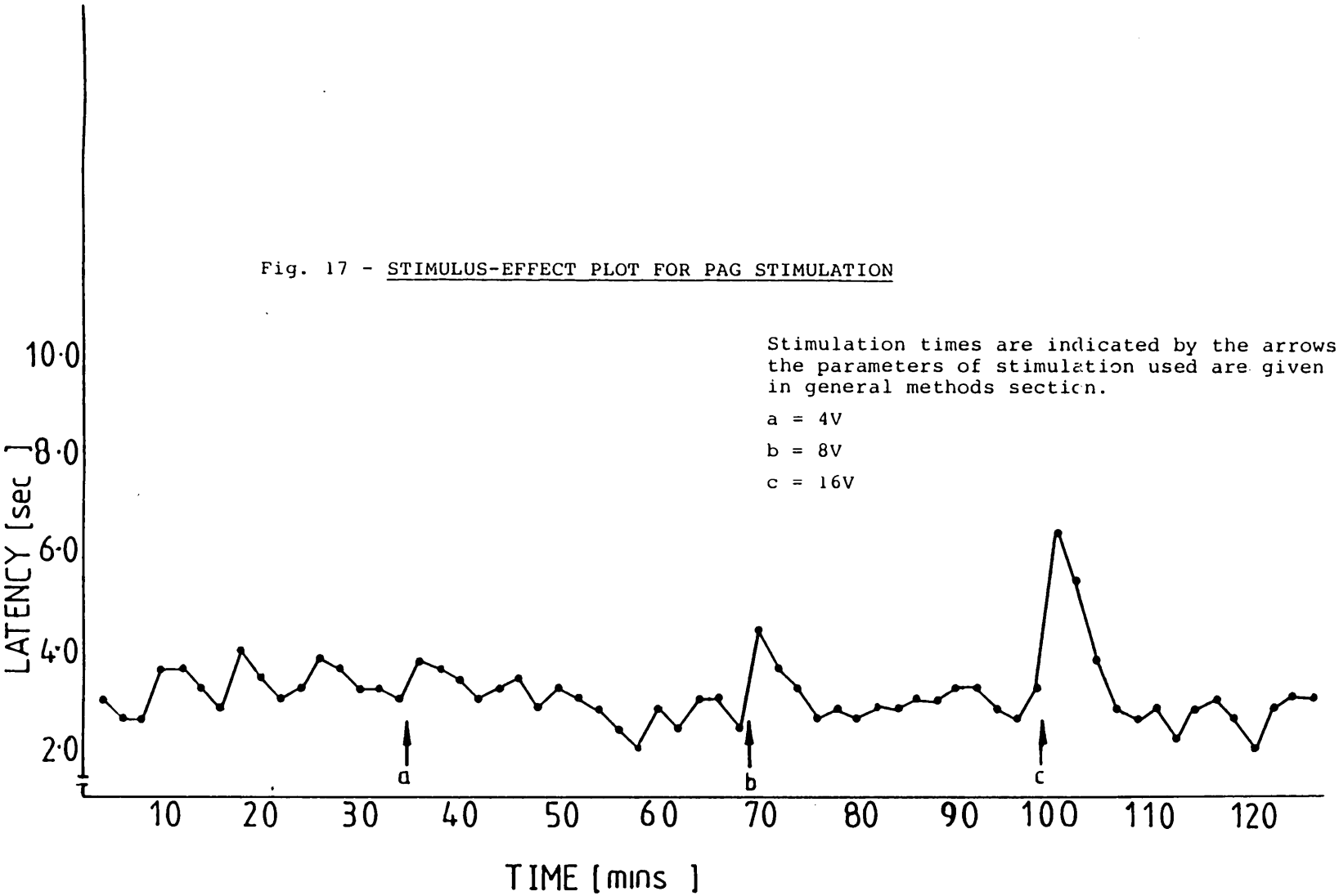


Fig. 18 - STIMULUS-EFFECT PLOT FOR PAG STIMULATION

a = 4v
b = 8v
c = 16v

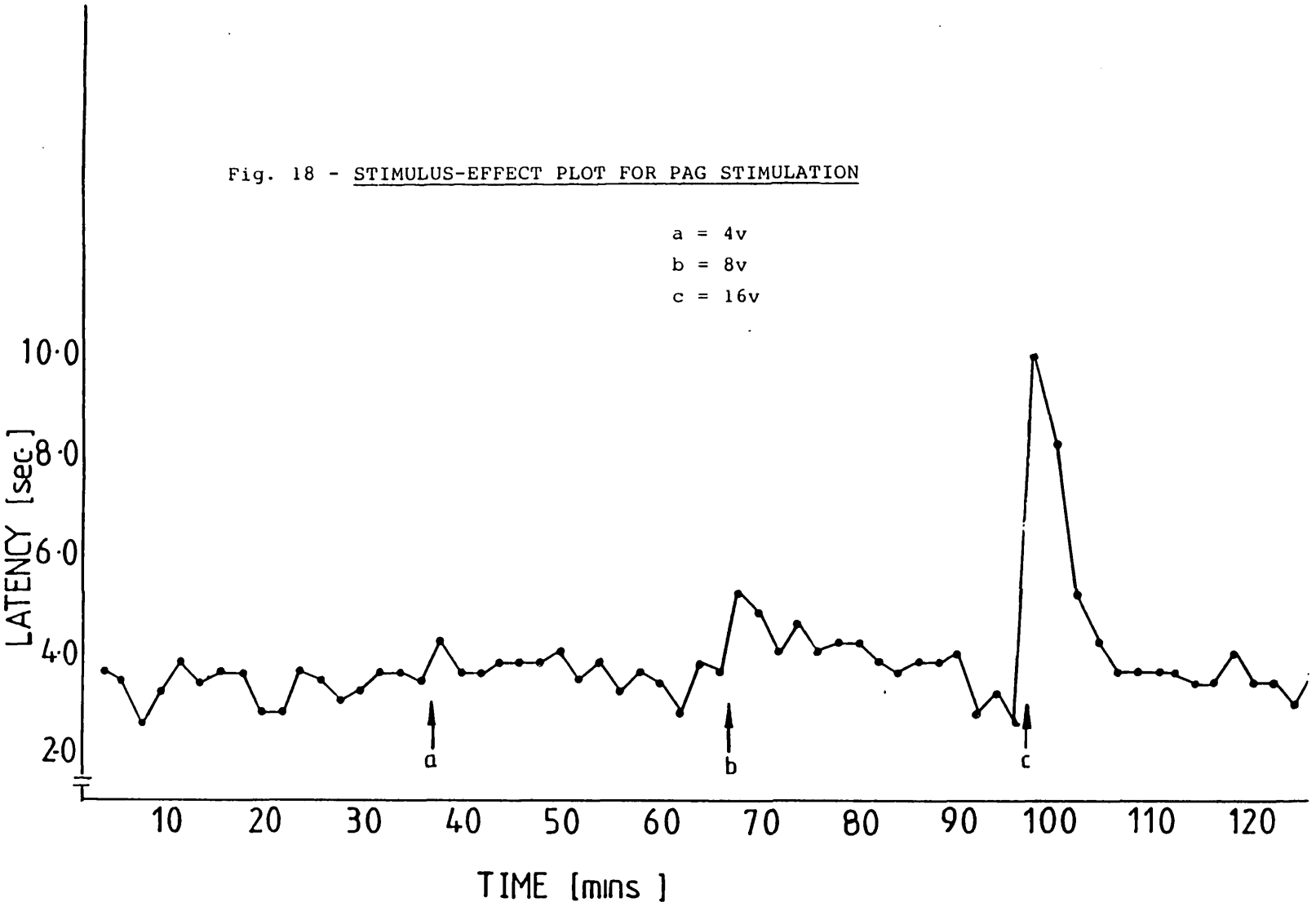
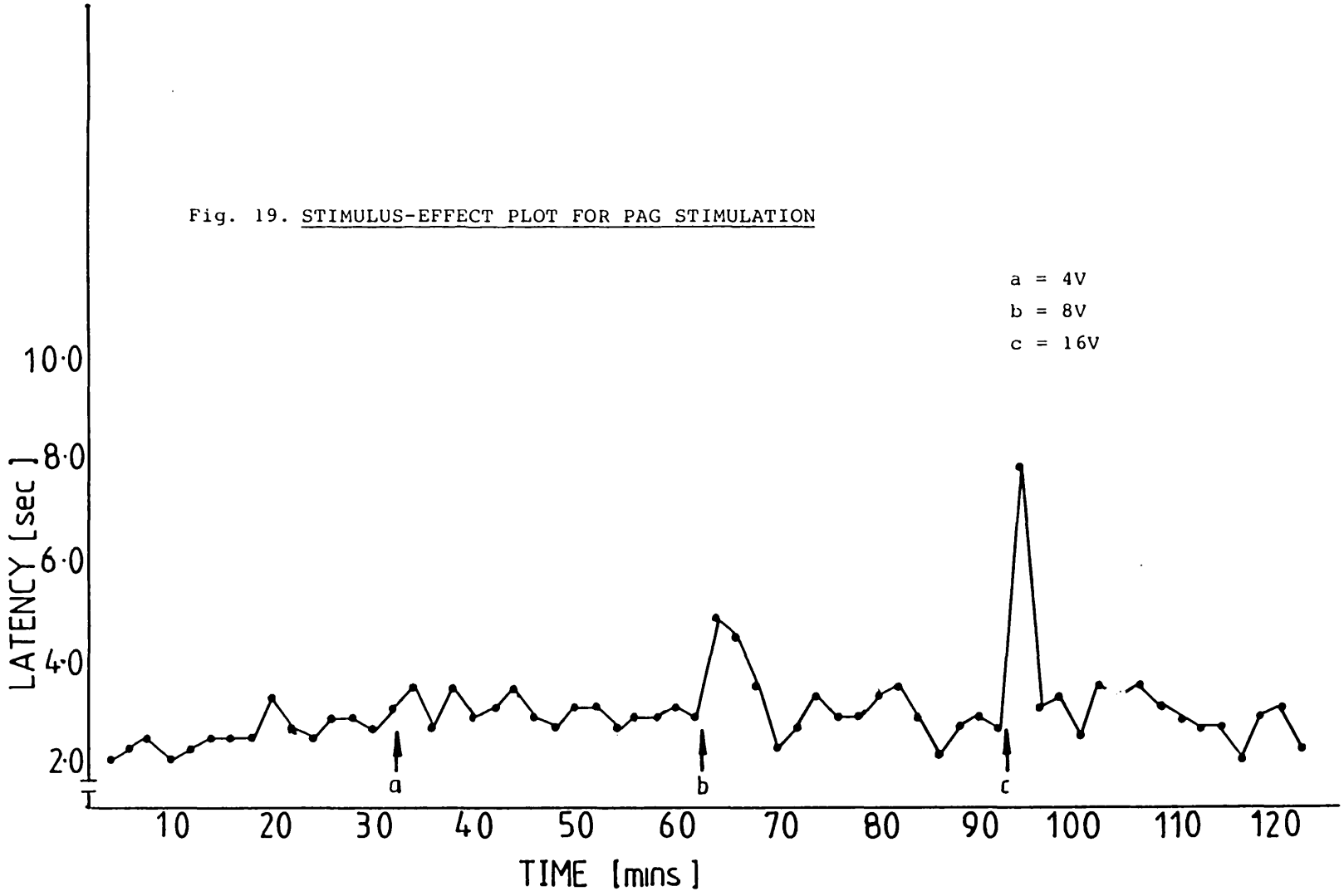


Fig. 19. STIMULUS-EFFECT PLOT FOR PAG STIMULATION



five trials immediately prior to brain stimulation.
T = latency obtained immediately post stimulation
cut-off latency = 10 seconds

RESULTS

Focal brain stimulation of the periaqueductal gray with intensities sufficient to produce reliable analgesia was often accompanied by pronounced tail rigidity and tail rotation. Stimulation of the PAG produced analgesia; the degree of analgesia produced varied according to the stimulus intensity used. Figs. 17, 18 and 19 show the raw tail flick latencies obtained for three rats at the intensities shown. In some rats the stimulus intensity used yielded a greater than 70% degree of analgesia.

Naloxone at 1.0 mg./kg. of body weight consistently reduced the degree of analgesia (fig. 20). Interestingly, naloxone at 10 mg./kg. of body weight produced both an antagonism of stimulation-induced analgesia (SIA) (fig. 21) and paradoxically, in some rats, produced a potentiation in the degree of analgesia obtained (fig. 22). As shown in fig. 23, the drug vehicle alone (saline) did not significantly alter the degree of analgesia.

DISCUSSION

The results show that administration of naloxone can alter the degree of analgesia produced by midbrain stimulation. The results also confirm that stimulation of the periaqueductal gray produces analgesia in a stimulus-dependent manner. The dose of naloxone employed appears to be critical, the lower dose (1.0 mg./kg.) consistently producing antagonism of SIA and the higher dose (10 mg./kg.) paradoxically producing both

Fig. 20 - ANTAGONISM OF STIMULATION INDUCED ANALGESIA BY NALOXONE - 1.0 mg./kg.

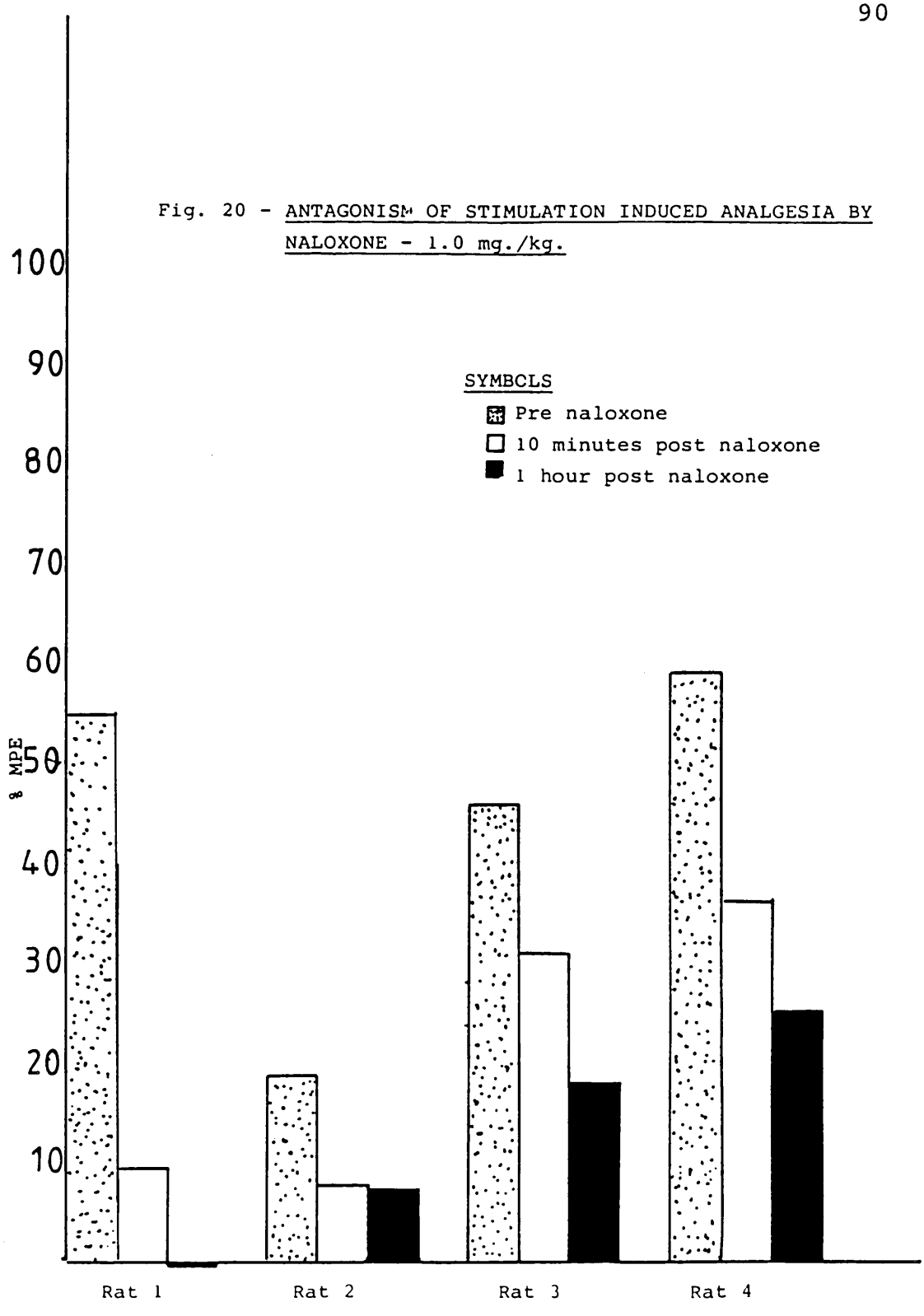


Fig. 21 - ANTAGONISM OF STIMULATION-INDUCED ANALGESIA BY NALOXONE - 10 mg./kg.

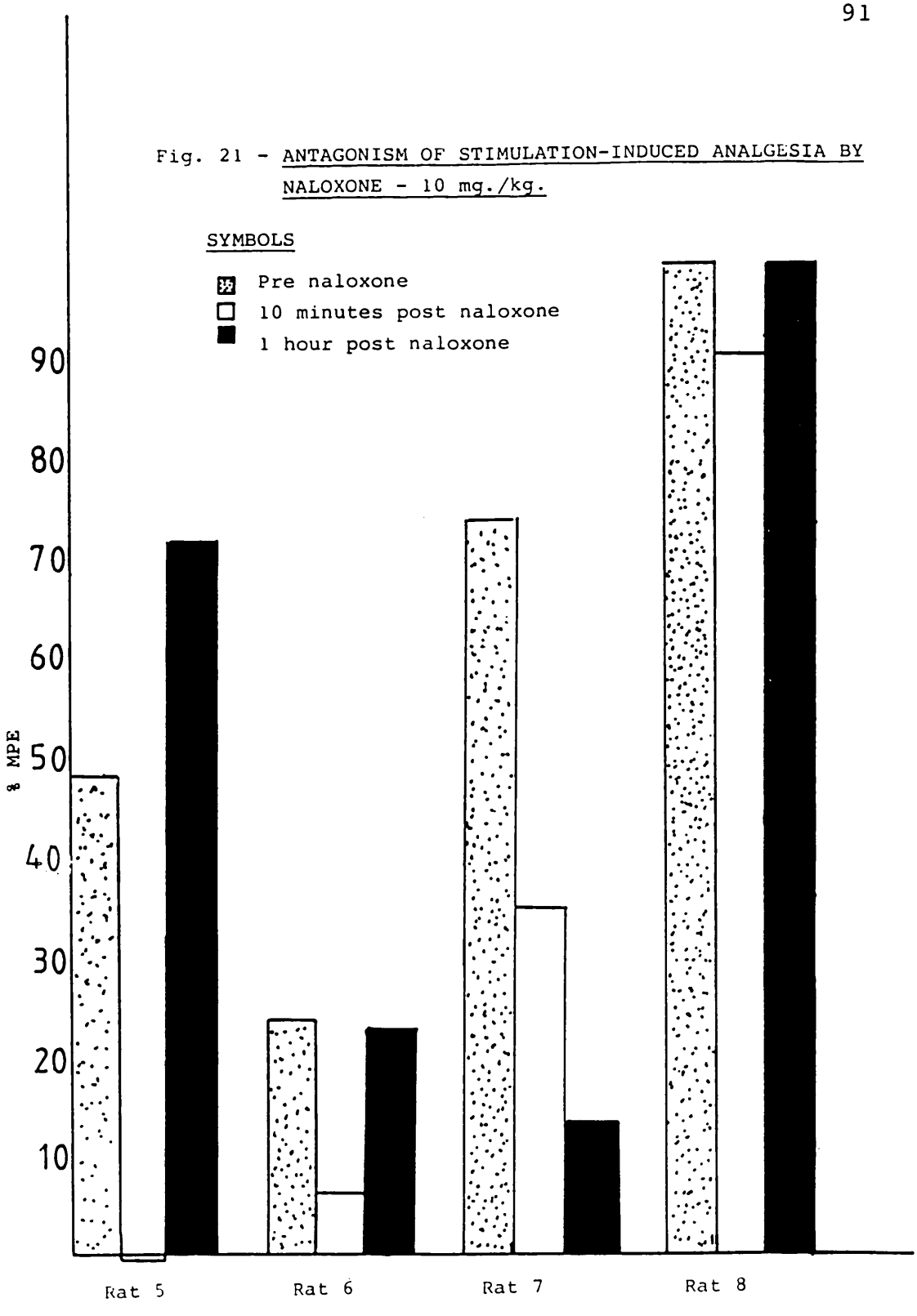


Fig. 22 - POTENTIATION OF STIMULATION-INDUCED ANALGESIA BY NALOXONE - 10 mg./kg.

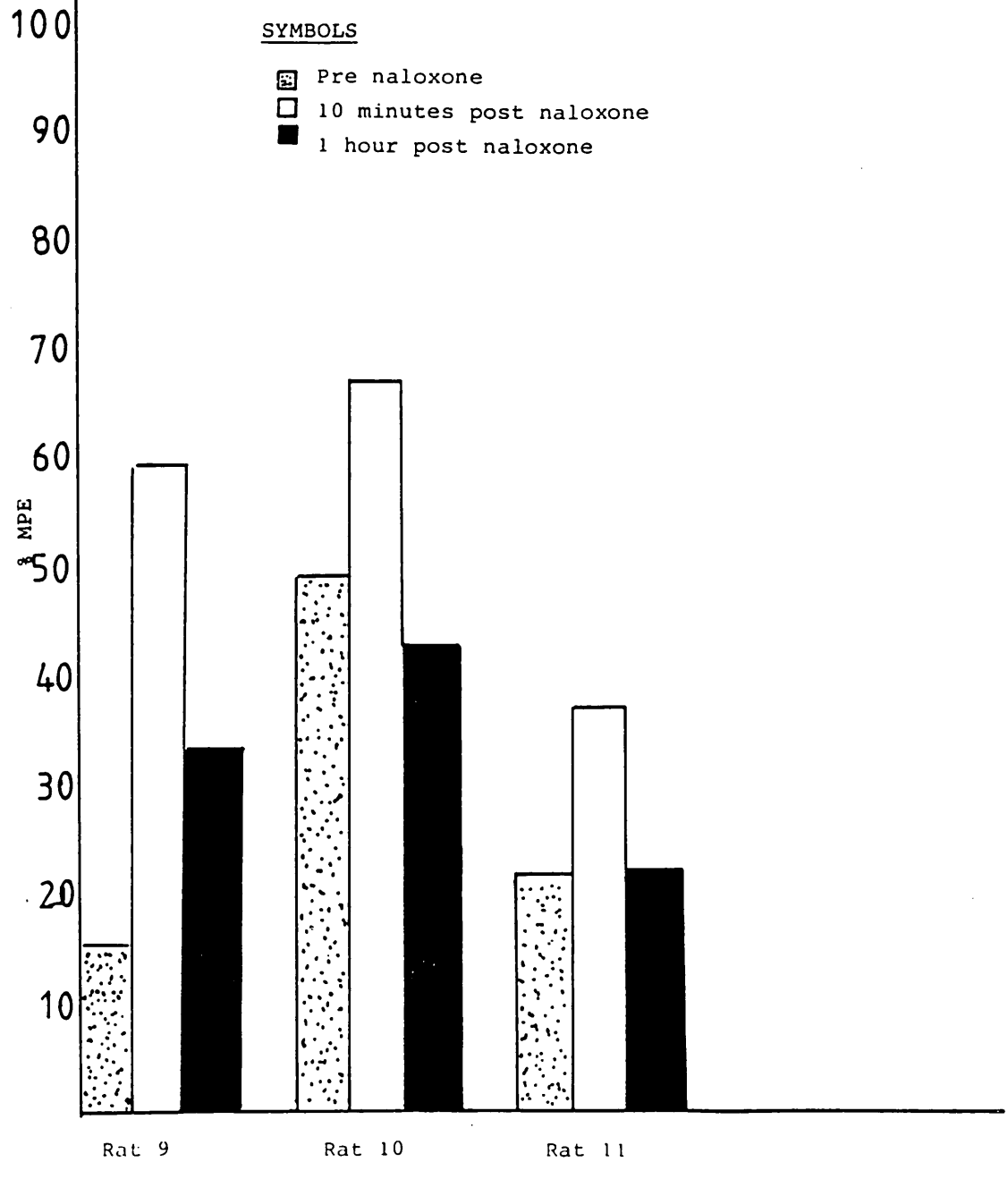
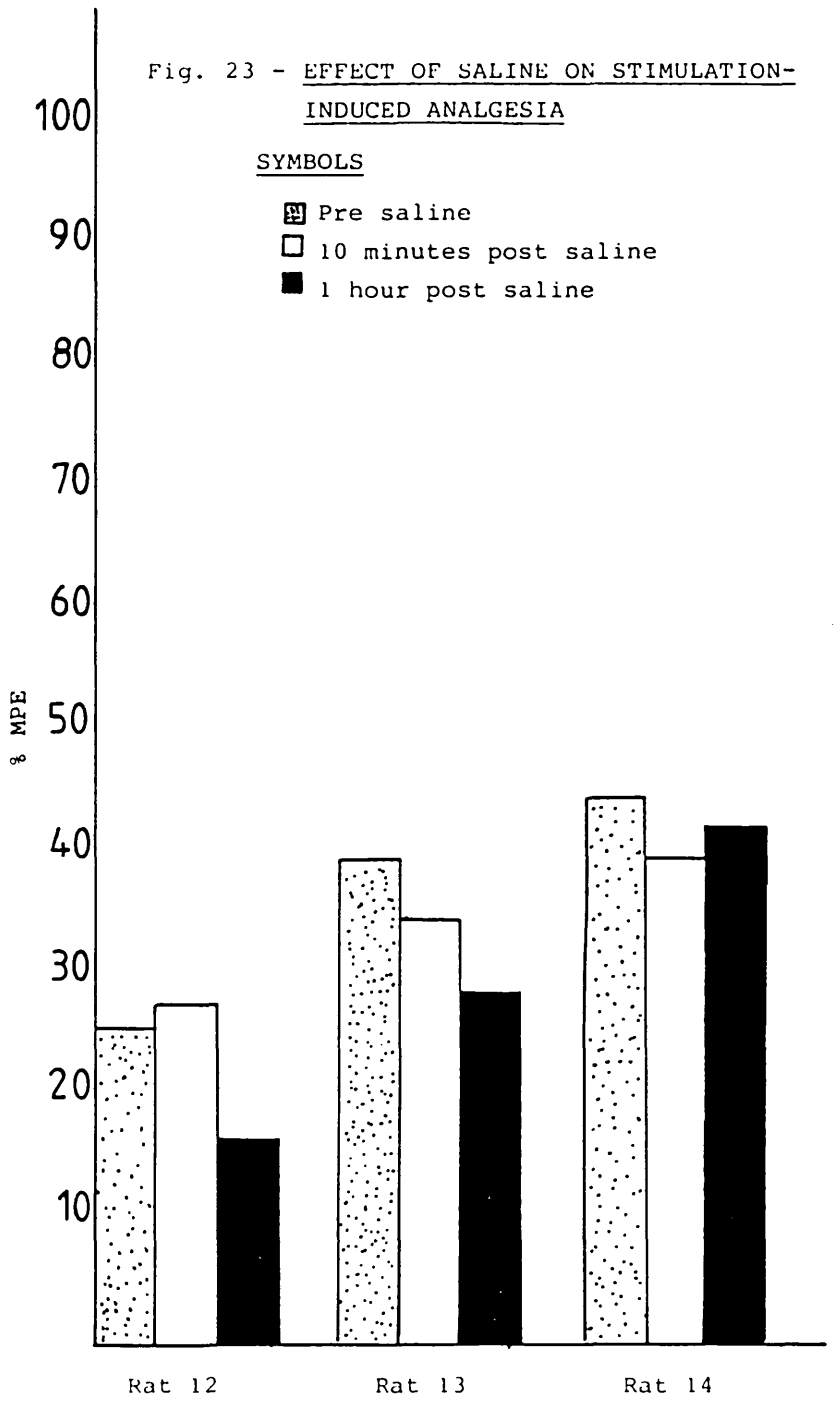


Fig. 23 - EFFECT OF SALINE ON STIMULATION-INDUCED ANALGESIA



antagonism and poteniation of SIA in different rats. In some cases the antagonism produced persisted even up to one hour after the administration of naloxone.

Previous studies concerning the effect of naloxone on SIA have yielded inconclusive findings. In view of the multiple indications that opioids are involved in mediating analgesic effects from the PAG, it is curious that reports on naloxone antagonism have shown that antagonism of analgesia induced from PAG stimulation in the rat is often weak (Akil et al. 1976), and has not been observed at all by some investigators (Pert and Walter 1976, Yaksh et al. 1976, Dennis et al. 1980). One of the factors contributing to the disparate findings between studies attempting to antagonise SIA with naloxone may be the use of different methodologies. Yaksh et al. (1976) who failed to find any antagonism with naloxone, assessed analgesia during stimulation. In the study by Pert and Walter (1976) and that by Akil et al. (1976), analgesia was assessed after stimulation. Also Liebeskind et al. (1982) have suggested that variations in electrode placement could contribute to inconsistent reports pertaining to naloxone antagonism of SIA from the caudal PAG.

Our finding of both antagonism and poteniation by the same (high) dose of naloxone in different rats indicates caution should be taken in interpreting data on naloxone antagonism. This dual effect could perhaps be explained since it has been demonstrated that several different types of opiate receptor exist, and that these receptors possess varying affinities for naloxone (Kosterlitz et al. 1977, Paterson et al. 1983, and Wood 1983). Thus it is possible that high doses of naloxone could also bind to other opioid receptors mediating

an agonistic effect. In fact, Liebeskind et al. (1982) have reported that doses of naloxone as low as 5 µg., administered systemically, significantly elevated stimulation-induced analgesia thresholds, which suggests that receptors with a high naloxone affinity are involved in stimulation-induced analgesia. In the present work it is important to note that consistent data were obtained for antagonism of SIA in all experiments at the lower dose of 1 mg./kg.

Interestingly, in comparison with our own findings, micro-injections of glutamate (a substance known to cause neural activation) into the PAG produce analgesia in the conscious rat (Urca et al. 1980). This effect of glutamate could be blocked by pretreatment with a low dose of naloxone (1.0 mg./kg.) However, in contrast a high dose of naloxone (10 mg./kg.) administered five minutes prior to glutamate caused a potentiation of the analgesic effect.

The anatomical specificity of the PAG in subserving glutamate-induced analgesia, stimulation-induced analgesia and morphine-induced analgesia, and the ability of naloxone at appropriate doses to block the analgesia produced by all three agents, suggests that their analgesic action is mediated by a system using opiate-like substances as neurotransmitters or neuromodulators. The paradoxical results obtained with naloxone on the analgesia produced by glutamate closely parallels our findings on the ability of naloxone to potentiate SIA at the higher dose used, and to consistently block SIA at the lower dose. Similar effects have been revealed in other studies investigating naloxone's ability to block SIA. Akil et al. (1976) found that low doses of naloxone (1 mg./kg.) can partially block SIA in the rat. In contrast, Yaksh et al. (1976) reported

that naloxone (20 mg./kg.) was ineffective in reversing this phenomenon. Similarly, Pert and Walter (1976) concluded that SIA was not mediated by opioids because a high dose of naloxone (10 mg./kg.) did not block SIA. These authors support their conclusion, however, by reporting that in many cases lower doses of naloxone were effective in blocking SIA, and that this lack of a dose response relation mitigates against an opioid basis alone in SIA. The similarity between the paradoxical naloxone results in this study and in studies investigating naloxone action on glutamate-induced analgesia, suggest a common mode of action for glutamate-induced analgesia and SIA. Moreover, these data raise serious doubts as to whether a lack of a dose-related naloxone blockade of a given phenomenon should be the basis for rejection of its opioid nature. This question is accentuated further by studies investigating the effects of naloxone in the otherwise untreated animal. Hyperalgesia induced by this drug can be obtained with relatively low doses, whereas high doses are often ineffective (Frederickson et al. 1977, Jacob and Ramabadrian 1978, Carmody et al. 1979, Woolf 1980). These opposing effects on the administration of naloxone alone at different doses have also been reported in human studies (Levine et al. 1979).

Thus, in summary, the demonstration that electrical stimulation of the mesencephalic periaqueductal gray can produce powerful analgesia effects (Reynolds et al. 1969, Mayer et al. 1971), has suggested the existence of an endogenous supraspinal pain-inhibitory system (Mayer et al. 1971). Naloxone has been regarded as a relatively pure narcotic antagonist, and as such has been widely used to investigate the possible involvement of endogenous opiates in a variety of physiological

and pharmacological processes. Yet despite the involvement of the PAG in both SIA and morphine-induced analgesia, the latter has been reported to be consistently, completely and dose-dependently antagonised by naloxone (Pert and Walter 1976), but the antagonism produced by the former has not been clearly defined (Yaksh et al. 1976). The often incomplete reversal of SIA by naloxone (Akil et al. 1976) has led to the suggestion that PAG stimulation also activates non-opioid analgesia systems (Akil et al. 1976).

Antagonism of analgesia with naloxone seems to be only a necessary and not a sufficient condition to demonstrate that such manipulations are related to opiate receptor mechanisms. In order to implicate an endogenous opiate in a process, evidence in addition to blockade by naloxone is required. Such evidence includes the demonstration of

1. cross tolerance with morphine;
2. similar responses with other opiate antagonists;
3. the lack of an effect with non-antagonist isomers;
4. the potentiation of a response with agents which inhibit breakdown of endogenous opioid peptides; and
5. a direct release of endogenous opioids.

The inconsistent findings observed using the higher dose of naloxone in our study illustrates the complexity of the systems involved in SIA. However, in any event, a lack of antagonism by naloxone should not be used to reject the involvement of endogenous opiate in SIA. These findings may be justified because there are multiple opiate receptors in nervous tissue (Kosterlitz and Paterson 1980, Iwamoto and Martin 1981, Paterson et al. 1983, Wood 1983). Naloxone may be interacting with the endogenous opiate systems in a manner

which has yet to be elucidated. In addition, naloxone is a competitive inhibitor of opiate responses, and the inhibitory concentration depends upon the level of agonist in the receptor environment. In spite of our findings at the high dose, the consistent antagonism of SIA by the lower dose of naloxone leads us to assume that endogenous opioids are involved in analgesia resulting from stimulation of the PAG.

A chapter on naloxone's action would be incomplete without mention of its immense clinical importance. Naloxone's primary clinical application is in reversing the reduced respiration caused by natural and synthetic opiate-like substances, i.e. naloxone can counteract a heroin overdose as well as reverse the effects of narcotics used in surgical anaesthesia. Naloxone can also reverse the effects on a newborn child of narcotics given to the mother during labour. Furthermore, naloxone is used in diagnosing comas of unknown origin to determine if narcotic overdose is involved. Thus, despite the caution that needs to be taken in studies on naloxone antagonism, naloxone has proved to be an essential tool in the discovery and investigation of endogenous opioids.

CHAPTER 5

EXPERIMENT TO DETERMINE IF STIMULATION OF THE PERIAQUEDUCTAL GRAY ALTERS THE LEVEL OF METHIONINE ENKEPHALIN RELEASED INTO THE THIRD VENTRICLE

METHOD

Experiments were performed on Sprague-Dawley rats weighing 280-320 g. After implantation of the bipolar electrode into the periaqueductal gray (PAG) and the push-pull cannula into the third ventricle, the animals were allowed one week to recover from surgery. On the day of implantation, the animals were transferred from the animal house to the experimental room, where they remained until the end of the experiment.

LOCAL PERFUSION

After a one week recovery period, the rats were screened for stimulation-induced analgesia at three intensities (details given in general methods section) using the tail flick test. Two weeks post surgery, rats were perfused with 0.9% saline containing 1.8 mM. calcium. The perfusing medium was pumped in and withdrawn by a peristaltic pump (Gilson) at a constant flow rate of 10 µl/minute through polyethylene tubing. After an initial washout period of thirty minutes, three consecutive sixty minutes samples were collected on ice. After the first sample was collected, the rat's brain (PAG) was electrically stimulated for a total of one minute at the predetermined intensity and the second sixty minute sample was collected. The perfusate was then freeze-dried. The above perfusion procedure was repeated seven and fourteen days later, and the individual perfusates pooled. After the final perfusion, the location of the cannula and electrode was histologically verified.

HPLC OF PERFUSATE SAMPLES

HPLC was carried out on reverse phase μ Bondapak C18 columns using the conditions given in general methods. Typically, samples were resuspended in the HPLC starting buffer, centrifuged for five minutes at approximately 1000 rpm and the supernatant applied to the u Bondapak C18 column. The eluant was monitored at 280 nm. and 254 nm. Fractions of 1.0 ml. were collected and then dried under vacuum. Synthetic methionine enkephalin standard was chromatographed under the same conditions.

RADIOIMMUNOASSAY

The levels of methionine enkephalin present in the perfusate were measured by a specific radioimmunoassay (RIA). The samples were oxidised after HPLC as follows:

(1) the dried fractions were resuspended with 80% methanol: water mixture;

(2) then 10 μl. of hydrogen peroxide was added to 10 mls of water and 50 μl. from this mixture was then added to the fractions containing the samples;

(3) the fractions were mixed and then dried down under vacuum.

The oxidation step was necessary to convert methionine enkephalin to methionine sulphoxide because the antibody recognition was optimal for the sulphoxide.

RESULTS

The levels of the peptide present in all samples assayed were below the detection limit of the assay.

I - THE OXIDATION OF METHIONINE ENKEPHALIN AND THE IDENTIFICATION OF THE PRODUCTS FORMED

At this stage, the possibility was considered that the oxidation procedure used was unsuccessful at producing the sulphoxide. This conversion step is a critical one since the assay utilises high titre antibodies to the methionine sulphoxide. A pilot experiment was then performed to confirm the identity of the oxidised product(s) formed. Methionine enkephalin was oxidised using the same concentration of hydrogen peroxide as used for sample oxidation; a time course experiment was conducted as shown in Table 2:

TABLE 2 - INCUBATION CONDITIONS USED FOR OXIDATION OF METHIONINE ENKEPHALIN.

SAMPLE	INCUBATION TIME (HOURS)	NMOLS OF METHIONINE ENKEPHALIN	NMOLS OF HYDROGEN PEROXIDE
1	0	50	96
2	1	50	96
3	4	50	96
4	0/N	50	96

The above samples were then chromatographed on the HPLC using the same conditions as for the perfusate samples. Figs. 24-27 show the HPLC profiles obtained. It can be seen that, rather surprisingly, only the overnight incubation produced 100% of the oxidised form (fig. 27).

The possibility also existed that the oxidation procedure generated the sulphone rather than the sulphoxide. The powerful technique of Fast Atom Bombardment Mass Spectrometry (FAB-MS) (Morris et al. 1982) was used by Dr. Anne Dell to analyse and confirm the identity of the oxidised product(s) ex HPLC. Using this technique the identity of the oxidised product was found to be the sulphoxide in all samples.

Fig. 24 - HPLC PROFILE OF OXIDISED METHIONINE ENKEPHALIN

(0 HOUR INCUBATION)

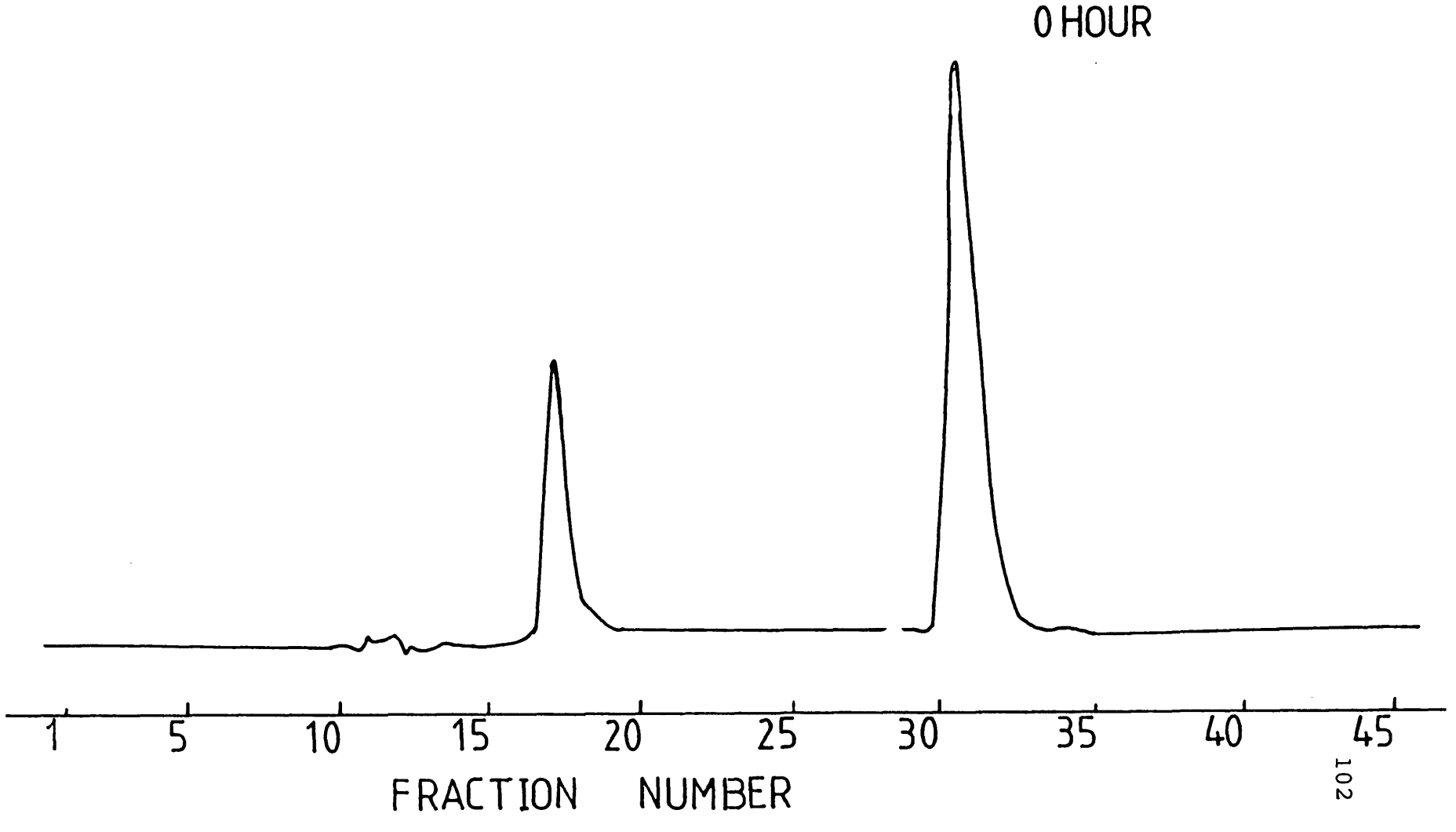


Fig. 25 - HPLC PROFILE OF OXIDISED METHIONINE ENKEPHALIN

(1 HOUR INCUBATION)

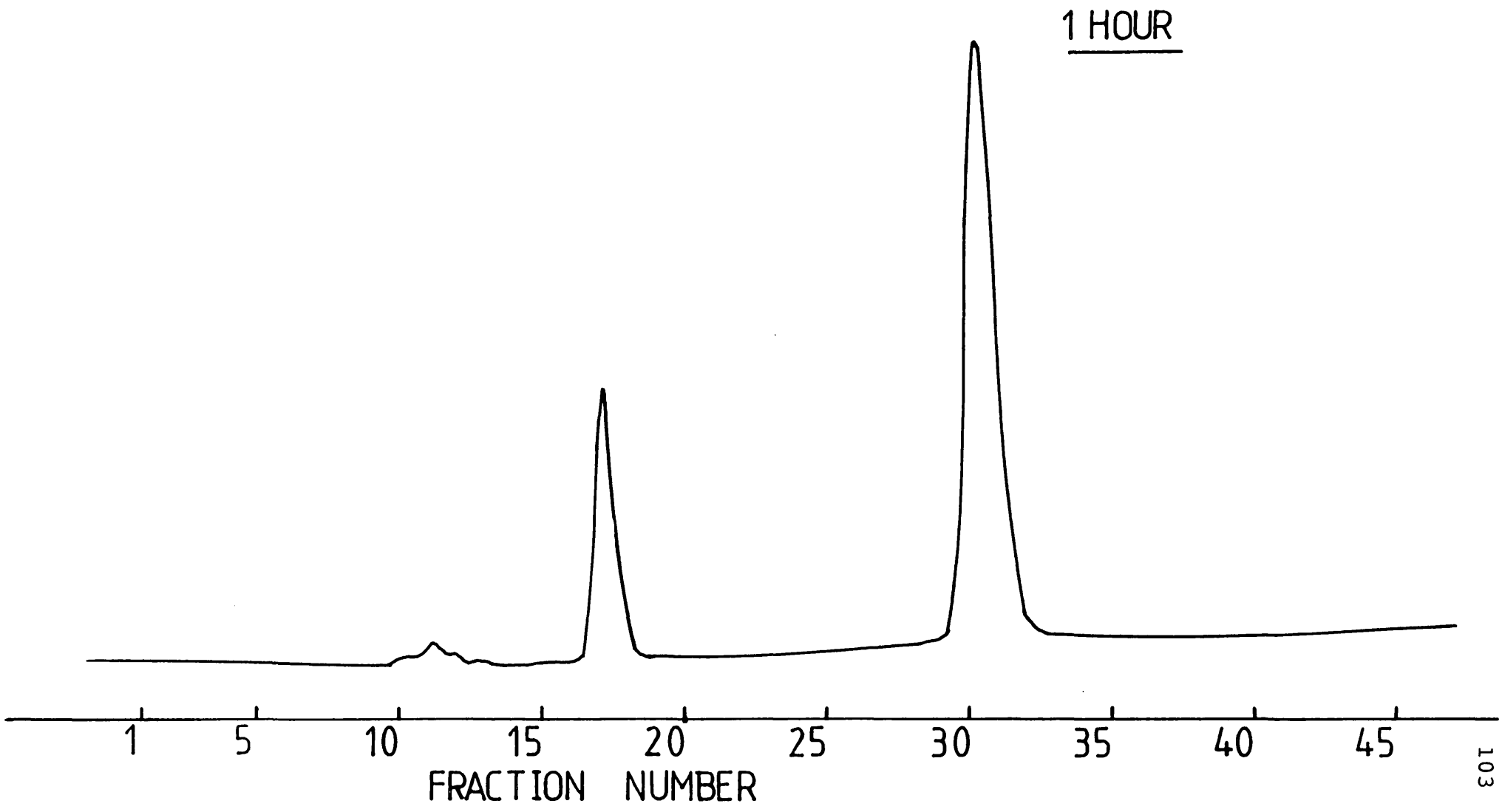


Fig. 26 - HPLC PROFILE OF OXIDISED METHIONINE ENKEPHALIN

(4 HOURS INCUBATION)

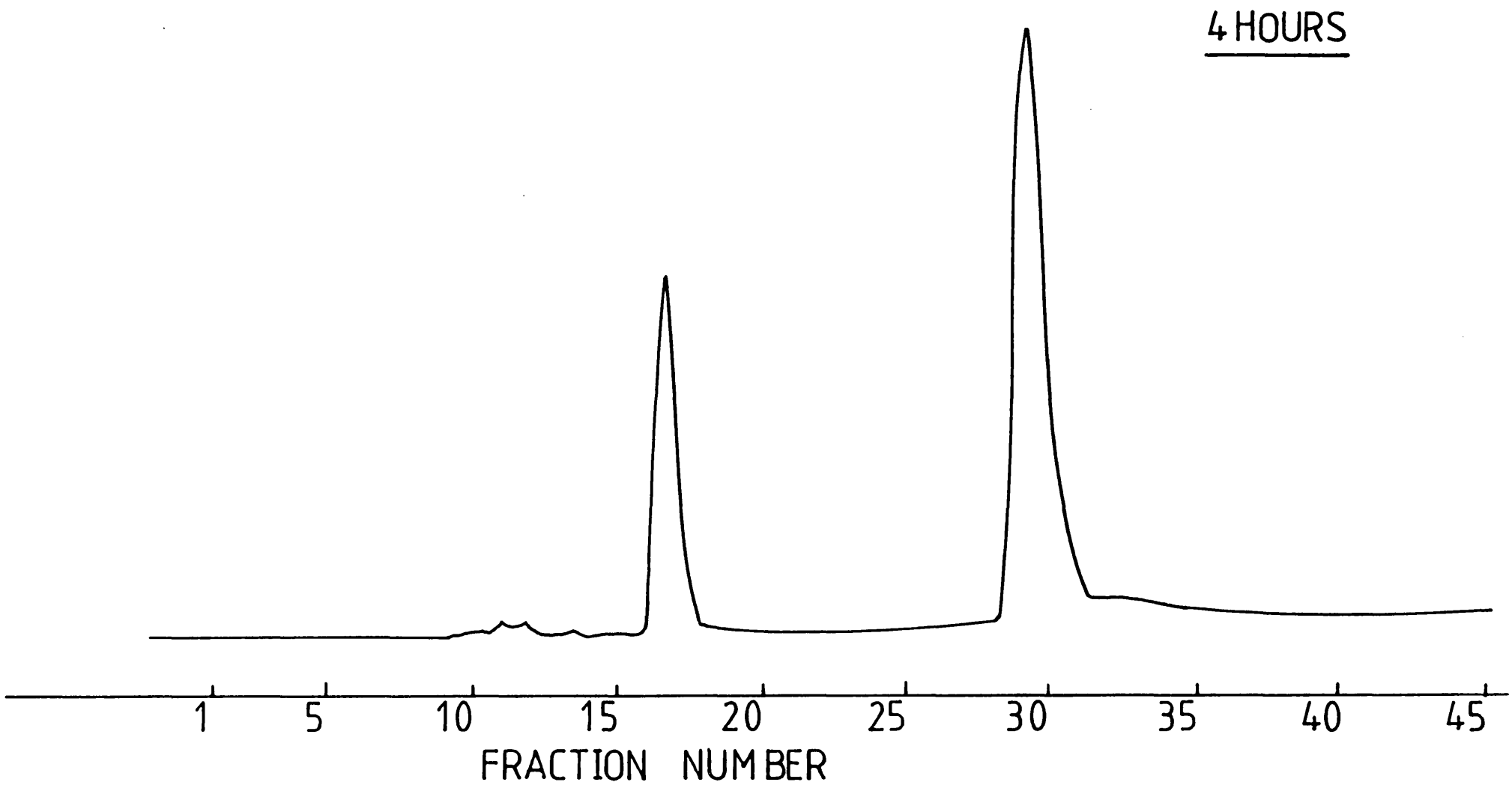
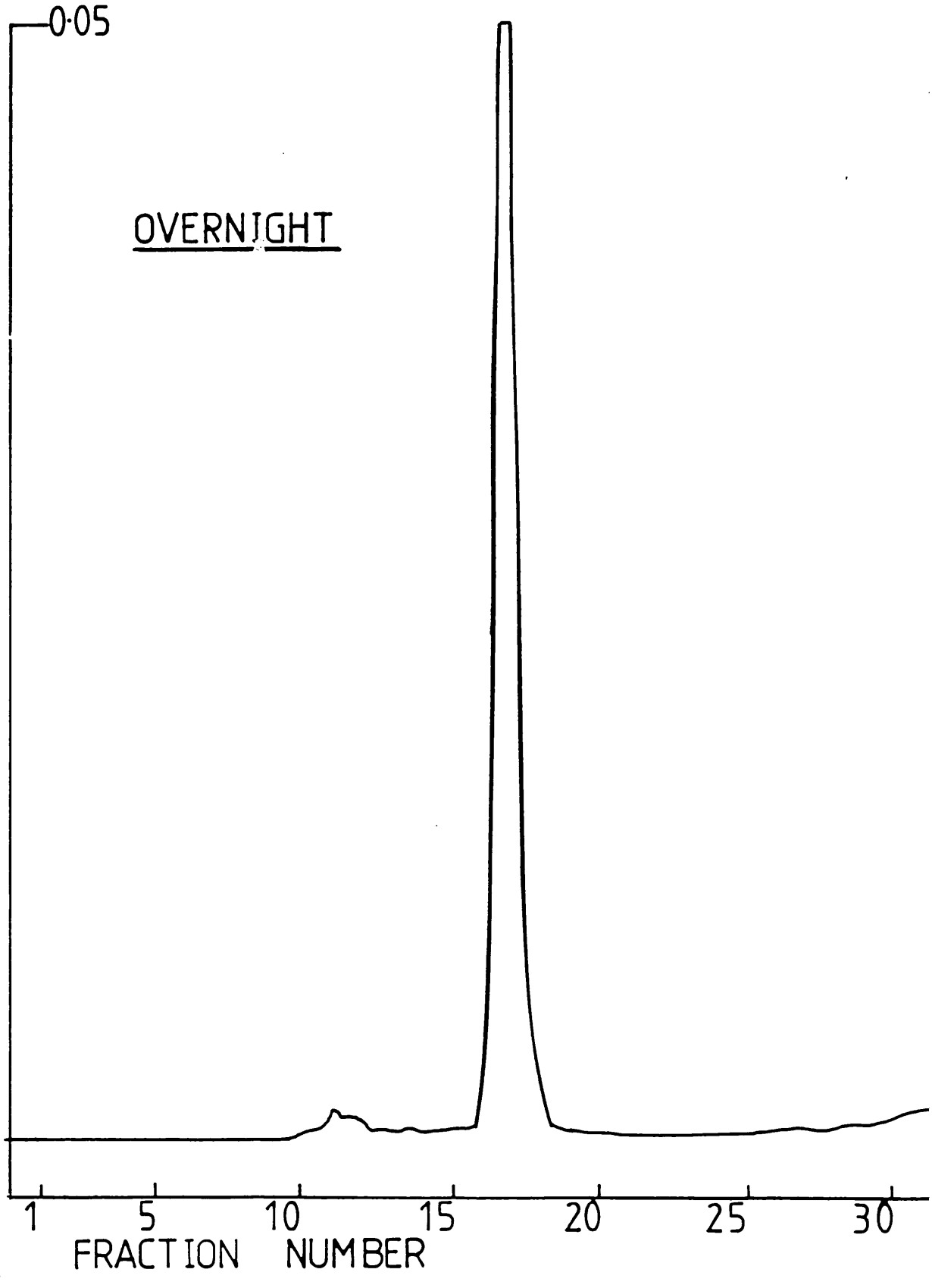


Fig. 27 - HPLC PROFILE OF OXIDISED METHIONINE ENKEPHALIN (OVERNIGHT INCUBATION)



It was concluded from the above experiment that no methionine enkephalin-like immunoreactivity was detected in the samples because there was only partial oxidation of the peptide (remembering that the oxidation reaction was previously terminated instantly). Further experiments were then performed in an identical manner to previous perfusions, except that the samples were now incubated overnight with hydrogen peroxide. Despite this alteration, the levels of the peptide in all the perfusate samples were, once again, below the detection limit of the assay.

An alternative explanation for these negative results could have been due to the rapid in situ degradation of any released methionine enkephalin. Thus, further experiments were then performed in order to investigate this possibility.

II - EXPERIMENT TO INVESTIGATE THE METABOLISM OF METHIONINE ENKEPHALIN IN SITU USING ^{3}H-METHIONINE ENKEPHALIN

CHEMICALS

Bacitracin, bestatin and bovine serum albumin were obtained from Sigma, tyrosyl 3,5^{3}H-enkephalin (methionine) from Amersham. Thiorphan was generously donated by Professor Roques.

METHOD

The purity of the tritiated enkephalin was checked using HPLC before it was used in the experiments. This step was necessary to purify the ^{3}H-YGGFM, as it was later discovered that methionine enkephalin sulphoxide was also present in the sample. The fractions containing the unoxidised enkephalin were pooled, dried and subsequently used for the experiment.

Fig. 28 - HPLC PROFILE DEMONSTRATING THE METABOLISM OF 3H-METHIONINE ENKEPHALIN IN SITU

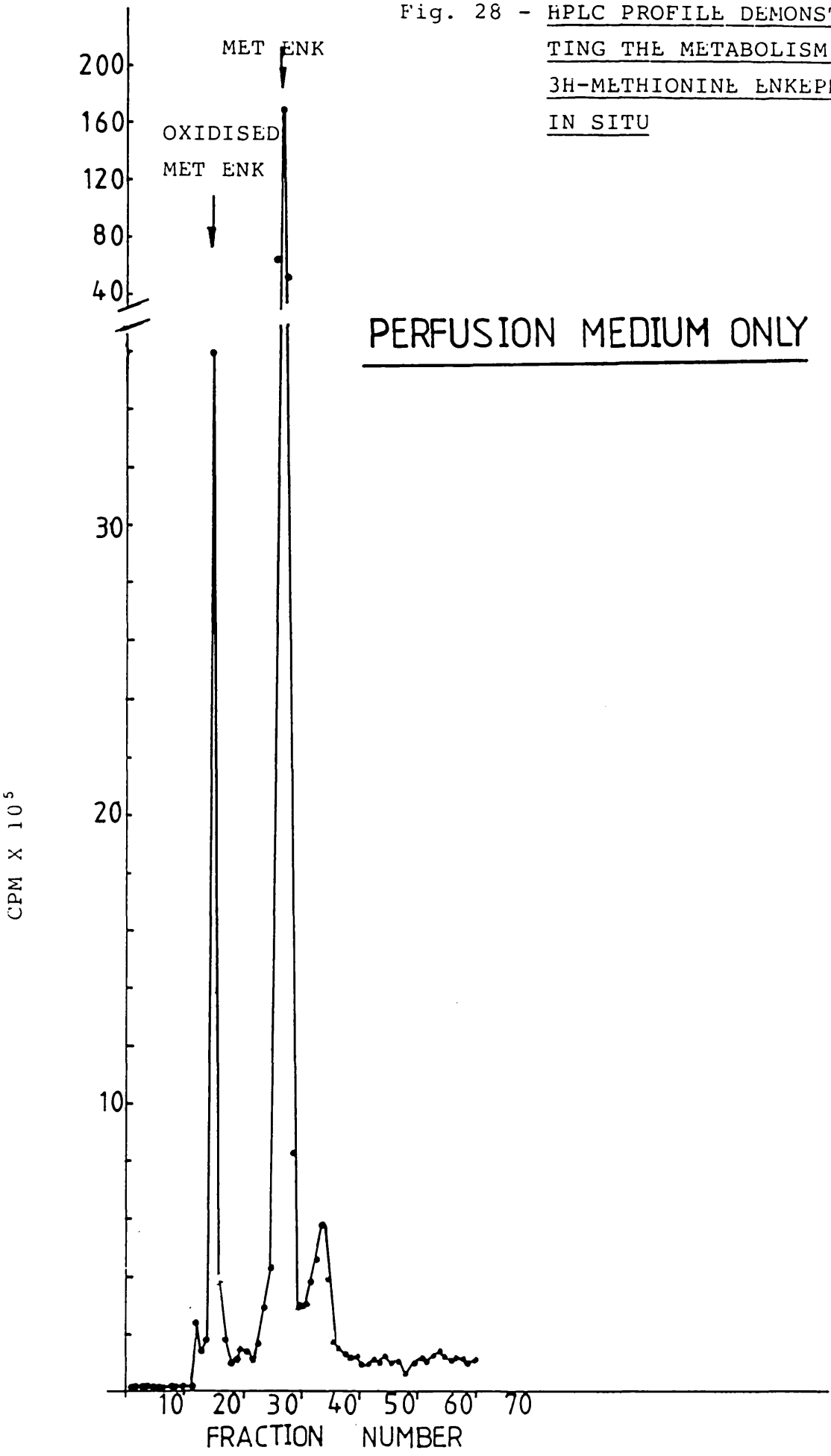


Fig. 29 - HPLC PROFILE DEMONSTRATING THE METABOLISM OF 3H-METHIONINE ENKEPHALIN IN SITU

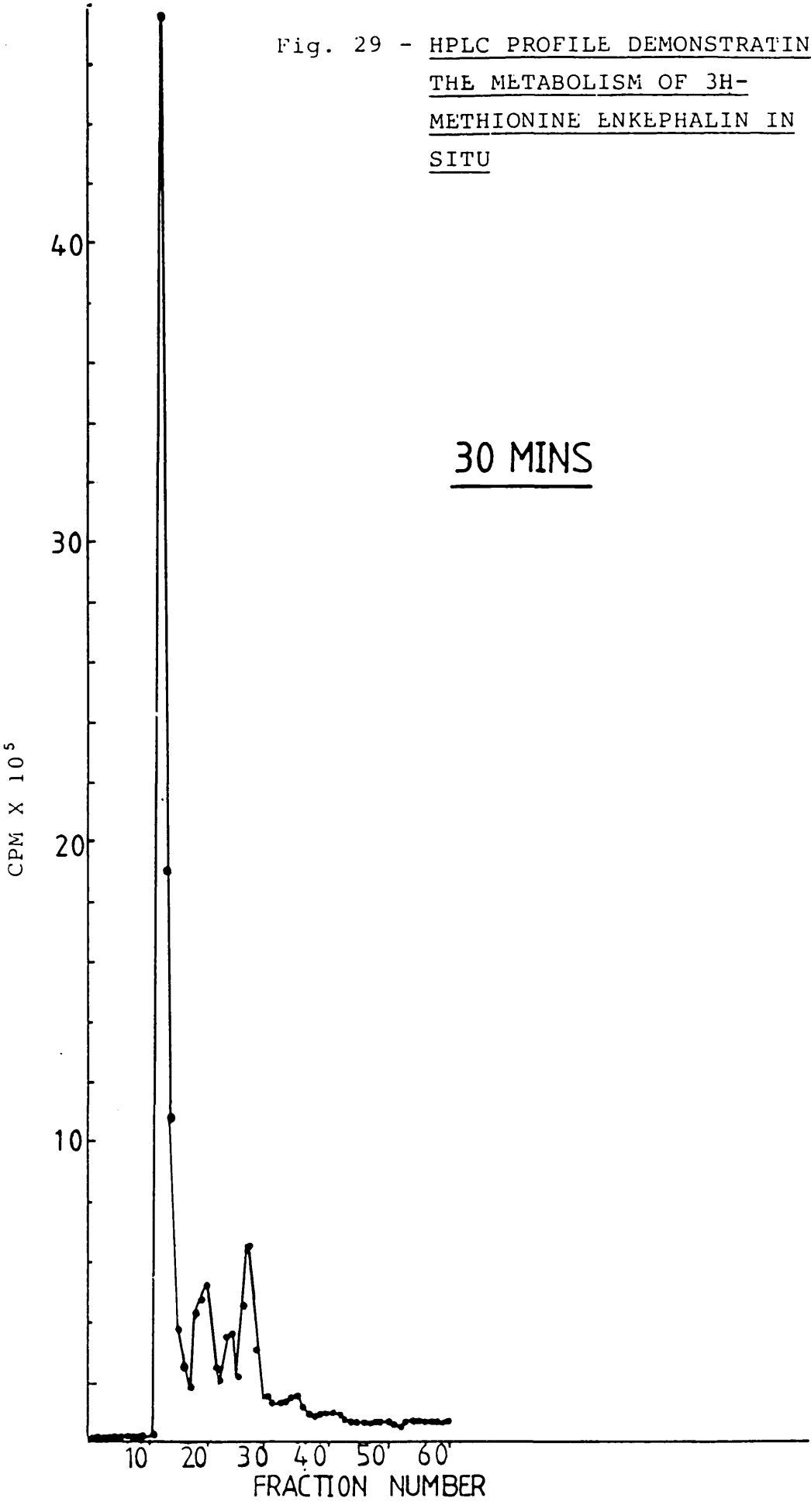


Fig. 30 - HPLC PROFILE DEMONSTRATING THE METABOLISM OF 3H-METHIONINE ENKEPHALIN IN SITU

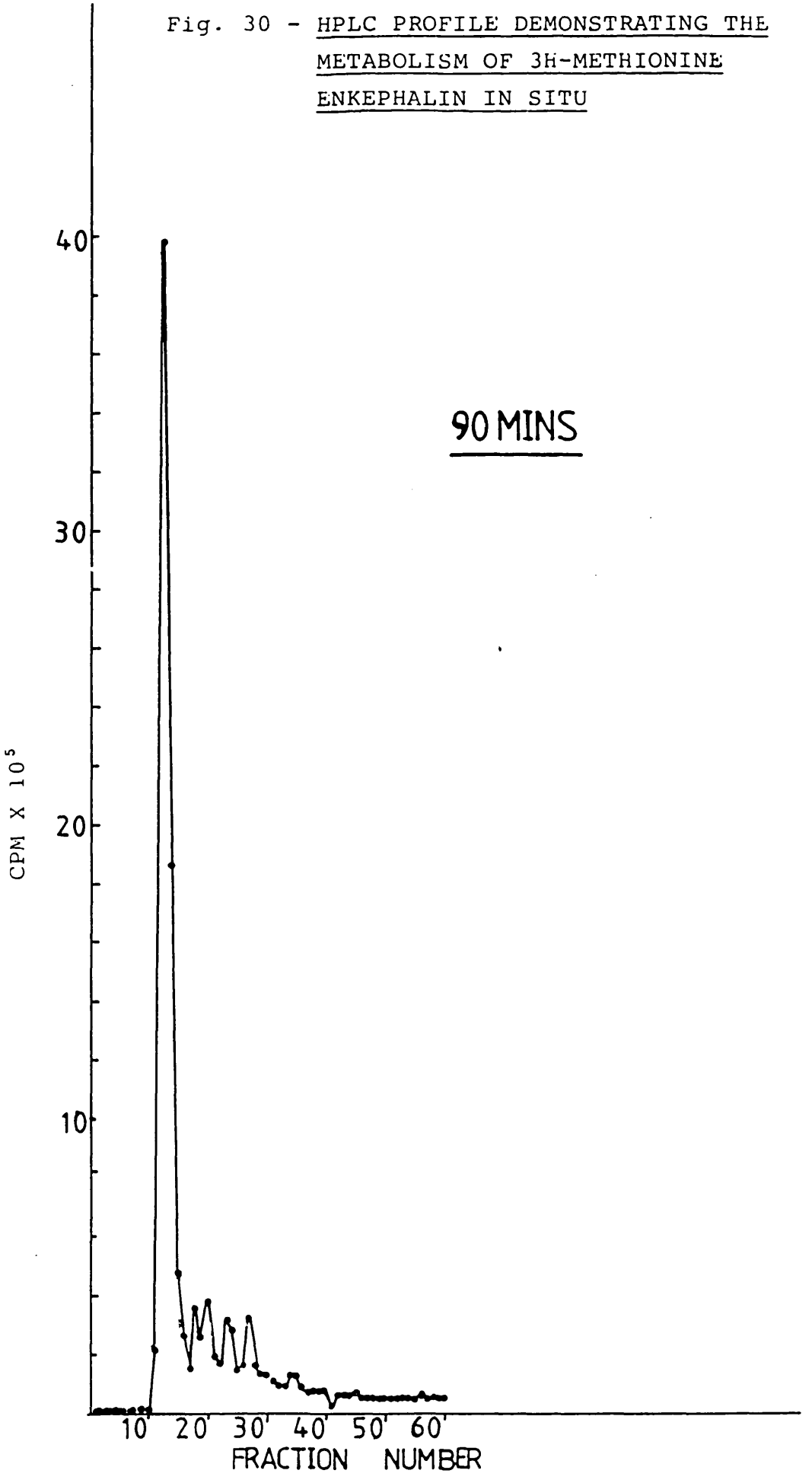
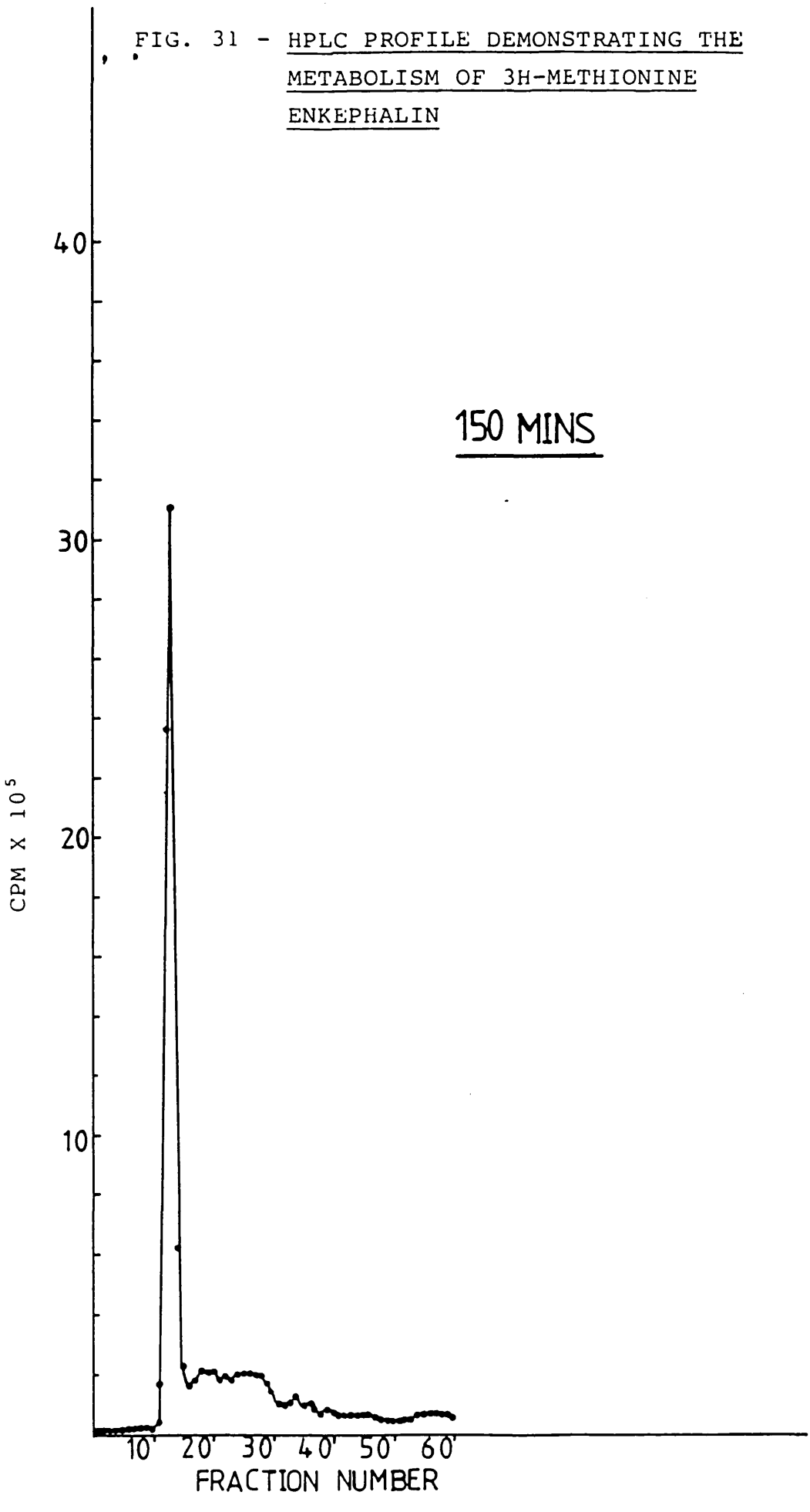


FIG. 31 - HPLC PROFILE DEMONSTRATING THE METABOLISM OF 3H-METHIONINE ENKEPHALIN



IN SITU METABOLISM OF ^{3}H-YGGFM

Rats were implanted with push-pull cannulae into the third ventricle as described previously. After a one week recovery from surgery, rats were perfused with artificial cerbrospinal fluid (CSF), (NaCl, 127 mM, KCl, 3.73 mM, $CaCl_2$, 1.8 mM, KH_2PO_4, 1.18 mM, $MgSO_4$, 1.18 mM, $NaHCo_3$, 20 mM) containing bovine serum albumin, (0.1% w/v), bacitracin (30 µg/ml) bestatin (20 µM) and thiorphan (0.1 µM). The rat was perfused for a total of four and a half hours at a rate of 10 µl/minute, and consecutive samples were collected on ice. The first one was a thirty minute collection, the others all being one hour collections. At the end of the perfusion, the samples were freeze-dried in silanised pear-shaped flasks. An aliquot was removed from the remaining perfusion medium alone (i.e. having not passed through the ventricles) at the end of the experiment, and was treated identically to the perfusate samples. This sample should give an indication of any degradative effects due simply to standing at room temperature for the duration of the experiment. The freeze-dried samples were analysed by HPLC to determine the proportion of intact ^{3}H-YGGFM per sample and total metabolites. The details of the HPLC gradient are given in general methods.

RESULTS

Typical levels of ^{3}H-YGGFM and ^{3}H-metabolites during the continuous in situ ventricular perfusion are shown in figs. 28-31. Figure 28 shows the HPLC profile obtained from chromatographying the perfusion medium alone. It shows quite clearly both intact ^{3}H-YGGFM and the sulphoxide form (presumably oxidised during the time of the perfusion). Inspection of the HPLC profiles obtained from chromatographing the samples

obtained, thirty minutes (fig. 29), ninety minutes (fig. 30), and 150 minutes (fig. 31) post the beginning of the perfusion, indicates that during the ^{3}H-YGGFM perfusion, the percentage of ^{3}H-YGGFM in the perfusate decreased dramatically with time. In fact, in the first thirty minutes of the perfusion most of the intact ^{3}H-YGGFM had disappeared. No attempt was made to determine the identity of the radiolabelled metabolites present in the perfusates. However, the shift of the radiolabelled compounds to the earlier fractions in the HPLC runs suggests that the metabolites formed were all more polar than the intact peptide and also indicates that perhaps tyrosine, tyr-gly-gly, or tyr-gly are the major metabolites. However, since ^{3}H-YGGFM labelled on the tyrosine residue was used in this experiment, only the ^{3}H-tyr-gly-gly, tripeptide, ^{3}H-tyr-gly dipeptide or the ^{3}H-tyr residue would have been observable as a cleavage product and not the dipeptide phe-met.

DISCUSSION

Aminopeptidases were the first enkephalin hydrolysing enzymes to be identified when it was realised that large amounts of tyrosine were released by breakdown of the penta-peptide in vivo and in vitro (Grynbaum et al. 1977, Hambrook et al. 1978). These enzymes all seem to be metallopeptidases and the most potent inhibitor presently available is bestatin (Barclay et al. 1980). Its structure suggests that it chelates the metal in the active site. Puromycin, a drug generally used for other purposes, also inhibits enkephalin hydrolysing aminopeptidases from several brain preparations (Malfroy et al. 1979) but is generally less potent than bestatin.

Dipeptidyl aminopeptidases hydrolysing the Gly^{2}-Gly^{3} peptide bond of enhephalin are also present in brain (Gorestein

et al. 1979) in membrane bound form but no selective inhibitor for these enzymes is known.

Angiotensin-converting enzyme (ACE), which catalyses the formation of the octapeptide angiotensin II from the decapeptide angiotensin I, was detected in brain tissue several years ago (Yang et al. 1973). However, its functional role is not clear because the presence of a renin angiotensin system in brain is still a matter of controversy. In 1979 Erdos et al. showed that purified ACE hydrolyses the enkephalins at the site of the Gly^3-Phe^4 peptide bond, an action potently inhibited by compounds like captopril.

Enkephalin dipeptidyl carboxy peptidase (enkephalinase) activity was first detected by Malfroy et al. (1978) in cerebral membranes used for opiate-receptor binding assays. Like ACE, enkephalinase is a metallopeptidase which cleaves the Gly^3-Phe^4 peptide bond but it clearly differs in its substrate specificity, its regional distribution and the effect of inhibitors (Schwartz et al. 1981). Thus captopril is 1000 times less potent on enkephalinase than on ACE, whereas the recently discovered inhibitor thiorphan is about 50 times more potent (nanomolar affinity) on enkephalinase than on ACE (Roques et al. 1980).

Thus it appears that both enkephalinase and the amino-peptidase pathways play a critical role in the inactivation of endogenous enkephalin. This is well illustrated by work done recently by De la Baume et al. (1983). They demonstrated the effect that peptidase inhibitors have on the recovery of endogenous enkephalins released by depolarisation of brain slices. It was shown that in the absence of any peptidase inhibitors only a small fraction (16%) of radioimmunoassayed

methionine enkephalin released from the tissue was recovered from the incubation medium in intact form. The presence of either thiorphan or bestatin enhanced the recovery by about twofold, but it was only when both inhibitors were present that the recovery was complete (De La Baume et al. 1983). They also showed that neither the aminopeptidase inhibitor puromycin nor the ACE inhibitor captopril significantly enhanced recovery.

In this experiment involving the perfusion of ^{3}H-YGGFM, we have included bacitracin, bestatin and thiorphan into the perfusion medium in an effort to reduce the rapid metabolism of enkephalin. Bacitracin, reported to be a non-selective inhibitor of a variety of peptidases (Desbuquois et al. 1974), including enkephalinase, inhibited the enkephalin hydrolysing amino peptidases from brain tissues with an IC 50 in the range of 10 µM (Malfroy et al 1978) and in a non-competitive manner. Bestatin, an inhibitor of bacterial origin, inhibits the enzyme activity from rat brain supernatant with an IC 50 value as low as 200 nm (Barclay et al. 1980). Thiorphan is a competitive inhibitor (K_i = 4nM) of enkephalinase (Roques et al. 1980).

It is apparent from the data presented that HPLC is particularly useful in the quantitative analysis of enkephalin and analogs. HPLC provides an unambiguous, reproducible separation of the parent enkephalins from metabolites and contaminants; thus allowing the investigator freedom to choose a detection system most suited to the situation in a particular study.

Caution must be exercised when radiolabelled peptides are used as the ligand and in opiate binding assays or for metabolic

studies; since in this study it was found that ^{3}H-YGGFM which exceeded 90% purity by the manufacturer's specification, contained significant amounts of contamination when analysed by HPLC and liquid scintillation spectrometry. Bioassays commonly used for opiate peptide detection may also be subjected to some misinterpretation, since we have found methionine enkephalin sulfoxide was formed in solution and this is known to have about half the potency of met enkephalin (Beddell et al. 1977).

The results from this experiment indicate that methionine enkephalin is metabolised rapidly, despite the fact that various peptidase inhibitors were present in the perfusion medium and thus this could be a probable reason why methionine enkephalin was not detected in the third ventricular perfusate samples. Supporting this finding, Craves et al. (1978) have shown that ^{3}H-YGGFM perfused through the lateral ventricle to the cisterna magna in the anaesthesised rat was initially cleaved either at the Gly -Gly or Gly -Phe peptide bond. Although Craves' report demonstrated the rapid metabolism of ^{3}H-YGGFM, the exact area in the ventricular system containing the peptidases could not be identified from this study; it could be that all the structures lining the ventricles contain high concentrations of the peptidases, or perhaps there is a sparse distribution. Our model demonstrates for the first time the in situ metabolism of ^{3}H-YGGFM at a specific site in the conscious unrestrained rat. This work well illustrates the potential use of the animal model for studying localised metabolism of peptides.

Since we have shown that ^{3}H-methionine enkephalin is rapidly metabolised in situ, despite the presence of various

potent inhibitors, and since there appears to be no detectable met enkephalin in the third ventricular perfusate samples, it was of interest to determine if exogenous application of met enkephalin into the third ventricle would in fact produce analgesia. In addition, to further confirm that met enkephalin is rapidly degraded in situ, experiments using D-Ala-Methionine enkephalinamide, a synthetic enkephalin analog that is less susceptible to degradation by brain enzymes, were conducted to investigate its analgesic action on central administration into the third ventricle.

III - EXPERIMENT TO INVESTIGATE THE ANTINOCICEPTIVE EFFECTS OF METHIONINE ENKEPHALIN AND D-ALA-METHIONINE ENKEPHALINAMIDE ADMINISTERED TO THE THIRD VENTRICLE.

MATERIALS AND METHOD

Rats were implanted stereotaxically with microinjection guide cannulae (Plastic Products) into the third ventricle. Each guide cannula was fitted with an indwelling occlusion stylet. The co-ordinates used were identical to those used for implantation of push-pull cannulae (see general methods section). One week post surgery, rats were tested in the tail flick test for analgesic activity following the intracerebroventricular infusion of met enkephalin, D-Ala-methionine enkephalinamide (DAME), or saline.

For these microinjection experiments the protocol was as follows: Rats were put in restrainers one hour prior to testing. During this time, the stylets were removed from the guide cannula and the injection cannula inserted into the permanently implanted guide cannula. This was done so that the animals would become familiar with this handling procedure, and thus cause the minimum disturbance at the time of actual

injection. Fifteen trials were then obtained (intertrial interval, ITI = 2.0 minutes) prior to infusion of drugs. Intraventricular injections were made with the injection cannula (22 gauge) connected by a 15 cm. length of PE 60 tubing to a 25 µl. Hamilton syringe.

DAME and methionine enkephalin were purchased from Sigma and the drug solutions were always prepared freshly just prior to use. Drugs were dissolved in water and further diluted in 0.9% saline. Either saline or the drug solution was back-loaded into the tubing and was injected in a volume of 5 µl. (at a rate of 5 µl./15 seconds).

The injection cannula was always placed into the guide cannula at least four minutes before the actual injection. This was done to reduce any effects resulting from simply placing the injection cannula into the guide cannula which might mask the actual drug effects. At the end of the first injection, the injection cannula was left in place in the guide cannula for at least five minutes. This extra post injection interval provided sufficient time for the drugs to diffuse into the ventricles and to prevent backflow of liquid up the guide cannula. The injection needle was then replaced with the stylet.

Immediately post injection of the drugs (or saline) thirty trials were conducted (ITI = 2.0 minutes) to determine the time course of action of the drugs. This procedure was repeated for two higher doses of the same drug. Thus typically, each rat received three doses in one session with an interval of one hour between each drug administration. Control rats were treated identically except that they were injected with physiological saline. One test session, which was approximately

four and a half hours in duration (including the one hour adaptation period) was conducted once only on each animal. Methionine enkephalin (dissolved in saline) was injected in doses of 50 µg., 100 µg. and 250 µg. DAME (dissolved in saline) was injected in doses of 10 µg., 25 µg. and 50 µg. Cannula placements were verified histologically at the end of the experiment.

CALCULATION OF RESULTS

The time course data are presented in terms of withdrawal latencies. However, to permit comparisons and construction of dose-response curves, response latencies were converted to percent maximal possible effect (% MPE). The formula for this normalization procedure is:

$$\% \text{ MPE} = \frac{\text{post drug latency} - \text{baseline latency}}{\text{cut off time} - \text{baseline latency}} \times 100$$

where post drug latencies (T) were obtained by calculating the mean latency at the following times (minutes) post injection:

0 - 10
10 - 20
20 - 30
30 - 40
40 - 50

Baseline latency (BL) = mean of five trials immediately prior to drug administration, and
cut off time = 10 seconds.

RESULTS

Tables 3, 4 and 5 show the data obtained for injection of methionine enkephalin, DAME and saline respectively. Figs 32-34 show the dose response curves (taken from the data in table 4) for DAME. As can be seen from tables 3-5, methionine enkephalin

Fig. 32 - DOSE RESPONSE CURVES FOR DAME

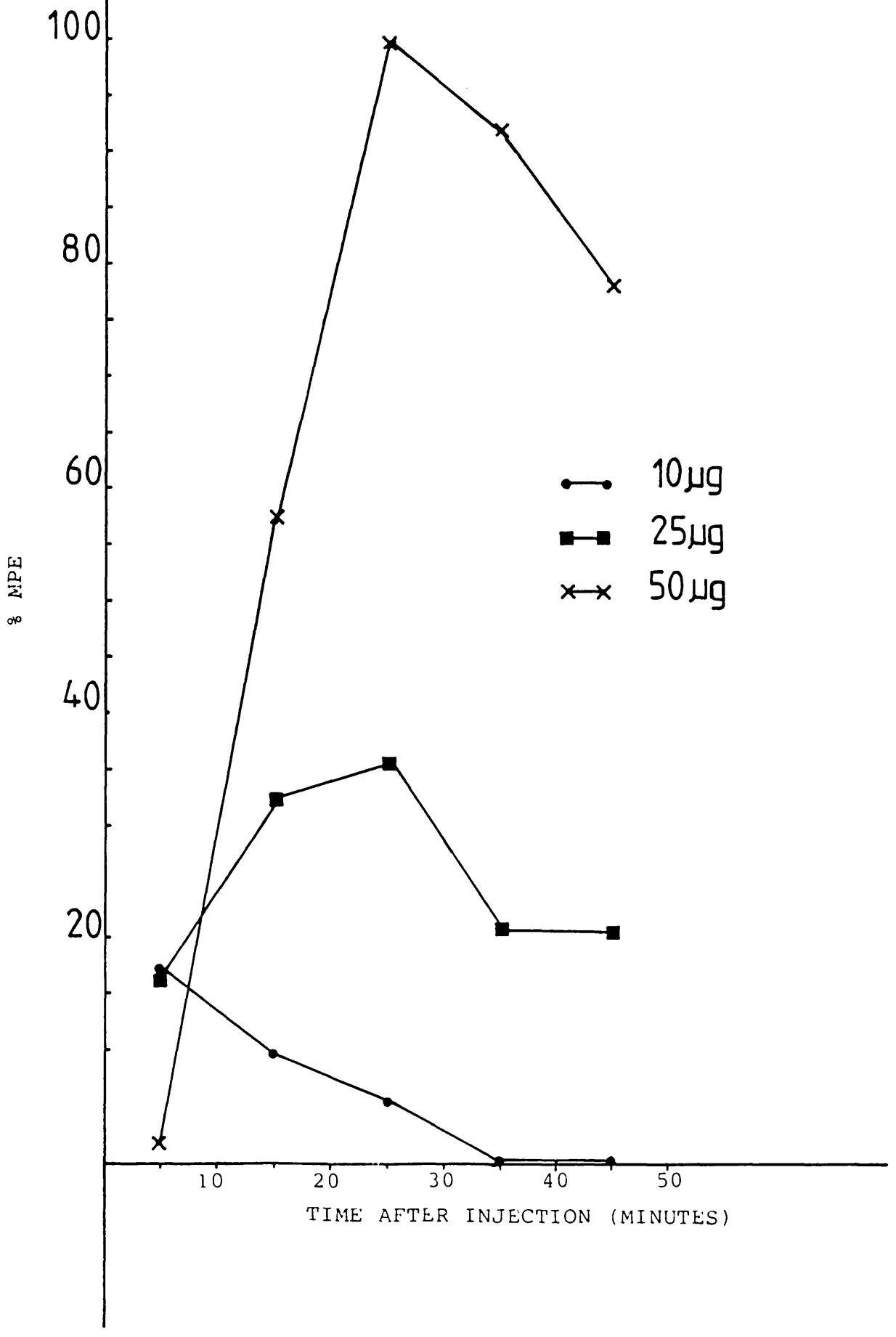


Fig. 33 - DOSE RESPONSE CURVES FOR DAME

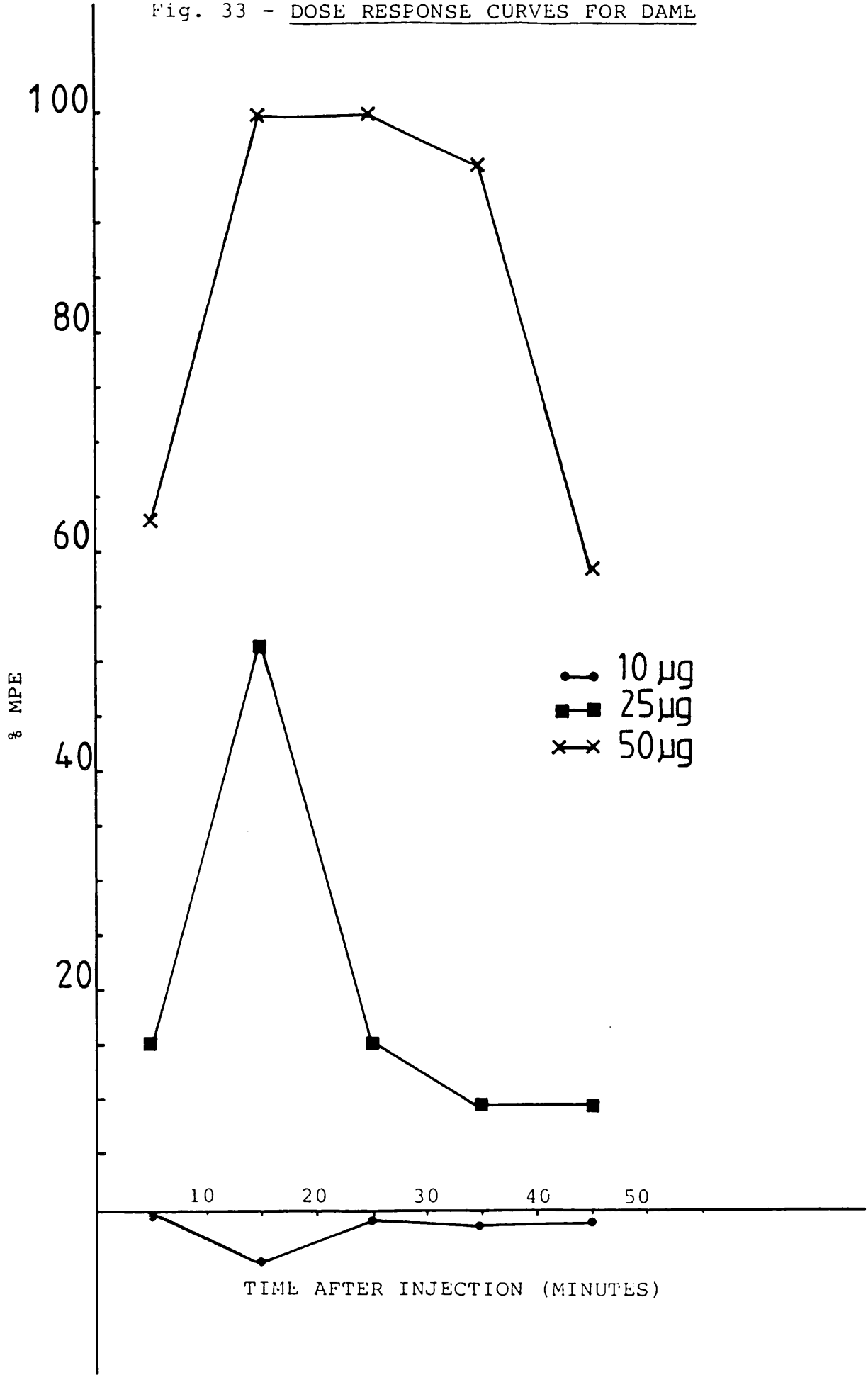


Fig. 34 - DOSE RESPONSE CURVES FOR DAME

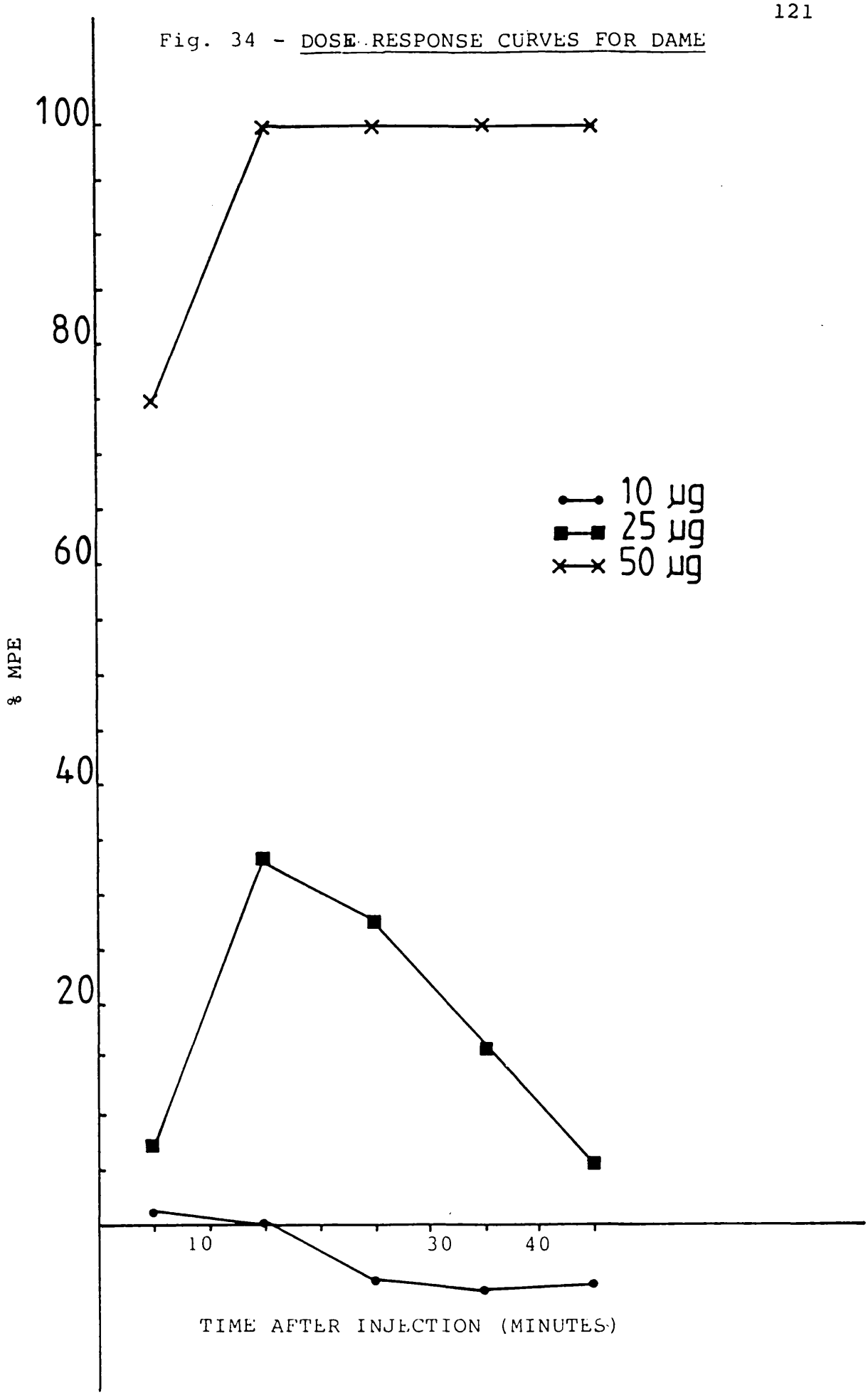


TABLE 3. % MPE VALUES OF ICV ADMINISTRATION OF METHIONINE ENKEPHALIN ON TAIL FLICK TEST AND THEIR MEAN ± S.E. MEAN OF LATENCIES

RAT NO.	MINUTES POST INJECTION										DOSE µg
	0-10		10-20		20-30		30-40		40-50		
	BL (±SEM)	T ±SEM	% MPE	T ±SEM	% MPE	T ±SEM	% MPE	T ±SEM	% MPE	T ±SEM	%MPE	
	2.52 (0.08)	2.84 (0.17)	4.2	2.64 (0.07)	1.6	2.52 (0.12)	0	2.16 (0.12)	-4.8	2.52 (0.05)	0	50
1	2.36 (0.15)	2.28 (0.21)	-1.0	2.36 (0.13)	0	2.56 (0.16)	2.6	2.44 (0.15)	1.0	2.28 (0.12)	-1.0	100
	2.16 (0.10)	2.48 (0.14)	4.0	2.28 (0.12)	1.5	2.04 (0.12)	-1.5	2.16 (0.12)	0	2.2 (0.06)	0.5	250
	2.84 (0.07)	3.12 (0.05)	3.9	2.92 (0.19)	1.1	2.80 (0.23)	-0.5	2.72 (0.14)	-1.6	3.08 (0.10)	3.3	50
2	3.24 (0.16)	3.00 (0.36)	-3.5	3.36 (0.15)	1.7	3.44 (0.15)	2.9	3.20 (0.15)	-0.5	3.00 (0.11)	-3.5	100
	3.20 (0.13)	3.72 (0.31)	7.6	3.20 (0.23)	0	3.48 (0.14)	4.1	3.32 (0.16)	1.7	3.28 (0.19)	1.1	250
	3.16 (0.12)	3.52 (0.15)	5.2	3.28 (0.14)	1.7	3.28 (0.10)	1.7	3.4 (0.13)	3.5	3.52 (0.14)	5.2	50
3	3.84 (0.17)	3.64 (0.29)	-3.2	3.36 (0.16)	-7.7	3.32 (0.14)	-8.4	3.52 (0.14)	-5.1	4.28 (0.19)	7.1	100
	3.00 (0.14)	3.16 (0.12)	2.2	3.24 (0.20)	3.4	2.96 (0.04)	-5.7	3.00 (0.28)	0	3.16 (0.07)	2.2	250

T = post drug latency

BL = baseline latency

TABLE 4. % MPE VALUES OF I.C.V. ADMINISTRATION OF DAME ON TAIL FLICK TEST AND THEIR MEAN ± S.E. MEAN OF LATENCIES

	MINUTES POST INJECTION											
	0 - 10			10 - 20		20 - 30		30 - 40		40 - 50		
RAT NO.	BL (±SEM)	T (±SEM)	% MPE	T (±SEM)	% MPE	T (±SEM)	% MPE	T (±SEM)	% MPE	T (±SEM)	% MPE	DOSE µg
	3.56 (0.12)	4.68 (0.41)	17.4	4.20 (0.28)	9.9	3.92 (0.29)	5.6	3.56 (0.27)	0	3.56 (0.33)	0	10
4	3.20 (0.18)	4.40 (0.35)	17.6	5.40 (0.2)	32.9	5.6 (0.3)	35.3	4.64 (0.13)	21.2	4.64 (0.15)	20.5	25
	3.88 (0.08)	4.0 (0.46)	1.9	7.40 (1.18)	57.5	10	100	9.52 (0.3)	92.1	4.36 (0.56)	78.4	50
	3.52 (0.19)	3.6 (0.24)	1.2	3.52 (0.27)	0	3.2 (0.19)	-4.9	3.12 (0.16)	-6.2	3.16 (0.15)	-5.5	10
5	3.56 (0.07)	4.04 (0.16)	7.4	5.72 (0.76)	33.5	5.32 (1.18)	27.3	4.6 (0.24)	16.1	3.93 (0.33)	5.7	25
	4.24 (0.17)	8.56 (1.02)	75	10.0	100	10.0	100	10.0	100	10.0	100	50
	3.08 (0.12)	3.04 (0.12)	-0.57	2.74 (0.2)	-4.9	3.0 (0.06)	-1.1	3.2 (0.13)	1.7	3.2 (0.26)	1.7	10
6	2.88 (0.21)	3.96 (0.48)	15.2	6.56 (0.82)	51.7	3.96 (0.23)	15.2	3.56 (0.16)	9.5	3.56 (0.15)	9.5	25
	3.68 (0.14)	7.68 (1.42)	63.3	10	100	10	100	9.72 (0.28)	95.5	7.4 (0.32)	58.5	50

T = post drug latency

BL = baseline latency

TABLE 5. % MPE VALUES OF ICV ADMINISTRATION OF SALINE ON TAIL FLICK TEST AND THEIR MEAN ± S.E. MEAN OF LATENCIES

	MINUTES POST INJECTION															
			0 - 10			10 - 20			20 - 30			30 - 40			40 - 50	
RAT NO.	BL (±SEM)		T (±SEM)		% MPE	T (±SEM)		% MPE	T (±SEM)		% MPE	T (±SEM)		% MPE	T (±SEM)		% MPE
	3.73	(0.17)	4.0	(0.14)	4.4	4.2	(0.17)	7.6	3.84	(0.12)	1.9	3.92	(0.22)	3.1	3.72	(0.4)	0
7	4.16	(0.17)	3.8	(0.33)	-6.1	3.68	(0.12)	-8.2	3.8	(0.26)	-6.1	4.16	(0.25)	0	3.96	(0.16)	-3.4
	4.04	(0.19)	3.6	(0.06)	-7.3	3.56	(0.42)	-8.0	4.16	(0.34)	2.0	4.16	(0.28)	2.0	4.6	(0,13)	9.3
	4.12	(0.16)	4.2	(0.2)	1.3	4.28	(0.21)	2.7	4.36	(0.18)	4.0	4.24	(0.12)	2.0	4.2	(0.09)	1.3
8	4.24	(0.07)	4.76	(0.1)	9.0	4.52	(0.08)	4.8	4.36	(0.04)	2.0	4.08	(0.4)	-2.0	4.52	(0.17)	4.8
	4.24	(0.18)	4.52	(0.34)	9.0	4.36	(0.2)	2.0	4.2	(0.28)	-0.6	4.52	(0.15)	4.8	4.48	(0.23)	4.1
	3.24	(0.22)	2.96	(0.31)	-4.1	3.08	(0.12)	-2.3	3.64	(0.07)	5.9	3.60	(0.09)	5.3	3.44	(0.17)	2.9
9	3.32	(0.19)	3.68	(0.14)	5.3	2.64	(0.23)	-10.0	3.64	(0.33)	4.7	3.2	(0.13)	-1.7	3.48	(0.05)	2.3
	3.20	(0.19)	3.68	(0.08)	7.0	3.50	(0.09)	8.8	3.52	(0.14)	4.7	3.84	(0.21)	9.4	3.8	(0.06)	8.8

T = post saline latency

BL = baseline latency

did not produce any significant increase in tail flick latency compared to saline-treated rats. However, DAME produced a potent analgesia comparable to that produced by peripheral administration of morphine. Indeed, its onset and duration of action mirrors that of morphine.

DISCUSSION

The discovery of opioid peptides in the brain (Hughes et al. 1975) and pituitary (Cox et al. 1975, Teschemacher et al. 1975) led to the natural assumption that they represented an endogenous system for producing analgesia (Goldstein, 1976). Several years later, however, this appears not to be so conclusive or straightforward. The evidence implicating these peptides in analgesia, however indirect, may be summarised as follows:

A. Since opiates are known to act at the specific opiate receptors at various levels of the spinal axis to produce analgesia (Simon 1976, Atweh and Kuhar 1977, Yaksh et al. 1977, Yaksh et al. 1978) and since the opioid peptides clearly interact with these same receptors, one has to suppose that if and when the peptides are released at the receptor sites, analgesia should result.

B. The distribution of enkephalinergic neurons at various levels of the cerebrospinal axis, in close association with pain pathways, especially in the substantia gelatinosa, seems to implicate them in some way in the modulation of pain. Enkephalins are in terminals of short neurons and in close relationship to the opiate receptor (Frederickson 1977, Hökfelt 1977).

C. Administration of enkephalins (at high doses) into the cerebroventricular system or at certain sites in brain or

spinal cord results in analgesia (as does administration of morphine by the same route)(Wei et al. 1975, Duggan et al. 1977, Feldberg et al. 1977, Li et al. 1977, Yaksh et al. 1977). The rapid breakdown of enkephalin probably accounts for its short activity and poor apparent potency, because these pentapeptides are highly potent in neuroblastoma-glioma hybrid cells that contain opiate receptors (Klee et al. 1976) and because enkephalin congeners stabilised against enzymatic attack are also highly potent in vivo (Dutta et al. 1977, Pert et al. 1977, Roemer et al. 1977, Yamushiro et al. 1977). D. Stimulation produced analgesia first suggested the possibility of endogenous opiate-like substances because this analgesia is partially blocked and reversed by the specific opiate antagonist naloxone (Akil et al. 1976).

A few months after the discovery of the enkephalins, Belluzi and co-workers (Belluzi et al. 1976) first reported analgesic activity following the central administration (lateral ventricle) of enkephalins to rats. The rats were tested in the tail flick test for analgesic activity following the intracerebroventricular infusion of methionine enkephalin (100 µg. and 200 µg. per rat i.c.v.). Statistically significant, dose-related increases in tail flick latencies were observed. The analgesia produced by the highest dose (200 µg. per rat) of methionine enkephalin appeared to be equivalent to that produced by a 10 µg. per rat dose of morphine sulphate. Thus although methionine enkephalin produced significant analgesia, it was approximately twenty times less potent than morphine when administered into the ventricles. The analgesia produced by methionine enkephalin exhibited a rapid onset (2 - 6 minutes) which also rapidly disappeared by 10 - 12

minutes post drug infusion. The delay between the time of administration and onset of activity was most likely attributed to diffusion time to the active sites and the short duration of activity was probably due to the rapid catabolism of enkephalins by brain enzymes.

Shortly thereafter, Malick and Goldstein reported the results of their studies (Malick et al. 1976, Malick et al. 1977) in which, for the first time, complete dose response curves were generated in order to compare the analgesic potencies of methionine enkephalin and morphine. Both morphine and methionine enkephalin exhibited potent, dose-related analgesic activity following infusion into the PAG. Methionine enkephalin was found to be approximately four times less potent than morphine on a weight basis. However, on a molar basis the potency of methionine enkephalin would be increased by a factor of two relative to morphine (i.e. $E.D._{50}$ values, methionine enkephalin = 0.27 μmol, morphine = 0.13 μmol). Thus Malick and Goldstein (1977) reported that methionine enkephalin was considerably more potent than indicated by Belluzi and co-workers (1976). This difference in potency can probably be attributed to the fact that Belluzi and co-workers utilised the intraventricular route of administration, which required diffusion to the site(s) of action; this could have markedly reduced the activity of the peptide since enkephalins have been shown to be rapidly inactivated by peptidases in brain and other tissues (Hambrook et al. 1976). Following intracerebral infusion (PAG), the analgesic activity of methionine enkephalin peaked before one minute post infusion but had a very short duration of activity (its activity had significantly diminished by seven minutes post infusion).

This is in agreement with the short duration of activity reported by Belluzi and co-workers (1976). In contrast, the activity of morphine did not peak until seven minutes post infusion, but persisted for as long as several hours post infusion, which is consistent with previous observations (Yaksh et al. 1976).

Thus, methionine enkephalin has been shown to produce significant analgesic activity in rodents following intra-ventricular administration (lateral ventricle). When it was infused directly at the site at which morphine was active (e.g. PAG), activity almost comparable to that observed with morphine was observed (Malick et al. 1976, Malick et al. 1977); however, if methionine enkephalin was placed into the ventricles, where it had to diffuse to the site of action, it appeared to be much less potent than morphine. This was probably due to rapid breakdown prior to the peptide reaching the receptor site.

Many synthetic analogs of methionine enkephalin have been synthesised and tested in a number of systems for their opiate activity. A wide range of potencies has emerged. Considerable information has been obtained as a result of this large research effort. The importance of the tyrosyl moiety in position 61; the increased potency due to substitution of D-Ala in position 62, which retards enzymatic degradation at the N-terminus; and the resistance to enzymatic breakdown at the carboxy terminus by the amide form of the peptides are examples of ways to increase the potency of the pentapeptide. Multiple changes are additive.

The examination of the analgesic potency of D-Ala-methionine enkephalin-amide (DAME) in vivo (figs. 32-34) demonstrates

that DAME has potent longlasting analgesic properties. DAME (50 µg.) elicited maximal analgesia fifteen to twenty minutes after microinjection into the third ventricle; in most cases this analgesic effect lasted for at least forty minutes.

The analgesic effects of DAME found in the present study closely parallel those reported by Pert et al. (1976), who found that DAME at 5 or 10 µg. produced a profound longlasting analgesia after unilateral intracerebral injection into the PAG of rats. Despite the fact that others (Belluzi et al. 1976), have shown it to be analgesic when administered into the lateral ventricle of rodents, methionine enkephalin, even at doses as high as 250 µg., failed to elicit any significant increase in tail flick latency (table 3). It seems conclusive that the difficulty of eliciting analgesia with enkephalin is due to rapid cleavage of the peptide. DAME, a very similar but less degradable analog of methionine enkephalin, is shown here to be a potent analgesic.

In addition, several other studies have shown that high doses (up to 200 µg.) of methionine enkephalin were necessary to produce a transient analgesia when administered into the lateral ventricle. The analgesic effect developed rapidly after a definite latent period (two to six minutes), and dissipated rapidly (ten to twelve minutes). However, Feldberg et al. (1977) have shown that methionine enkephalin when administered into the third ventricle, had no analgesic effect when infused in doses of between 30 and 400 µg. He also found that the pentapeptide amide produced no analgesia when infused into the third ventricle at a dose of 200 µg; but that administration of N-methyl methionine enkephalin amide into the third ventricle in doses of 150 and 180 µg. produced strong

analgesia. This clearly demonstrates that both ends of the peptide had to be blocked for the pentapeptide to be analgesic.

The delay in onset of action when DAME is administered into the third ventricle (table 4), suggests that slow diffusion of the peptide to distantly situated receptor sites is occurring and that this slow diffusion to the site of action would further contribute to the inactivation of methionine enkephalin when administered to the third ventricle. Extensive studies on the distribution of the enkephalins in the brain have shown that the cell bodies and terminals are broadly distributed, with the highest concentration in the striatum (particularly the globus pallidus), periaqueductal gray, anterior hypothalamus and amygdala. The proximity to lateral ventricle of structures known to be rich in opiate receptors suggests that this injection site might favour the occurrence of analgesia. It would be interesting to perfuse both the lateral ventricle and the third ventricle simultaneously, and then compare the levels of peptide present at both sites before stimulation, during stimulation and post stimulation.

Yaksh and Elde (1980) were the first to report the release of methionine enkephalin-like immunoreactivity from the rat spinal cord in vivo using an elegant spinal perfusion technique. The spinal perfusions were conducted by inserting a polyethylene (P.E.10) catheter through a slit in the atlanto-occipital membrane 10-11 cm. to the lumbar level of the spinal cord. Artificial CSF (containing bacitracin and bovine serum albumin) was then infused through this catheter at a rate of 100 µl. per minute and collected at the level of the cisterna magna through a short stainless steel cannula inserted adjacent to the inflow catheter. One ml. samples

were collected every ten minutes. These ten minute samples were then dried and assayed for methionine enkephalin-like immunoreactivity (MELI). The data reported on the resting levels of MELI in the perfusate samples was of the order of 0.4 ng./ml. / 10 minute, and that for the levels in the presence of 40 mM. potassium to the superfusate was increased 310%.

Others have found it difficult to demonstrate both the in vitro and in vivo release of methionine enkephalin. For instance, the release of enkephalin from the globus pallidus, the region in the brain containing the highest concentration of enkephalin, was investigated by Iversen et al. (1978) and Bayon et al. (1978). In these studies methionine enkephalin was released from perfused slices of rat globus pallidus in a calcium-dependent manner. However, considerable degradation (63%) occurred even in the presence of bacitracin. Substantial degradation of tissue stores (approximately 60%) took place even in the presence of aminopeptidase inhibitors, regardless of whether the tissue was stimulated to release the peptide or not. This rapid catabolism of the endogenous stores (as also suggested by our studies) raises some questions concerning the absolute values of peptide release observed in such studies, but the primary significance of those studies was in demonstrating a potassium-stimulated, calcium-dependent release.

Recently Glowinski (1981) has demonstrated the in vivo release of methionine enkephalin from the globus pallidus in the anaesthesised cat using push-pull cannula (basal release = 20-30 pg./15 minutes and release in the presence of 60 mM. potassium resulted in a 20-30 fold increase). However,

there are no reports on the release of methionine enkephalin into the ventricles of the rat; the only report on its in vivo release in the rat are those in which the spinal cord (Yaksh and Elde 1980) and the nucleus reticularis gigantocellularis (Kuraishi and Takagi 1981) were perfused. Unfortunately both of these studies lacked chemical specificity since no chromatographic separation of the collected perfusate was attempted.

In summary, despite the lack of reports on the conclusive identification of the in vivo release of methionine enkephalin, it was reasonable to expect that release of enkephalin would have allowed its detection in the novel experiment reported here. However, the negative data suggests that either methionine enkephalin is not released in detectable quantities into the third ventricle during PAG stimulation in the rat, or that it is released but is rapidly degraded close to the site of its release. This implies that its rate of metabolism far exceeds its rate of diffusion (away from its site of release) to the site(s) of action. In addition, the fact that the analgesic effects resulting from PAG stimulation are short lived (approximately six minutes in duration) is in agreement with its rapid metabolism.

A fascinating concept has recently emerged implicating increased opioid activity with nociceptive stimulation. Cesselin et al. (1982) have reported that tooth pulp stimulation (which activates only A and C fibres) in halothane-anaesthesised cats, induced a longlasting (approximately three hours) increase in the levels of methionine enkephalin-like material in the cisternal CSF. His data strongly suggest that the activity of enkephalinergic neurons was increased

following nociceptive stimulation. In fact, other groups have also shown that stressful situations with nociceptive stimuli (in particular inescapable footshocks) can induce subsequent analgesia (Madden et al. 1977, Lewis et al. 1980). Cesselin et al. (1980) reported the first evidence of a change in the activity of enkephalinergic systems in the spinal cord of chronically-suffering rats. Thus marked increase in enkephalin levels was detected both in the cervical and dorsal enlargements of the cord in polyarthritic rats. These findings are rather intriguing since traditionally only analgesic treatments or manipulations have been correlated with increased opioid activity.

The opioid peptides have been classically associated with analgesia. Non opioid peptides are now becoming linked with analgesia. It was of interest to investigate the antinociceptive activity of one of these peptides, namely cholecystokinin octapeptide (CCK_8). This peptide is generally associated with satiety rather than analgesia; however, recently it has been implicated in analgesia. It has been shown to be present in the PAG (Skirboll et al. 1982), and in addition it has been shown to produce analgesia when injected into the PAG (Jurna and Zetler 1981). The next Chapter will, using the model developed, investigate the role of endogenous and exogenous CCK_8 in analgesia.

CHAPTER 6

I. EXPERIMENT TO INVESTIGATE THE ANTINOCICEPTIVE EFFECTS OF CCK_8 ADMINISTERED TO THE THIRD VENTRICLE.

METHODS

The implantation and experimental procedures used were identical to those used for investigating the antinociceptive effects of methionine enkephalin. Solutions of CCK_8 (sigma) were always prepared freshly prior to use. CCK_8 was dissolved in water and further diluted in 0.9% saline. Either saline or the drug solution was backloaded into the tubing, and was injected in a volume of 5 µl. (at a rate of 5 µl./15 seconds).

As with previous experiments, the injection cannula was always placed into the guide cannula at least four minutes before the actual injection. Similarly, once the drug solution or saline were injected, the injection cannula remained in situ for at least five minutes post injection. The time course used was identical to that used previously. Briefly, fifteen trials were obtained (after a one hour adaptation period) prior to drug infusion, then the drug or saline was injected. Immediately post injection of the drug (or saline), thirty trials were conducted (ITI = 2.0 minutes) to determine the time course of action of the drug. This procedure was repeated for two higher doses of the same drug. Thus typically, each rat received three doses in one session with an interval of one hour between each drug administration. Control rats were treated identically except that they were injected with physiological saline. One test session, which was approximately four and a half hours in duration (including the one hour adaptation period) was conducted once only on each animal. CCK_8 (dissolved in saline) was injec-

ted in doses of 0.1 µg., 1.0 µg., and 5.0 µg. Cannula placements were verified histologically at the end of the experiment.

CALCULATION OF RESULTS

The response latencies were converted to percent maximal possible effect (% MPE) as previously. Post drug latencies (T) were obtained by calculating the mean latency at the following times (minutes) post injection:

0 - 10
10 - 20
20 - 30
30 - 40
40 - 50

Baseline latency (BL) = mean of five trials immediately prior to drug administration and the cut off time = 10 seconds.

RESULTS

Tables 6 and 7 show the data obtained for injection of CCK_8 and saline respectively. As can be seen from table 6, CCK_8, at the doses used, did not produce any significant change in the tail flick latency compared to saline-treated rats.

DISCUSSION

Subcutaneous (S.C.) administration of CCK_8 has been shown to produce naloxone-sensitive antinociceptive effects in mice (Zetler, 1980). These effects were dose-dependent and lasted for about one hour. On a molar basis CCK_8 was thirty-four times more potent in the hot plate test than morphine. This high potency and relatively long duration of effect of CCK_8 is rather surprising, because many peptides survive only a short time after systemic administration. In addition, Jurna and Zetler (1981) injected CCK_8 directly into the PAG of rats (doses used

TABLE 6. % MPE VALUES OF I.C.V. ADMINISTRATION OF CCK_8 ON TAIL FLICK TEST AND THEIR MEAN ± S.E.M. OF LATENCIES.

	0-10		10-20		20-30		30-40		40-50 MINS POST INJECTION			
RAT	BL (± SEM)	T (± SEM)	% MPE	T (± SEM)	% MPE	T (± SEM)	% MPE	T (± SEM)	% MPE	T (± SEM)	% MPE	DOSE (µg)	VOLUME INJECTED µl
	2.88 (0.19)	3.04(0.12)	2.2	2.56(0.22)	-4.5	2.68(0.22)	-2.8	2.56(0.18)	-4.5	2.52(0.08)	-5.0	0.1	5
1	2.92 (0.08)	2.56(0.32)	-5.1	2.56(0.07)	-5.1	2.88(0.28)	-0.5	3.4 (0.35)	6.7	3.08(0.19)	2.2	1.0	5
	3.16 (0.22)	2.96(0.19)	-2.9	2.88(0.24)	-4.1	3.16(0.34)		2.96(0.49)	-2.9	3.68(0.15)	7.6	5.0	5
	3.68(±0.21)	2.72(0.27)	-2.9	3.36(0.36)	-5.1	3.32(0.16)	-5.7	3.48(0.27)	-3.2	3.24(0.32)	-6.9	0.1	5
2	3.36(±0.56)	3.88(0.1)	-15.2	3.64(0.17)	4.2	4.04(0.12)	10.2	3.28(0.52)	-1.2	3.56(0.27)	3.0	1.0	5
	3.44(±0.28)	3.44(0.07)	7.8	3.16(0.33)	-4.2	3.64(0.17)	3.0	3.8 (0.26)	5.5	3.32(0.19)	-1.8	5.0	5
	3.6 (0.33)	3.52(0.29)	-1.2	3.0 (0.11)	-9.4	3.6 (0.14)		3.16(0.27)	-6.8	3.48(0.27)	-3.1	0.1	5
3	3.7 (0.2)	3.28(0.19)	-6.6	3.72(0.33)	0.3	3.2 (0.26)	-7.9	2.84(0.15)	-13.6	2.96(0.12)	-11.7	1.0	5
	3.56 (0.23)	3.6 (0.25)	0.6	4.32(0.3)	11.8	3.4 (0.23)	-2.5	3.48(0.14)	-1.2	3.28(0.22)	-4.3	5.0	5
	2.84 (0.18)	3.28(0.31)	6.14	2.44(0.56)	-5.6	2.24(0.37)	-8.4	3.24(0.53)	5.5	3.04(0.27)	2.8	0.1	5
4	3.12 (0.37)	3.32(0.44)	2.9	3.4 (0.55)	4.1	3.08(0.36)	-0.6	3.3 (0.36)	2.9	3.36(0.1)	3.5	1.0	5
	3.00 (0.45)	2.96(0.52)	-0.5	3.48(0.43)	6.8	3.00(0.4)		4.12(0.21)	16.0	3.4 (0.42)	5.7	5.0	5

T = post drug latency (sec)

BL = baseline latency (sec)

TABLE 7. % MPE VALUES OF I.C.V. ADMINISTRATION OF SALINE ON THE TAIL FLICK TEST AND THEIR MEAN ± S.E.M. OF LATENCIES.

	0-10		10-20		20-30		30-40		40-50 MINS POST INJECTION		
RAT	BL (± SEM)	T (± SEM)	% MPE	T (± SEM)	% MPE	T (± SEM)	% MPE	T (± SEM)	% MPE	T (± SEM)	% MPE	VOLUME INJECTED µl
	3.52 (0.08)	3.40 (0.2)	-1.8	3.4 (0.06)	-1.8	3.44 (0.12)	-1.2	3.88 (0.1)	5.5	3.5 (0.05)	0.3	5
5	3.52 (0.08)	3.92 (0.14)	6.2	3.36 (0.1)	-2.5	3.28 (0.08)	-3.7	3.76 (0.04)	3.7	3.76 (0.12)	3.7	5
	3.76 (0.12)	3.6 (0.17)	-2.5	3.72 (0.1)	-0.6	3.20 (0.13)	-8.0	3.4 (0.14)	-5.7	3.56 (0.07)	-3.2	5
	3.8 (0.09)	3.36 (0.20)	-7.0	3.04 (0.25)	-12.2	3.44 (0.16)	-5.8	3.2 (0.17)	-9.6	3.36 (0.23)	-7.0	5
6	3.6 (0.11)	3.64 (0.13)	0.6	3.44 (0.23)	-2.5	3.48 (0.14)	-1.8	3.84 (0.07)	3.7	3.52 (0.16)	-1.2	5
	3.32 (0.15)	3.48 (0.26)	2.4	3.60 (0.09)	4.2	3.36 (0.07)	0.5	3.44 (0.23)	1.7	3.76 (0.12)	6.5	5
	3.6 (0.13)	3.12 (0.12)	-7.5	3.6 (0.09)	0	3.32 (0.15)	-4.3	3.16 (0.15)	-6.8	3.44 (0.31)	-2.5	5
7	3.4 (0.11)	3.32 (0.1)	-1.2	3.48 (0.1)	1.2	3.4 (0.17)	0	3.32 (0.1)	-1.2	3.2 (0.09)	-3.0	5
	3.16 (0.1)	3.56 (0.13)	5.8	3.48 (0.14)	4.6	4.05 (0.2)	13.0	3.92 (0.22)	11.1	3.64 (0.07)	7.0	5
	3.56 (0.12)	3.56 (0.13)	0	3.2 (0.28)	-5.5	3.12 (0.23)	-6.8	3.12 (0.12)	-6.8	3.36 (0.25)	-3.1	5
8	3.24 (0.13)	3.40 (0.37)	2.3	3.48 (0.16)	3.5	2.88 (0.27)	-5.3	3.52 (0.05)	4.1	3.2 (0.23)	-0.5	5
	3.56 (0.31)	3.52 (0.12)	-0.6	2.88 (0.14)	-10.0	3.32 (0.1)	-3.7	3.0 (0.26)	-8.0	2.84 (0.17)	-11.0	5

T = post saline latency (sec.)

BL = baseline latency (sec.)

were in the ng range) and found that it consistently produced antinociceptive effects. These studies support the concept that endogenous CCK_8 plays a role in the regulation of nociceptive activity in the CNS. However, in our study (using higher doses than mentioned above), administration of CCK_8 to the third ventricle did not produce analgesia. In the current work, it seems probable that rapid metabolism occurred before the peptide reached its site of action.

We have previously shown that the rapid metabolism of ^{3}H-methionine enkephalin could have prevented both its detection in the third ventricular perfusate samples, and the production of analgesia when microinjected into the third ventricle. We further confirmed these findings by demonstrating that D-Ala methionine enkephalinamide, a more stable analogue of methionine enkephalin, produced potent analgesia when microinjected into the third ventricle. It was probable, therefore, that CCK_8, another small peptide, was also metabolised rapidly in situ. Unfortunately no stable analogue for CCK_8 was available. It was considered worthwhile to investigate the metabolism of CCK_8 in vivo before proceeding to further experiments. This experiment will now be described.

II. EXPERIMENT TO INVESTIGATE THE METABOLISM OF CCK_8 IN SITU USING ^{3}H-CCK_8.

METHODS

Since ^{3}H-CCK_8 itself was not available, the labelled CCK_8 used for this experiment was Cholecystokinin octapeptide [propionyl -^{3}H] propionylated which was obtained from Amersham Radiochemicals. Despite the manufacturer's specification of purity of the labelled peptide, the purity of the compound was

rechecked using reverse phase HPLC just prior to the experiment. The fractions containing the labelled peptide were pooled, dried and subsequently used for the experiment.

PERFUSION OF RAT WITH ^{3}H-CCK$_{8}$

The procedure used for this experiment was identical to that used to demonstrate the in situ metabolism of ^{3}H-methionine enkephalin. Rats were implanted with push-pull cannulae into the third ventricle. After a one week recovery period, rats were perfused with artificial CSF (details given in Chapter 5), containing bovine serum albumen (0.1% w/v), bacitracin (30 µg./ml.), bestatin (20 µm.) and thiorphan (0.1 µm.). The rat was perfused for a total of four and a half hours at a rate of 10 µl. per minute and consecutive samples were collected on ice. The first sample was a thirty minute collection and the others were all one hour collections. At the end of the perfusion, the samples were freeze-dried in silanised flasks. An aliquot was removed from the remaining perfusion medium alone (i.e., not having passed through the ventricles) at the end of the experiment and was treated identically to the perfusate samples. This sample should give an indication of any degradative effects due simply to standing at room temperature for the duration of the experiment. The freeze-dried samples were analysed by HPLC to determine the proportion of intact ^{3}H-CCK$_{8}$ per sample and total metabolites. The details of the HPLC gradient are given in the general methods section.

RESULTS

Examination of the HPLC profile obtained from purifying the labelled peptide, revealed rather surprisingly, 3 peaks,

labelled A, B and C in figure 35. Closer inspection of the structure of both the labelled and unlabelled octapeptide, (fig. 36), enables us to recognise peak A as the main ^{3}H-CCK$_{8}$ (Cholecystokin octapeptide ^{3}H propionylated) and peaks B and C as most probably being the oxidised ^{3}H-CCK$_{8}$ (the presence of two methionine residues could theoretically generate a minimum of two oxidised products). Due to the uncertainty of the identity of the peaks, fractions 60 - 90 were pooled, dried and used for the rest of the experiment.

Typical levels of ^{3}H-CCK$_{8}$ and ^{3}H-metabolites during the continuous in situ ventricular perfusion are shown in figs. 37 - 41. Figure 42 shows the HPLC profile obtained from chromatographing the perfusion medium alone. Inspection of the HPLC profiles from chromatographing the samples obtained, 0.5 hour (fig. 37), 1.5 hours (fig. 38), 2.5 hours (fig. 39), 3.5 hours (fig. 40) and 4.5 hours (fig. 41) post the beginning of the perfusion indicates that during the ^{3}H-CCK$_{8}$ perfusion, the percentage of peak A, B and C decreased rather sharply with time, but not so rapidly as in the ^{3}H-enkephalin experiment shown earlier (Chapter 5). In addition, the presence of two other peaks, D and E, were also noticed. Interestingly, the percentage of peak D present in the perfusate samples at 0.5 hour was observed also to decrease with time, while conversely, the percentage of peak E was shown to have markedly increased with time. Figure 43 illustrates the loss of peak A, B, C and D, and the formation of peak E with time. From this curve it is clear that a metabolite of ^{3}H-CCK$_{8}$ has formed (peak E) which after a lag period (approximately one hour) increased linearly and reached a maximum at 3.5 hours post the beginning of the perfusion at which time it remained at a

Fig. 35 - HPLC PROFILE OF ^{3}H-CCK_8 (PROPIONYLATED)

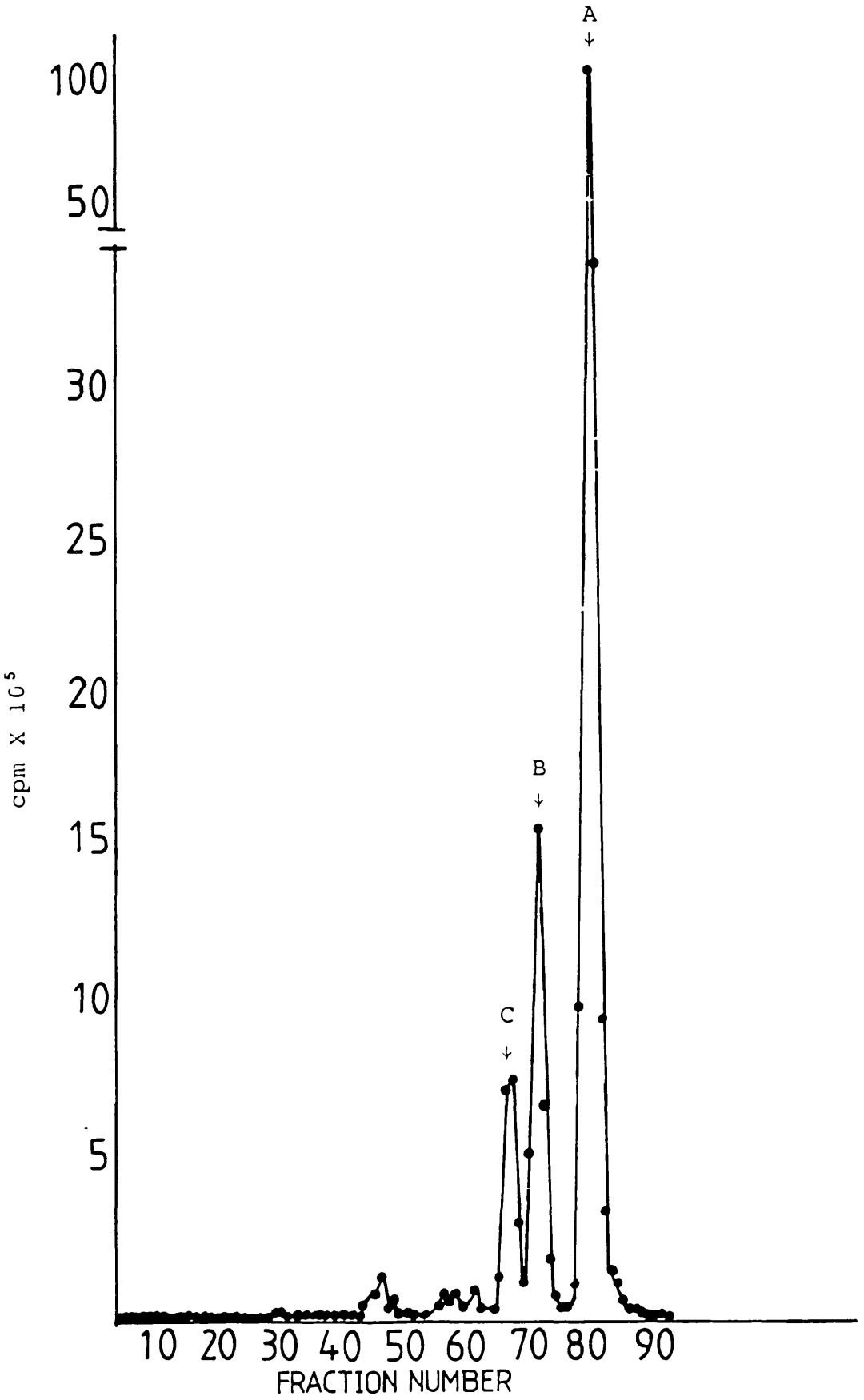
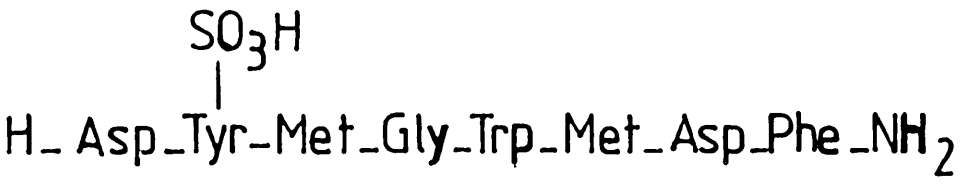
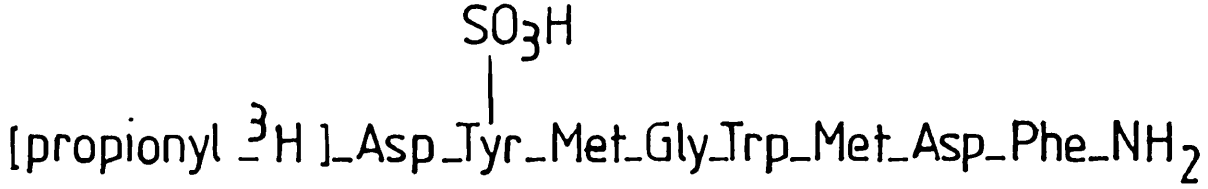


Fig. 36 - STRUCTURE OF CCK_8 AND ^3H-CCK_8 (PROPIONYLATED



CCK_8



^3H-CCK_8

Fig. 37 - HPLC PROFILE DEMONSTRATING THE METABOLISM OF ^{3}H-CCK$_{8}$ IN SITU

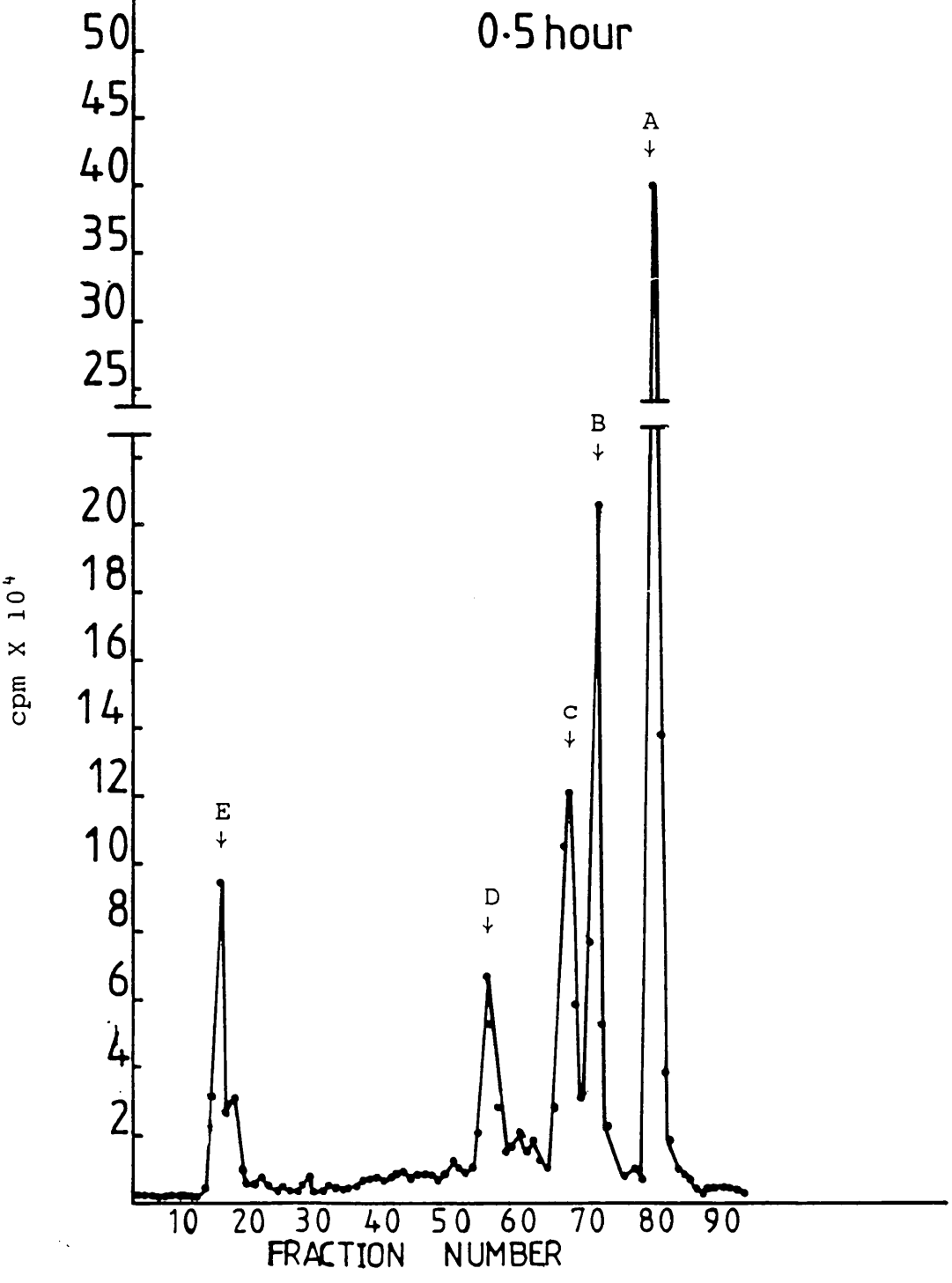


Fig. 38 - HPLC PROFILE DEMONSTRATING THE METABOLISM OF ^{3}H-CCK$_{8}$ IN SITU

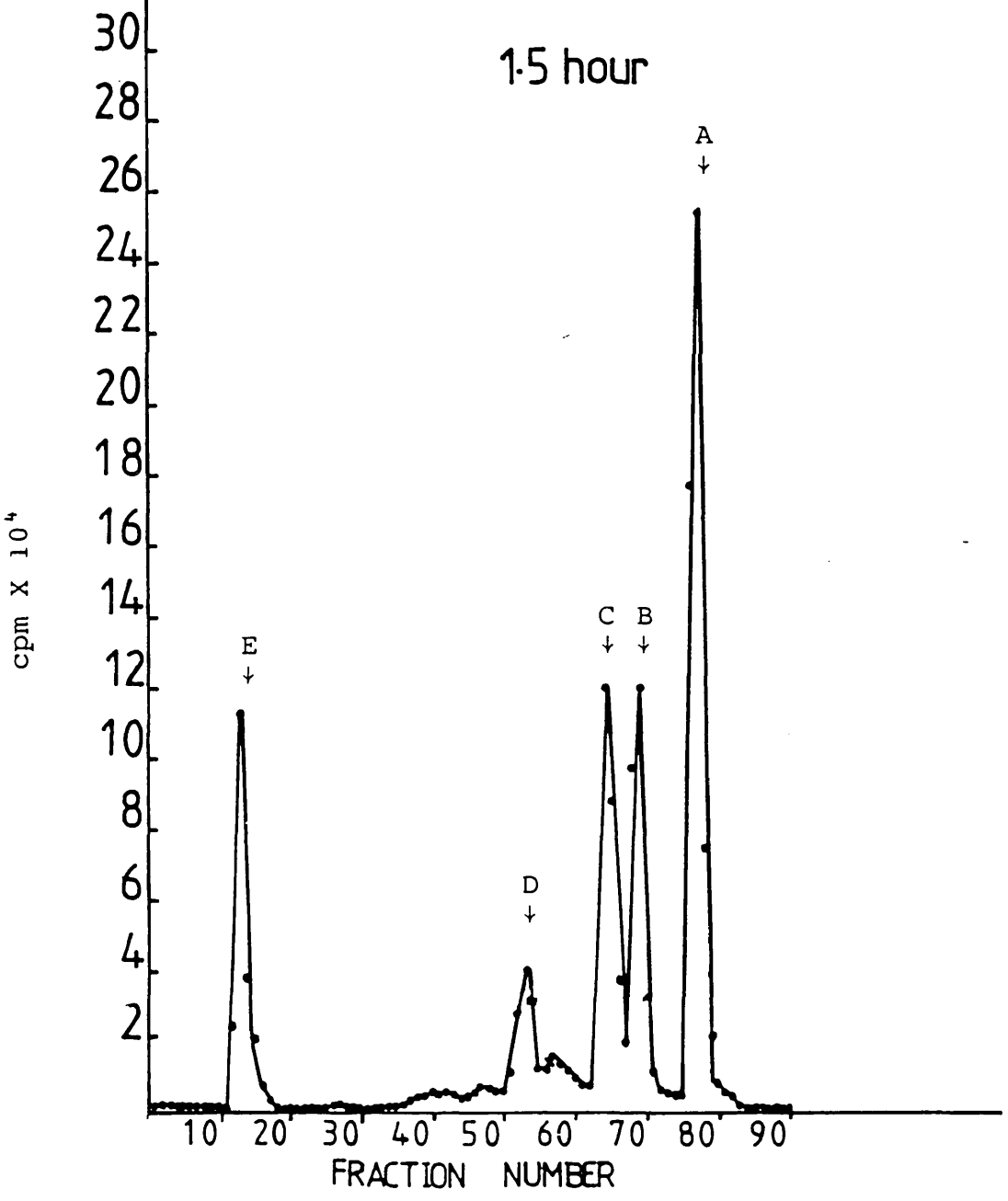


Fig. 39 - HPLC PROFILE DEMONSTRATING THE METABOLISM OF ^{3}H-CCK_8 IN SITU

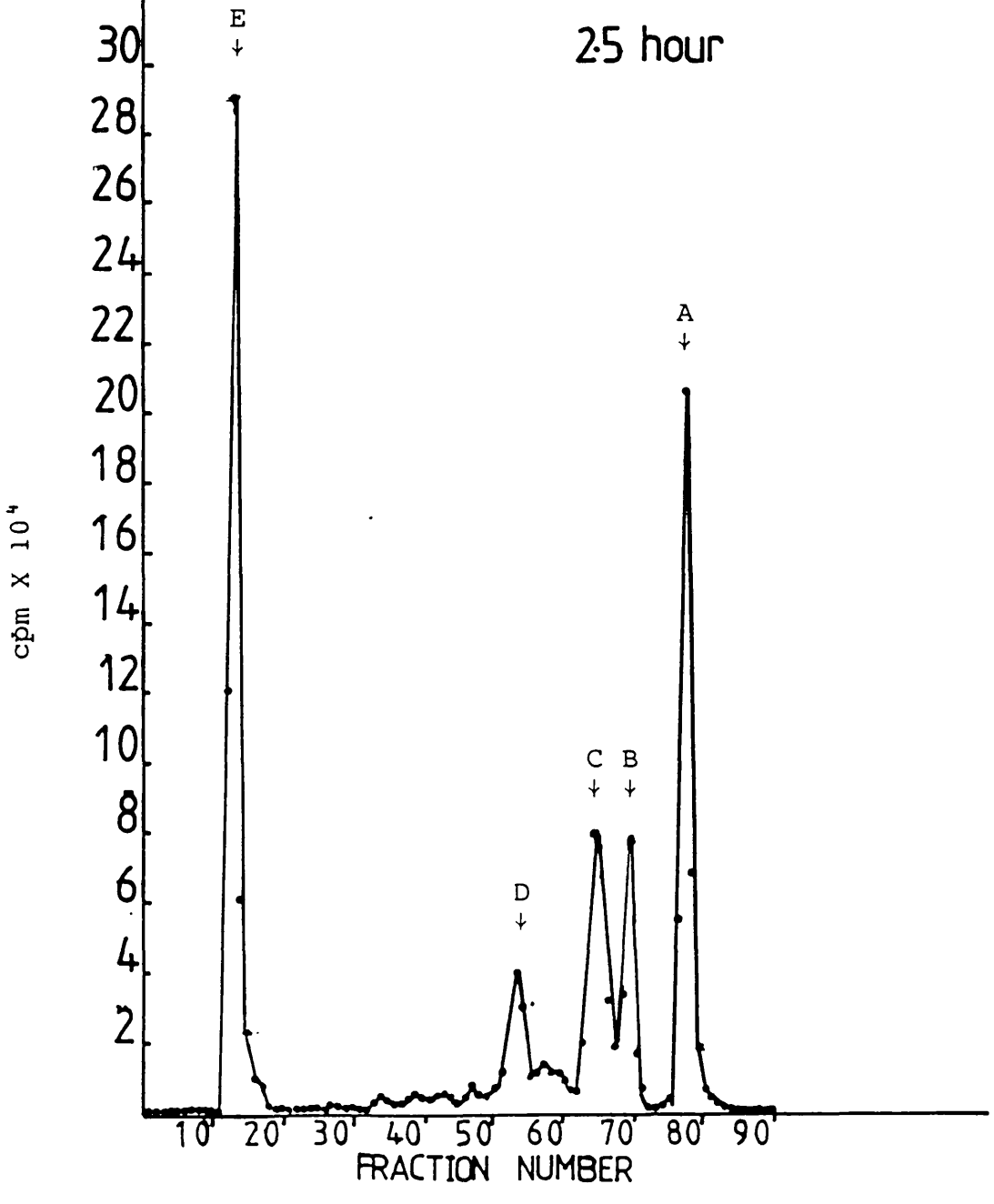


Fig. 40 - HPLC PROFILE DEMONSTRATING THE METABOLISM OF ^{3}H-CCK$_{8}$ IN SITU

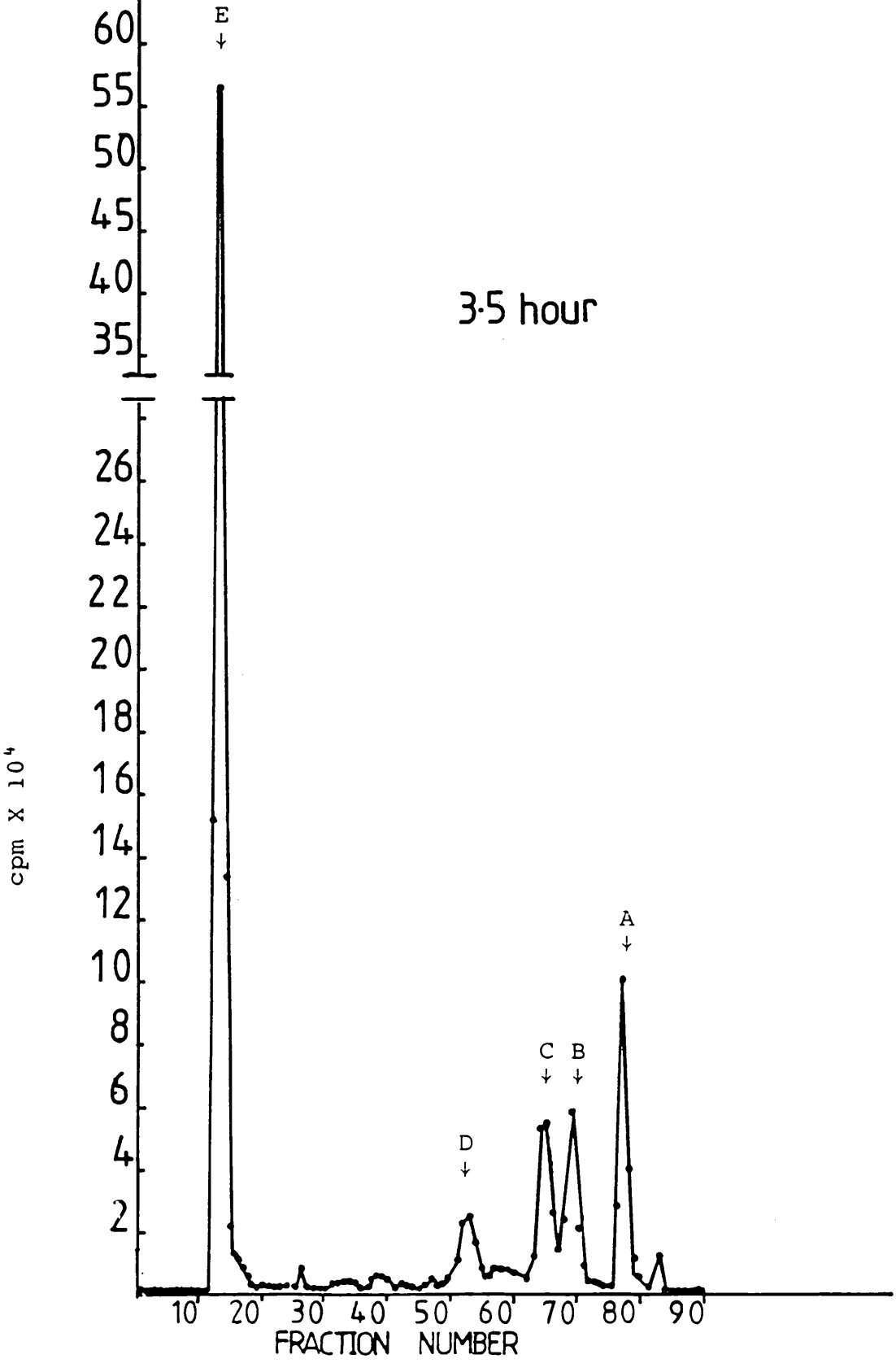


Fig. 41 - HPLC ELUTION PROFILE DEMONSTRATING THE METABOLISM OF ^{3}H-CCK$_{8}$ IN SITU

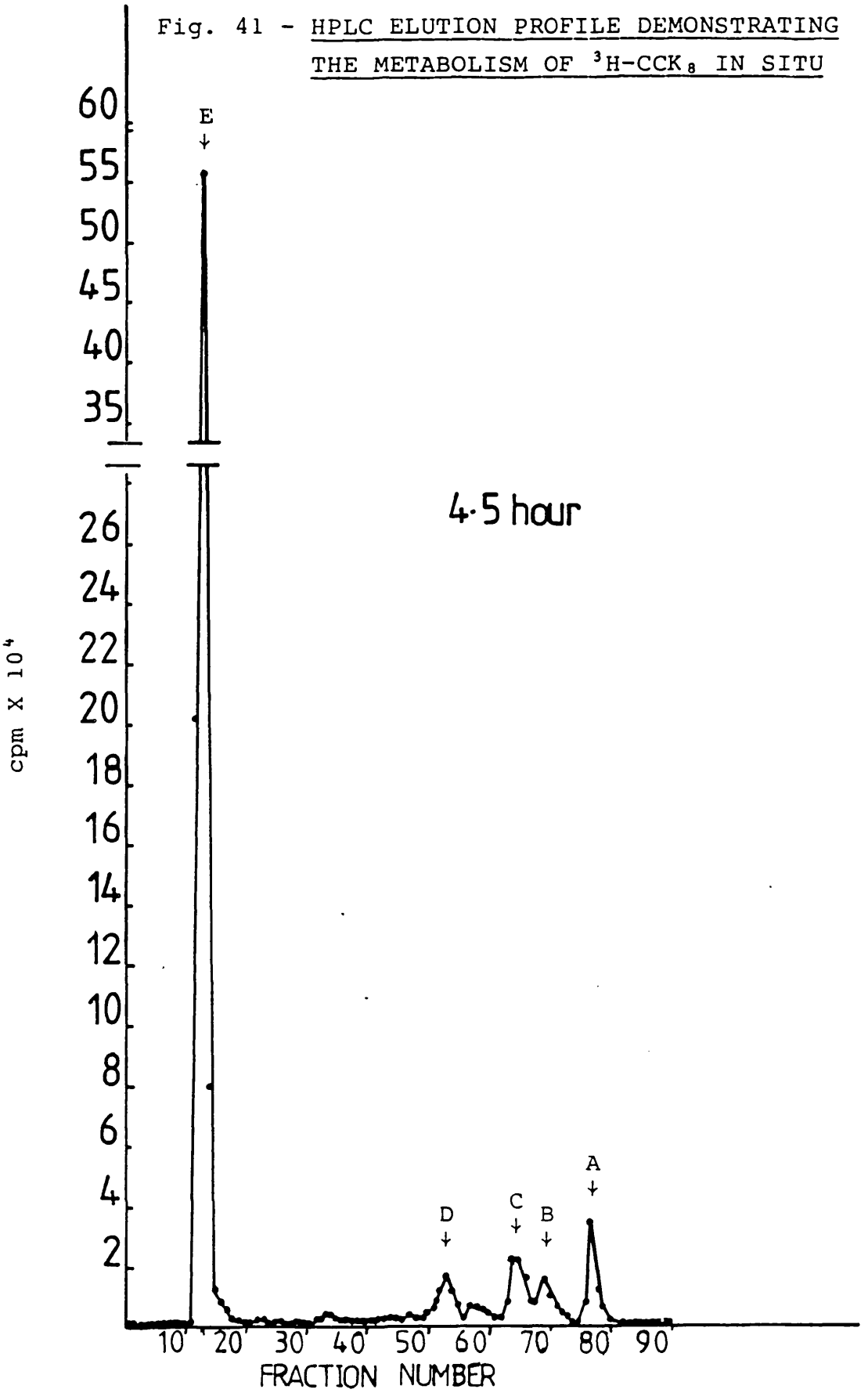


Fig. 42 - HPLC PROFILE OF PERFUSION MEDIUM ALONE

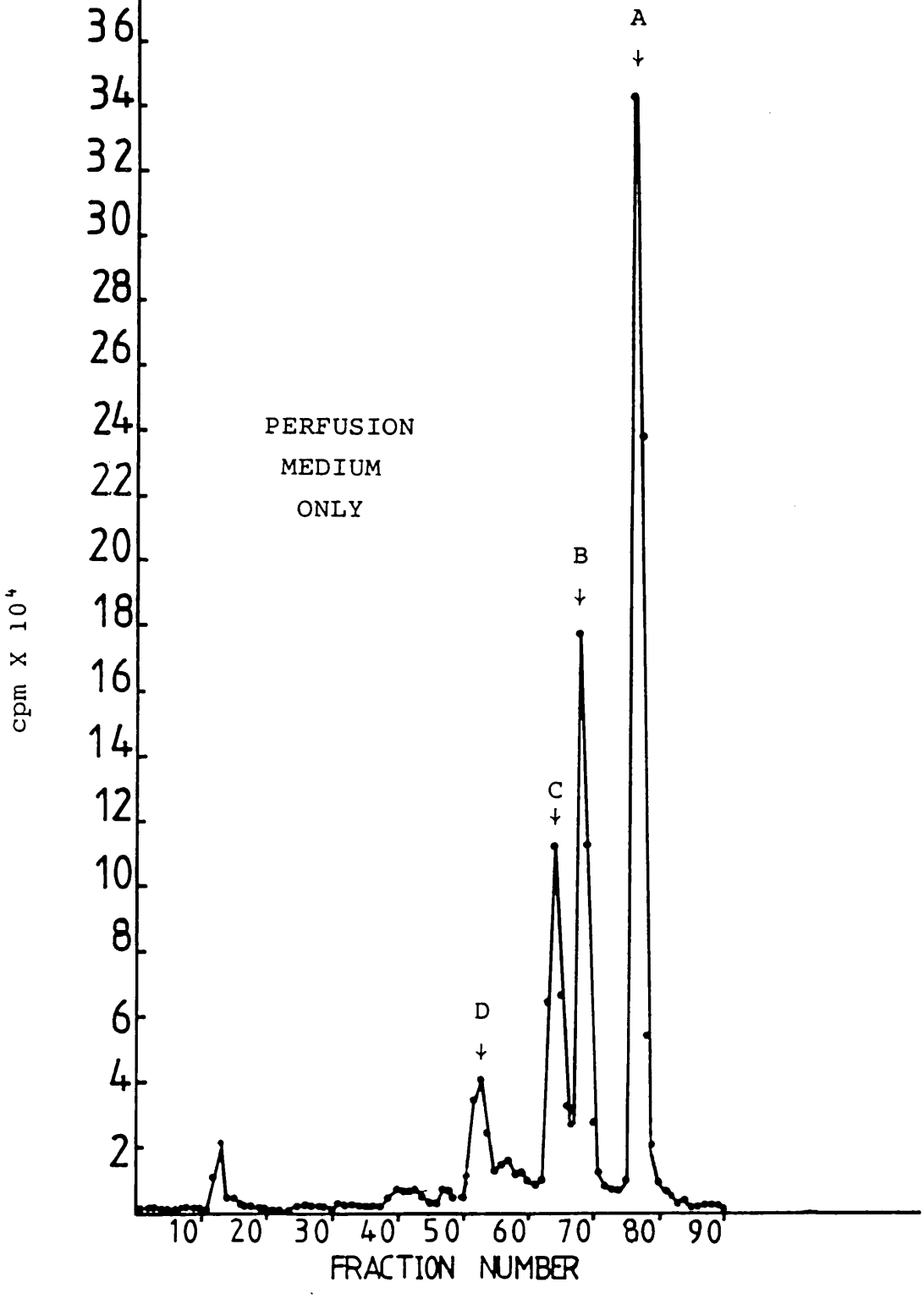
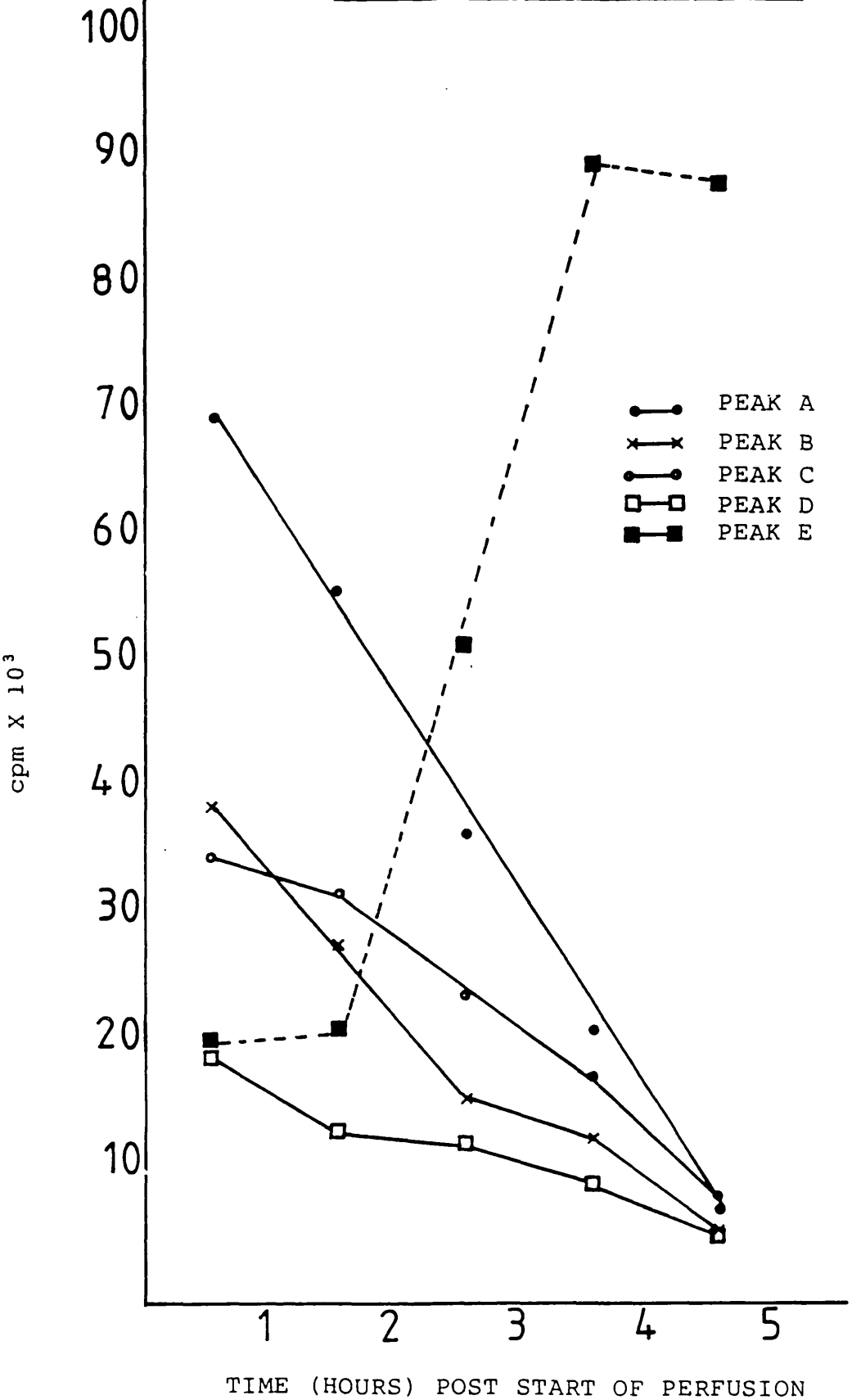


Fig. 43 - THE TIME COURSE OF FORMATION OF METABOLITE OF ^{3}H-CCK_8 IN VIVO



plateau. Also this curve shows that in contrast to peaks B, C and D, the percentage of peak A declined linearly and rapidly with time.

DISCUSSION

The inactivation mechanisms of classical neurotransmitters are well understood, and selective inhibitors of these mechanisms are routinely used in research. In contrast, very little is known about the mechanisms of peptidergic neurotransmission, and consequently there are only a limited number of "tools" available for manipulation of the system. For instance, the mechanism responsible for the synaptic inactivation of classical neurotransmitters has been rather easy to identify because they generally display a high degree of chemical specificity. Acetylcholine, for example, is probably the sole neurotransmitter hydrolysed by acetylcholinesterase, and cathecholamines are taken up in a unique fashion by the high affinity transport systems in the neurons. The identification of a physiologically relevant inactivating "neuropeptidase" is much more difficult because peptidases generally display a broad substrate specificity which depends on the aminoacids present at the labile peptide bonds. Consequently, a given neuropeptide can be hydrolysed by a variety of enzymes, and in addition a given peptidase can act on several neuropeptides.

A method that will allow investigation of inactivation mechanisms of neuropeptides is the use of push-pull cannula, labelled peptides combined with HPLC procedures. This model enables studies on metabolism of neuro-peptides at specific sites in the CNS and importantly the use of the model allows (unlike in vitro methods) the study to be made under the

appropriate physiological conditions. In this research we have shown for the first time the in situ metabolism of the small peptide, ^{3}H-CCK_8. The results clearly show that its rate of degradation is much slower compared to the rate of methionine enkephalin metabolism at the same site. This may not be too surprising since the labelled CCK_8 used for this experiment was blocked at the N-terminus (see fig. 36). There was at the time of purchase no other tritiated labelled CCK_8 available. Yet despite the fact that the free N-terminal amino acid group was blocked, conversion of the ^{3}H-CCK_8 to a more polar compound occurred. Figure 43 depicts the loss of ^{3}H-CCK_8 and the formation of its metabolite. It is not certain from this study whether peak E represents one product or if in fact more than one cleavage product has eluted in the same area. There are no reports on peptidases specific for the cleavage of CCK_8, and no information can be obtained on the nature of the peptidase(s) involved in CCK_8 metabolism in this study. However, recently Deschodt-Lanckman et al. (1981) have reported that bacitracin, 1,10-phenanthroline and p-chloro-mercuri benzoate all greatly reduced the action of amino peptidases on CCK_8 in the rat brain synaptosomal fractions, and that N-terminal acetylation totally protected the octapeptide from degradation. Yet in spite of the use of bacitracin, bestatin and thiorphan in the perfusion medium in our experiment, substantial inactivation of a ^{3}H-CCK_8 similarly N-terminally blocked, occurred.

It is known that as well as passive diffusion there are two active mechanisms whereby the action of a classical neuro-transmitter can be terminated after its synaptic release. These are enzymatic degradation (e.g., inactivation of acetyl-

choline by acetylcholinesterase) and high-affinity uptake systems (e.g., inactivation of biogenic amines, γ amino-butyric acid and amino acid transmitters). Since there is no evidence to indicate the inactivation of CCK_8 by uptake mechanisms, it is reasonable to assume that enzymatic degradation plays a key role in its inactivation. This rationale is further corroborated by our findings in this study which demonstrate quite unmistakably, the loss of the ^3H-CCK_8 parent peptide peak, concomitant with the formation of a metabolite (fig. 43).

The next section will describe an experiment conducted to determine the level of CCK_8 present in cisternal CSF. The rationale used in the experiment was based on the assumption that if it were possible to detect CCK_8 in the cisternal CSF of the rat, it may also be possible to detect it in the third ventricular perfusate samples.

III. DETERMINATION OF CCK_8 IMMUNOREACTIVITY IN CISTERNAL CSF.

METHOD

Male Sprague-Dawley rats were anaesthetised with nembutal, and a midline incision was made over the occipital area. Following the incision, the animals were placed in a lateral position with the head flexed downward at the neck. A 25 gauge needle was prepared by reducing the length of the bevel and drilling a small hole near the tip of the bevel. This needle was then attached to a length of tubing. While the head of the rat was kept in a flexed position the needle was inserted into the cisternal space, and CSF was then transferred to sterilin tubes on ice. The cisternal CSF from 9 - 11 rats were pooled and subsequently freeze-dried. This experiment was

repeated four times.

HPLC

The dried perfusate samples were resuspended in HPLC starting buffer and chromatographed on reverse phase μ Bondapak C18 columns. (The conditions and details are given in the general methods section).

RIA

The samples eluting in the same position as the synthetic CCK_8 standard were removed and assayed for CCK_8.

RESULTS

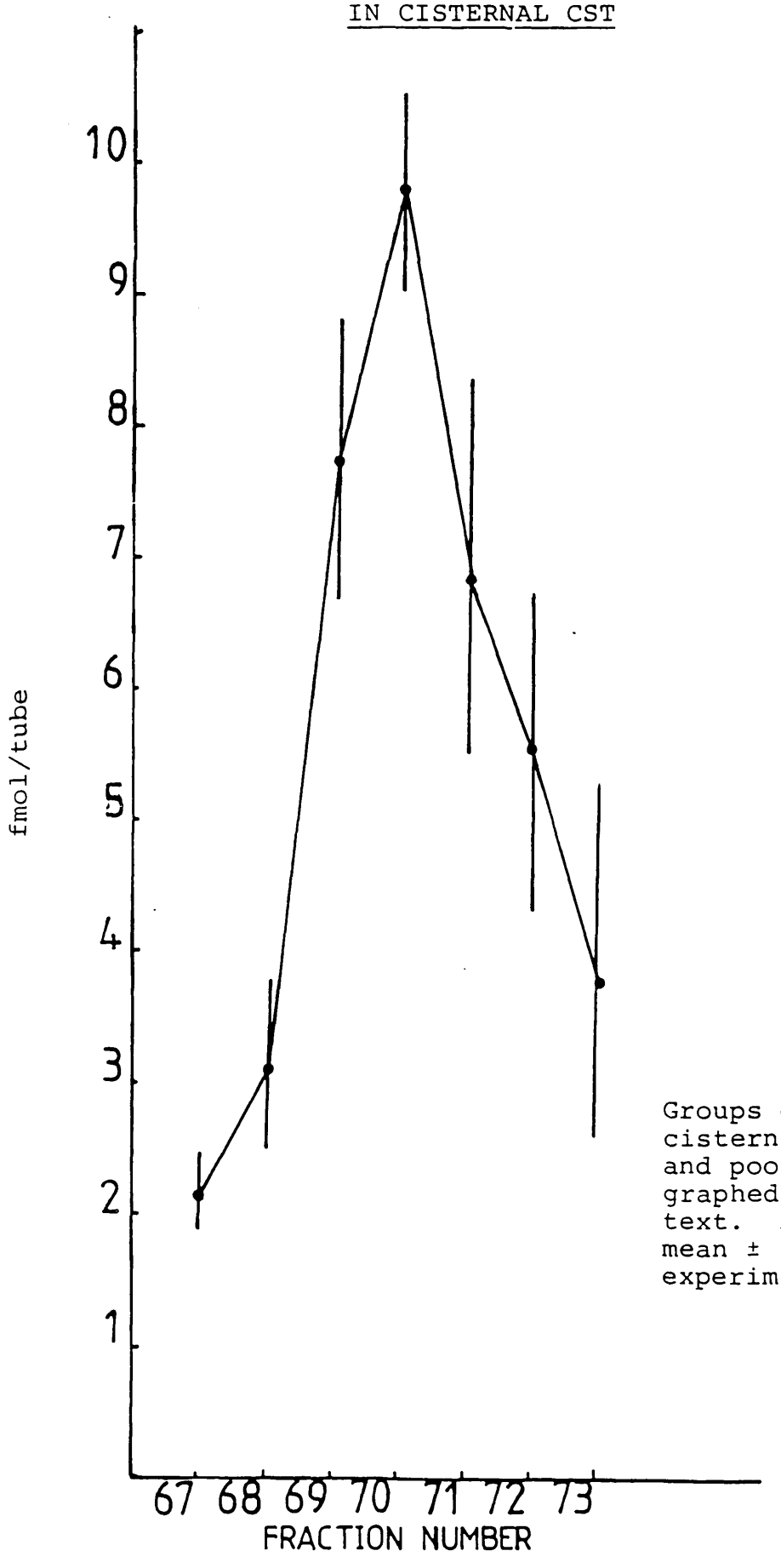
Fig. 44 shows the HPLC elution profile of CCK_8 immunoreactivity present in the cisternal CSF. It can be seen that the level of CCK_8 present in rat cisternal CSF is approximately 10 fmol./ml.

DISCUSSION

We considered it important to demonstrate CCK_8 presence in the CSF before proceeding to investigate its levels in the third ventricular perfusate samples. This is the first demonstration of the presence of CCK_8 in rat cisternal CSF. We have shown that it is present in very low but detectable quantities (10 fmol./ml). Although the CSF was withdrawn at the level of the cisterna magna, the precise anatomical origin of CCK_8 is not known. However, our finding is in agreement with the levels reported in human lumbar CSF (Rehfeld and Kruse-Larsen 1978, Gerner and Yamada 1982).

Encouraged by its detection in cisternal CSF, experiments were then conducted to determine the role of CCK_8 in electrically induced analgesia.

Fig. 44 - HPLC ELUTION PROFILE OF CCK_8 IMMUNOREACTIVITY IN CISTERNAL CST



Groups of 9-11 rats cisternal CSF collected and pooled and chromatographed as described in text. Each value is the mean ± sem from four experiments.

IV. EXPERIMENT TO DETERMINE IF STIMULATION OF THE PERIAQUEDUCTAL GRAY ALTERS THE LEVEL OF CCK_8 RELEASED INTO THE THIRD VENTRICLE.

METHOD

The experimental procedure used was identical to that used to investigate methionine enkephalin involvement in electrically induced analgesia. Briefly, rats were implanted with bipolar electrode and push-pull cannula into the PAG and the third ventricle respectively. After a one week recovery period, rats were screened for stimulation-induced analgesia at three intensities (details given previously) using the tail flick test. At two, three and four weeks post surgery, the rats were perfused with 0.9% saline containing 1.8 mm calcium at a rate of 10 µl./minute and the perfusate pooled. Three consecutive one hour samples were collected for each animal which corresponded to prestimulation, during stimulation and post stimulation samples.

HPLC OF PERFUSATE SAMPLES

The samples were chromatographed on a reverse phase µ Bondapak C18 column as described previously. Fractions of 1.0 ml. were collected and then dried under vacuum. Synthetic CCK_8 standard was chromatographed under the same conditions.

RIA

The level of CCK_8 present in the perfusate samples were measured by radio immunoassay.

RESULTS

Representative data obtained from this experiment are shown in figs. 45 - 49. Figs. 45, 46 and 47 show the HPLC immunoreactive profile obtained from three analgesic rats.

Fig. 45 - HPLC IMMUNOREACTIVE PROFILE OF CCK_8 (ANALGESIC RAT)

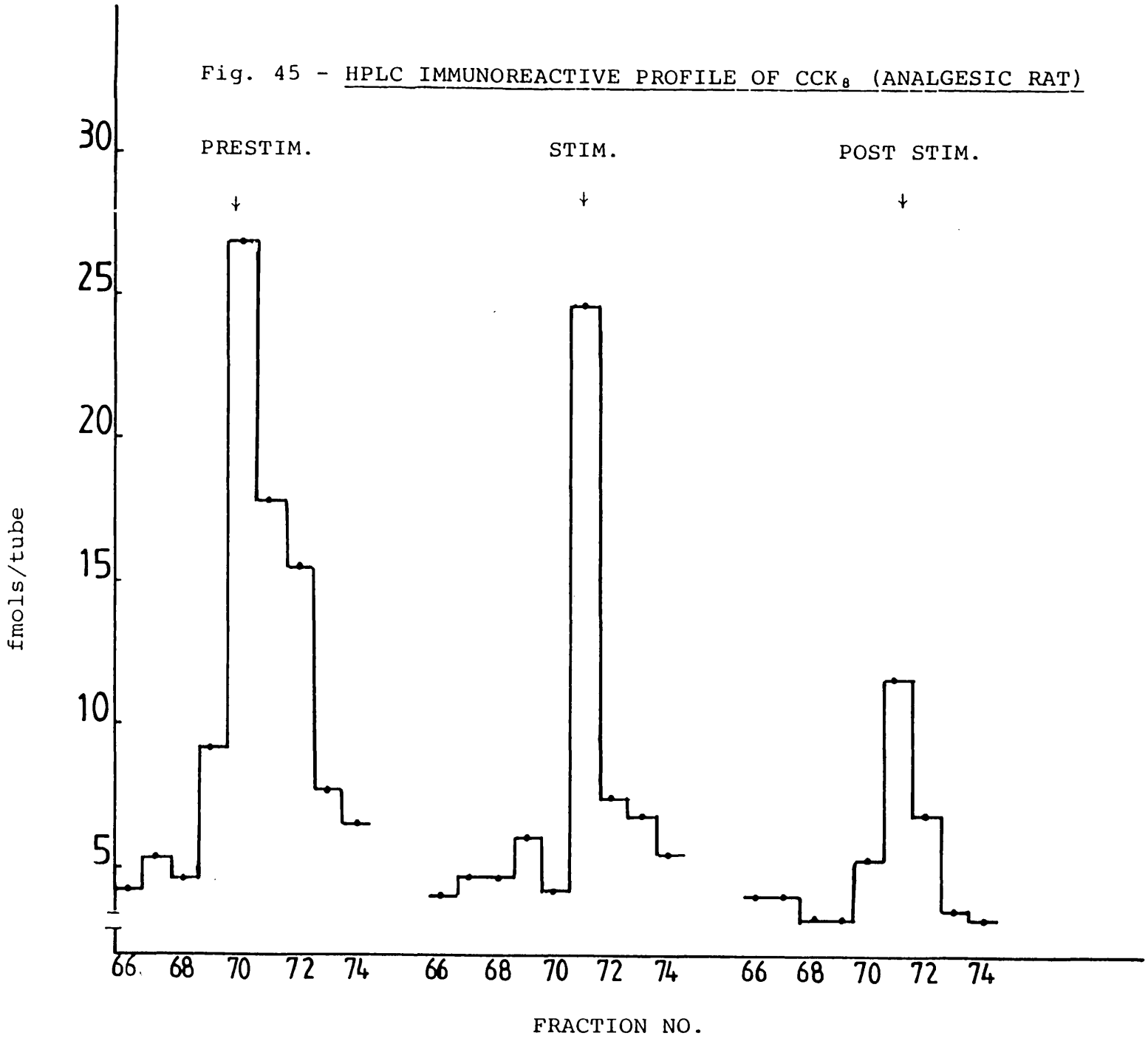


Fig. 46 - HPLC IMMUNOREACTIVE PROFILE OF CCK_8 (ANALGESIC RAT)

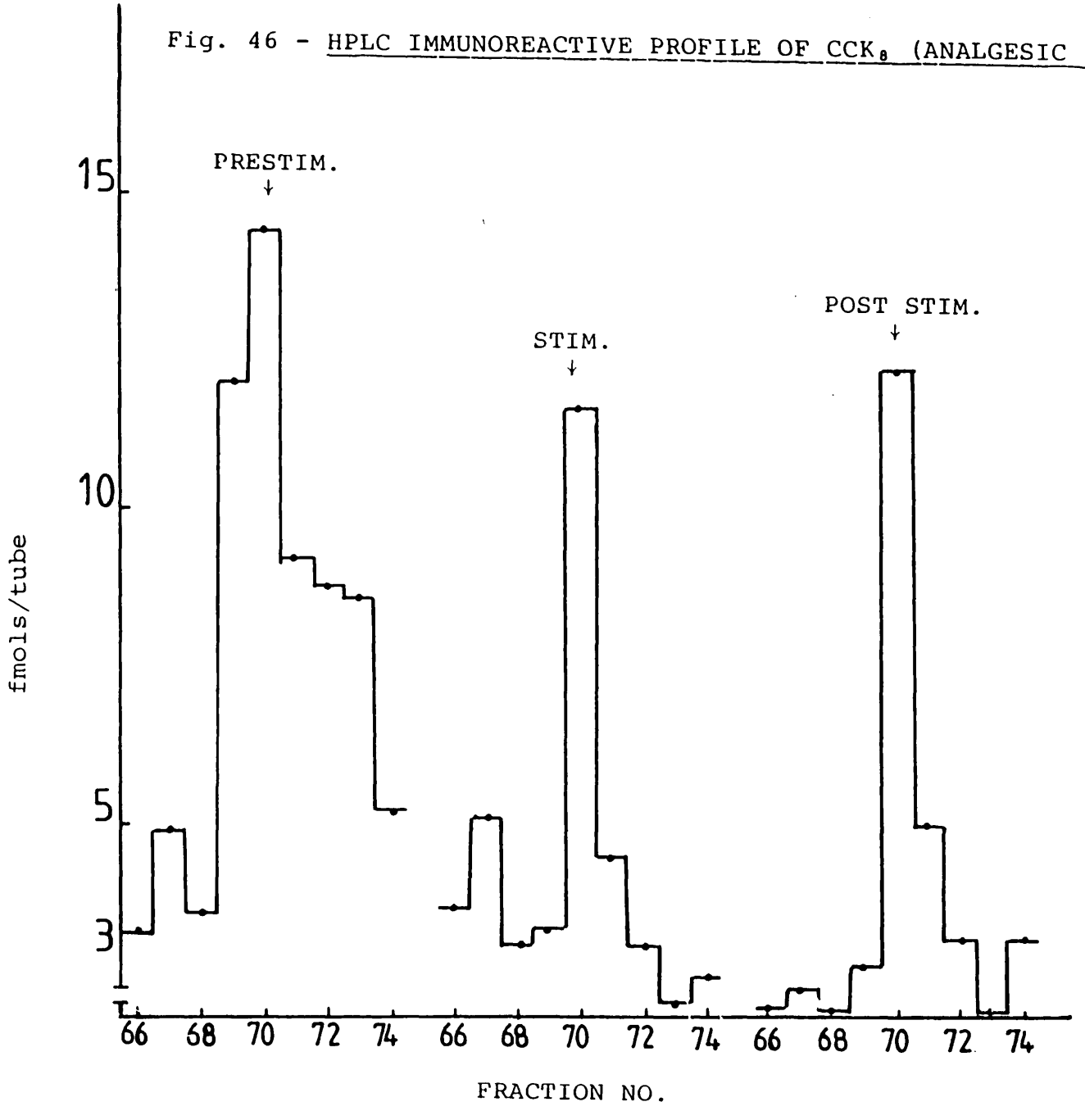


Fig. 47 - HPLC IMMUNOREACTIVE PROFILE OF CCK_8 (ANALGESIC RAT)

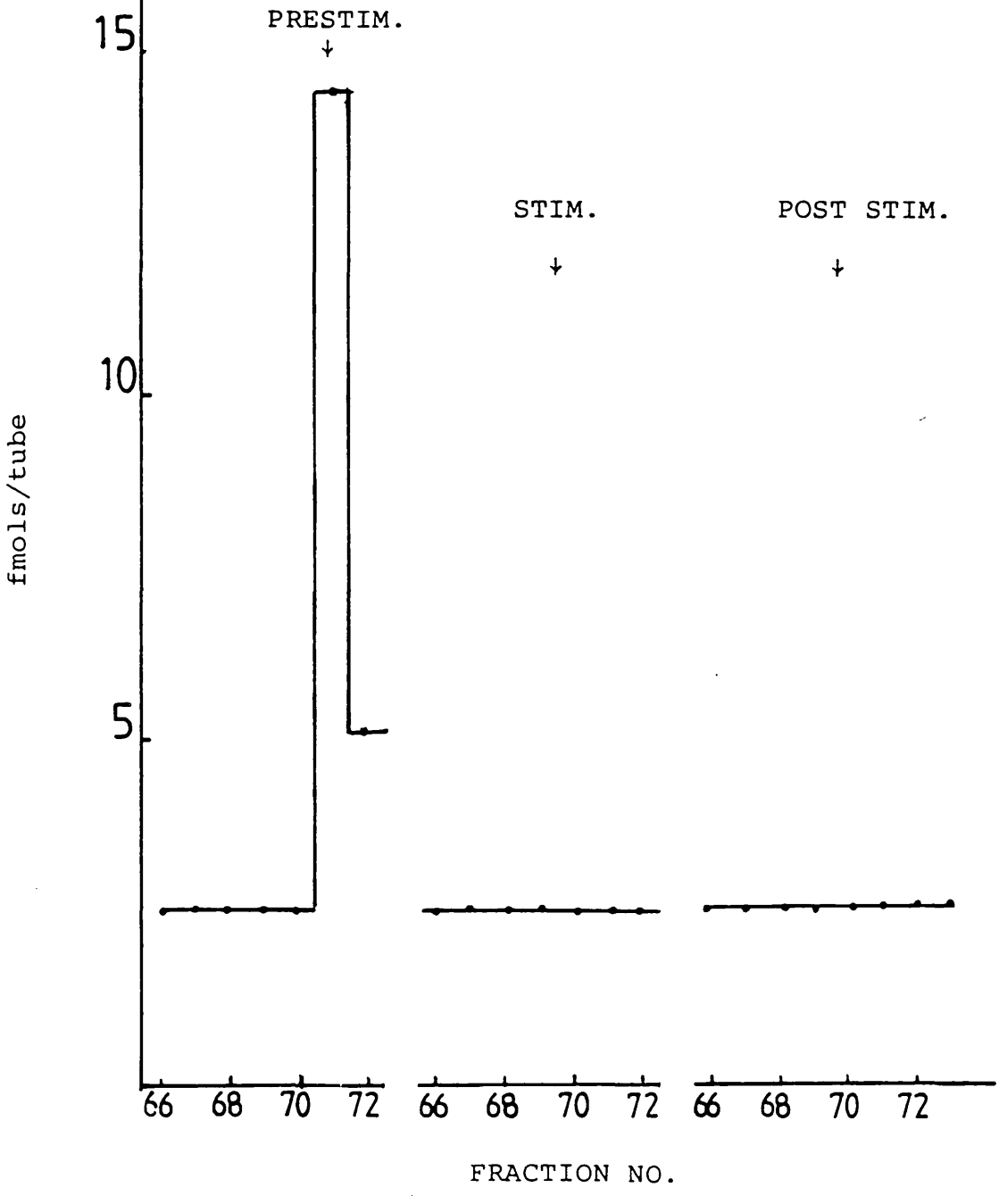


Fig. 48 - THE EFFECT OF TIME ON THE SPONTANEOUS RELEASE OF CCK_8 (CONTROL RAT)

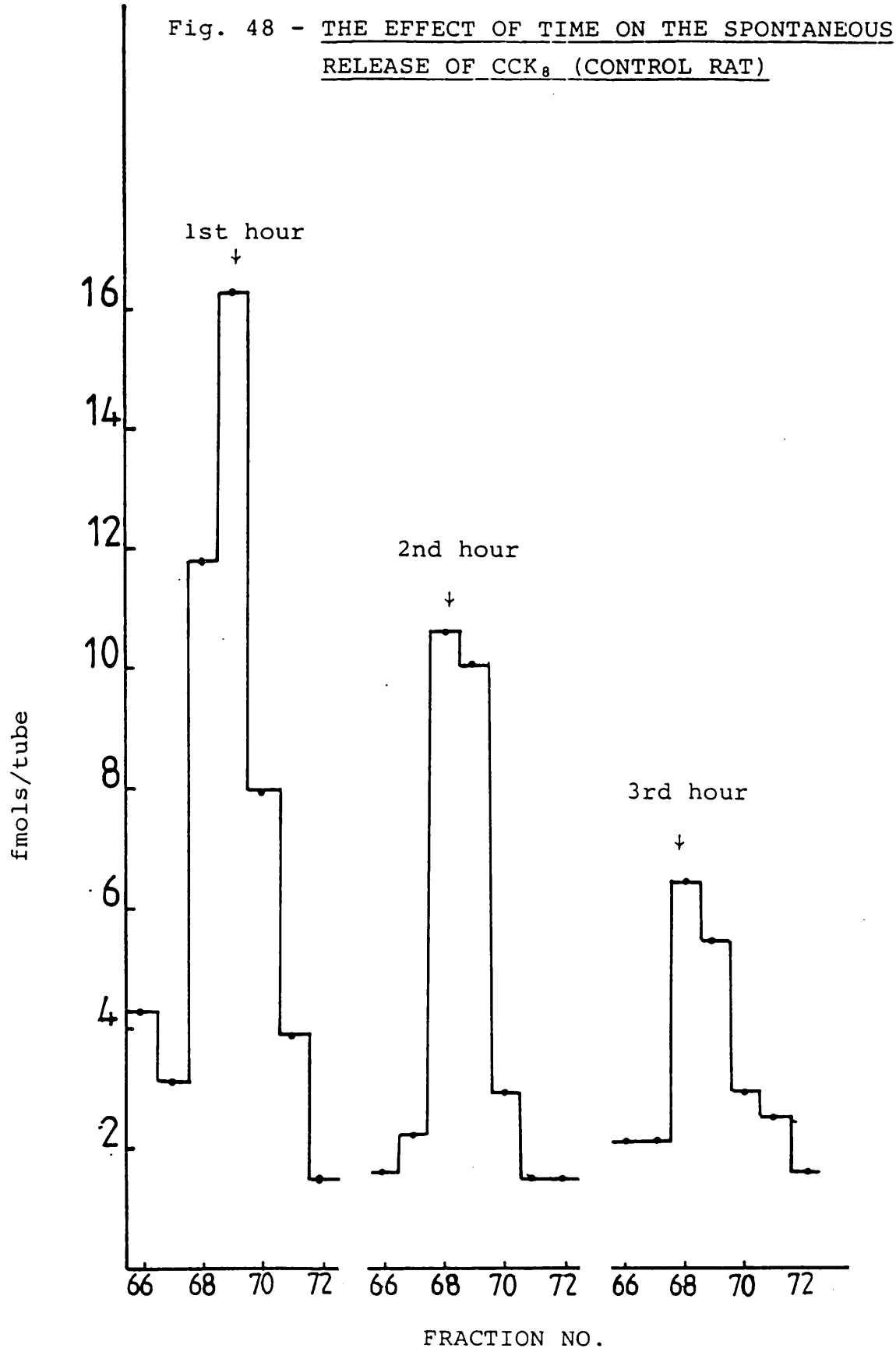
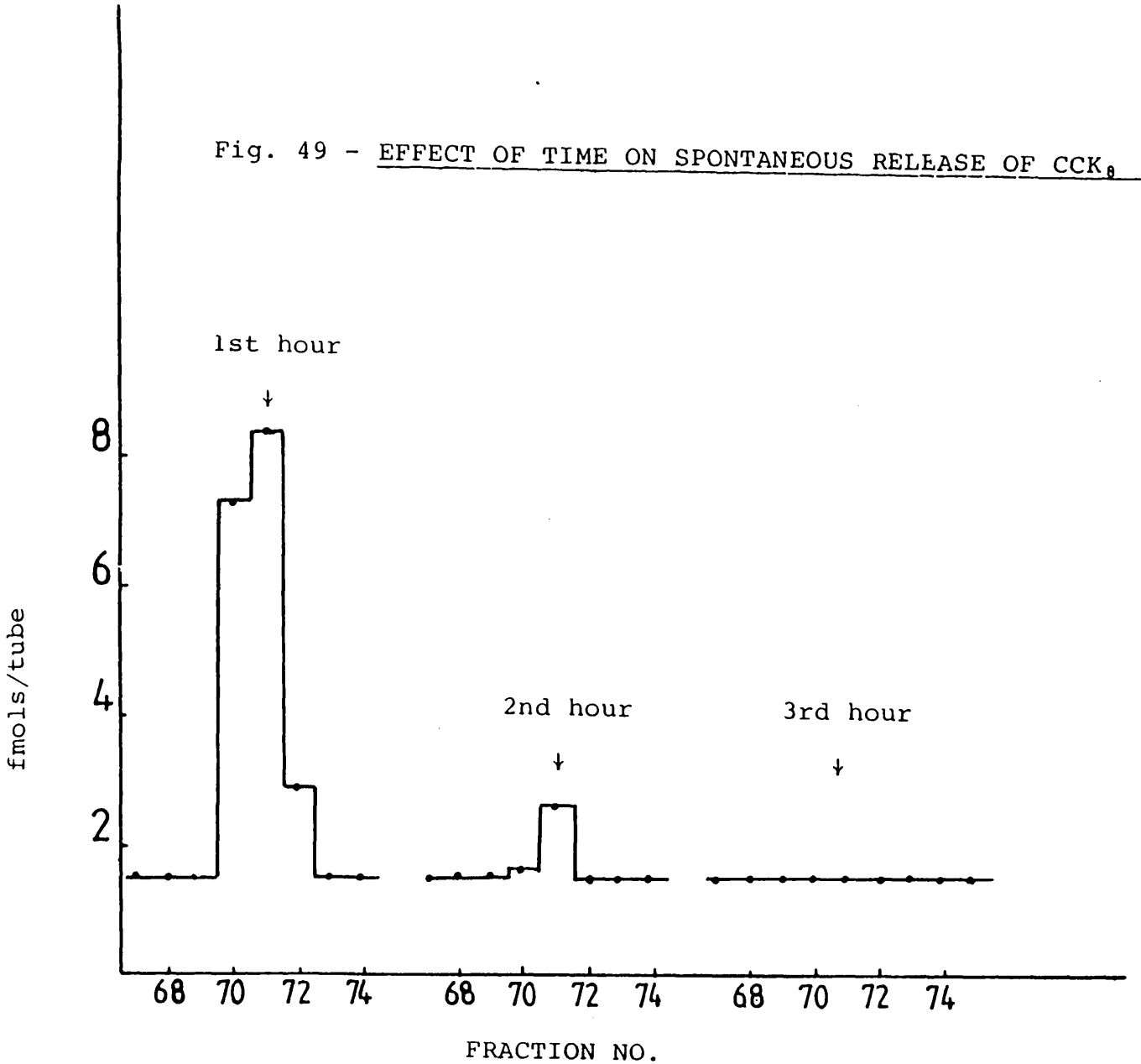


Fig. 49 - EFFECT OF TIME ON SPONTANEOUS RELEASE OF CCK_8 (CONTROL RAT)



Figs. 45 and 47 were obtained from rats that exhibited 100% degree of analgesia. Fig. 46 represents a rat that exhibited 15% degree of analgesia. Figs. 48 and 49 show the HPLC profile obtained from two rats that were not electrically stimulated. Inspection of the results demonstrates that CCK_8 is present in third ventricular perfusate samples in quantities of 8 - 25 fmol./ml. of perfusate. Also the results indicate that the levels of CCK_8 appear to be decreasing with respect to time.

DISCUSSION

Cholecystokinin (CCK) peptides are present in both the brain and gastrointestinal tract. Although many of the functions of intestinal CCK are well documented, those for brain CCK peptides are only recently being investigated. The primary form of CCK in the brain is CCK - octapeptide (CCK_8). The physiological evidence that CCK_8 may be involved in nociception first came from microinjection studies (Zetler 1980). This was recently further substantiated when CCK_8 was shown, both by RIA and immunohistochemistry, to be present in the PAG of rats (Skirboll et al. 1982).

Analysis of the current data suggests that CCK_8 is not released into the third ventricle during PAG stimulation, but that it is present there; probably being released from structures surrounding it. When one compares the data obtained with the analgesic animals to that obtained from animals <u>not</u> electrically stimulated, a rather surprising similarity in trend for the peptide level to decrease with time, is observed. There are two possible explanations for this phenomenon occurring. Firstly, it is possible that there is a dilution

effect occurring, resulting from the use of the push-pull cannula. This is an inherent problem that is presumably most noticeable when dealing with very small quantities of peptide (lower femtomole). Secondly, the peptide stores may have been depleted faster than they were being replenished, that is, CCK_8 might be removed from the third ventricle (by as yet unknown mechanisms) faster than it is being replaced. In addition, these effects might in fact be occurring simultaneously, thus masking the actual effects of PAG stimulation.

Interestingly, our results show that the levels of CCK_8 present in third ventricular perfusate are similar to the levels reported in the cisternal CSF. The physiological role of CCK_8 at both of these regions is not clear from our studies. However, recently it has been demonstrated that lateral ventricular infusion of antibody to CCK_8 in amounts that would bind a few picograms of CCK_8 per minute, caused a significant increase in food intake in satiated sheep (Della-Fera et al. 1981).

This work highlights the difficulties involved in working with femtomole quantities of peptides, amounts that are very near to the detection limit of the assay. However, despite the drawbacks, we can infer that unlike the results obtained in the enkephalin experiment, the negative data reported here are not due to rapid enzymatic destruction of the peptide. We can also infer that electrical stimulation of the PAG does not increase the levels of CCK_8 in the third ventricle.

A useful animal model has been developed in this project to investigate the analgesic mechanisms resulting from electrical stimulation of the PAG. The ultimate function of any model is to reproduce accurately the natural process or

processes that are being investigated. It is envisaged that information obtained from the use of such models of antinociception will enable a better understanding of the mechanisms involved during analgesia; thus contributing to a better interpretation of data obtained in similar studies conducted in humans. But how closely does our animal model reflect the situation in humans? The work in this thesis has demonstrated the following:

(a) The use of the model has enabled a reproducible generation of analgesic states in a quantifiable manner in the conscious unrestrained, and therefore unstressed, rat.

(b) Despite indirect evidence that methionine enkephalin, a peptide that has mainly inhibitory actions, is directly involved in analgesia, we have demonstrated that, for reasons mentioned in earlier chapters, it is extremely difficult to detect its presence in third ventricular perfusate samples in the rat.

(c) Cholecystokinin octapeptide, a peptide that has mainly excitatory actions, although present in both cisternal and third ventricular fluid, is not, apparently, altered upon electrical stimulation of the PAG.

A natural progression from work in this thesis would be to compare data on <u>human</u> CSF using HPLC/RIA approach. Time and difficulties obtained permitted only an initial experiment on one series of human CSF samples. These samples were obtained from Dr. John Miles at the Pain Foundation Clinic in Liverpool several months before they were used for the analytical investigation. The CSF was obtained from a chronic pain patient receiving electrical brain stimulation (PAG) for the relief of the pain. It was hoped that such research would

answer the question concerning the validity of the animal model to reflect accurately changes occurring in humans.

The next chapter describes experiments using the human CSF samples. The CSF was sampled from the third ventricle.

CHAPTER 7

INVESTIGATION INTO NEUROCHEMICAL CHANGES OCCURRING IN CSF OBTAINED FROM A CHRONIC PAIN PATIENT

METHOD

On arrival, the CSF samples were freeze-dried in silanised pear-shaped flasks and kept at -20 C until the time of use. The samples comprised CSF obtained at the following times post stimulation of the PAG:

(1) 0 hours
(2) 0.5 hours
(3) 5 hours

The samples were chromatographed using the reverse phase HPLC system described previously.

RIA FOR METHIONINE ENKEPHALIN

The samples for methionine enkephalin assay were resuspended in an 80% MEOH:water mixture and then incubated overnight in hydrogen peroxide (details given in Chapter 5) to oxidise it to methionine enkephalin sulphoxide. All fractions were then dried and assayed for methionine enkephalin sulphoxide in an identical manner to the rat perfusate samples.

RIA FOR CCK_8

The samples for CCK_8 determination were also assayed in an identical manner to the rat perfusate samples.

DETERMINATION OF 5HIAA AND HVA

Concurrent analysis of endogenous concentrations of the biogenic amine metabolites was conducted using HPLC with electrochemical detection (LCEC).

RESULTS

CCK

Figs. 50, 51 and 52 show the HPLC profile obtained from chromatographing the CSF samples. These figures also show the data obtained for CCK_8 determinations in the samples. As can be seen, CCK_8 is present in the ventricular CSF samples in very low but detectable quantities (approximately 2 fmol./ml.). The levels do not change upon electrical stimulation of the PAG (fig. 51).

5HIAA AND HVA

Fig. 53 shows the data obtained for 5HIAA and HVA determination. Inspection of this data reveals instantly that both 5HIAA and HVA are present in much higher quantities compared with the peptide CCK_8. In addition, it is clear that at thirty minutes post stimulation, the levels of HVA have increased significantly compared to prestimulation levels, and interestingly this increased level has returned to prestimulation levels at five hours post stimulation. The data for 5HIAA also shows a trend to increase upon stimulation of the PAG, and in addition the level remains elevated even at five hours post stimulation.

METHIONINE ENKEPHALIN

No methionine enkephalin immunoreactivity was detected in the CSF samples.

DISCUSSION

It is well established that the concentrations in CSF of 5hydroxy indoleacetic acid (5HIAA) and homovanillic acid (HVA) reflect, to a certain extent, turnover of their parent amines

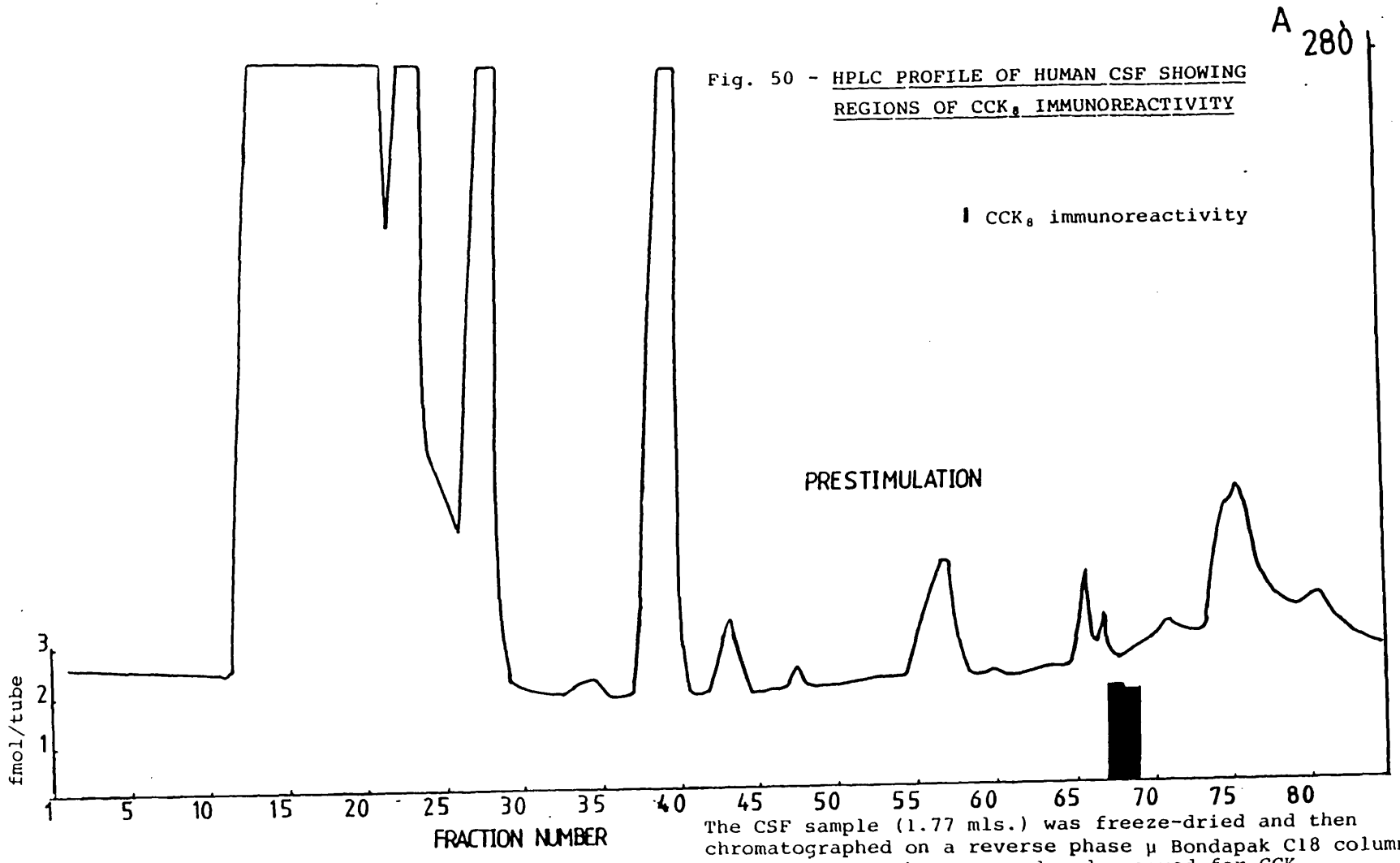
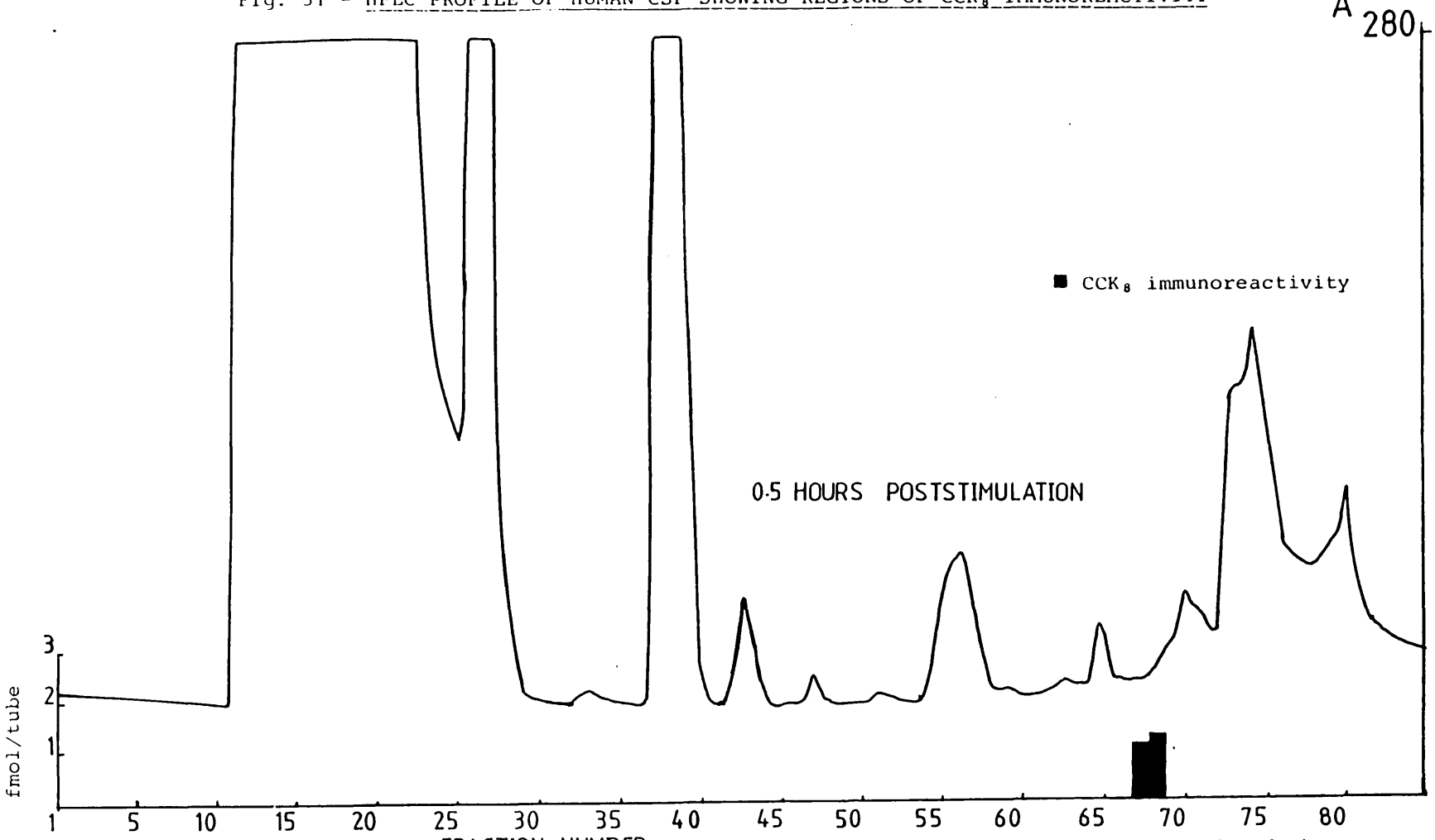


Fig. 50 - HPLC PROFILE OF HUMAN CSF SHOWING REGIONS OF CCK_8 IMMUNOREACTIVITY

▮ CCK_8 immunoreactivity

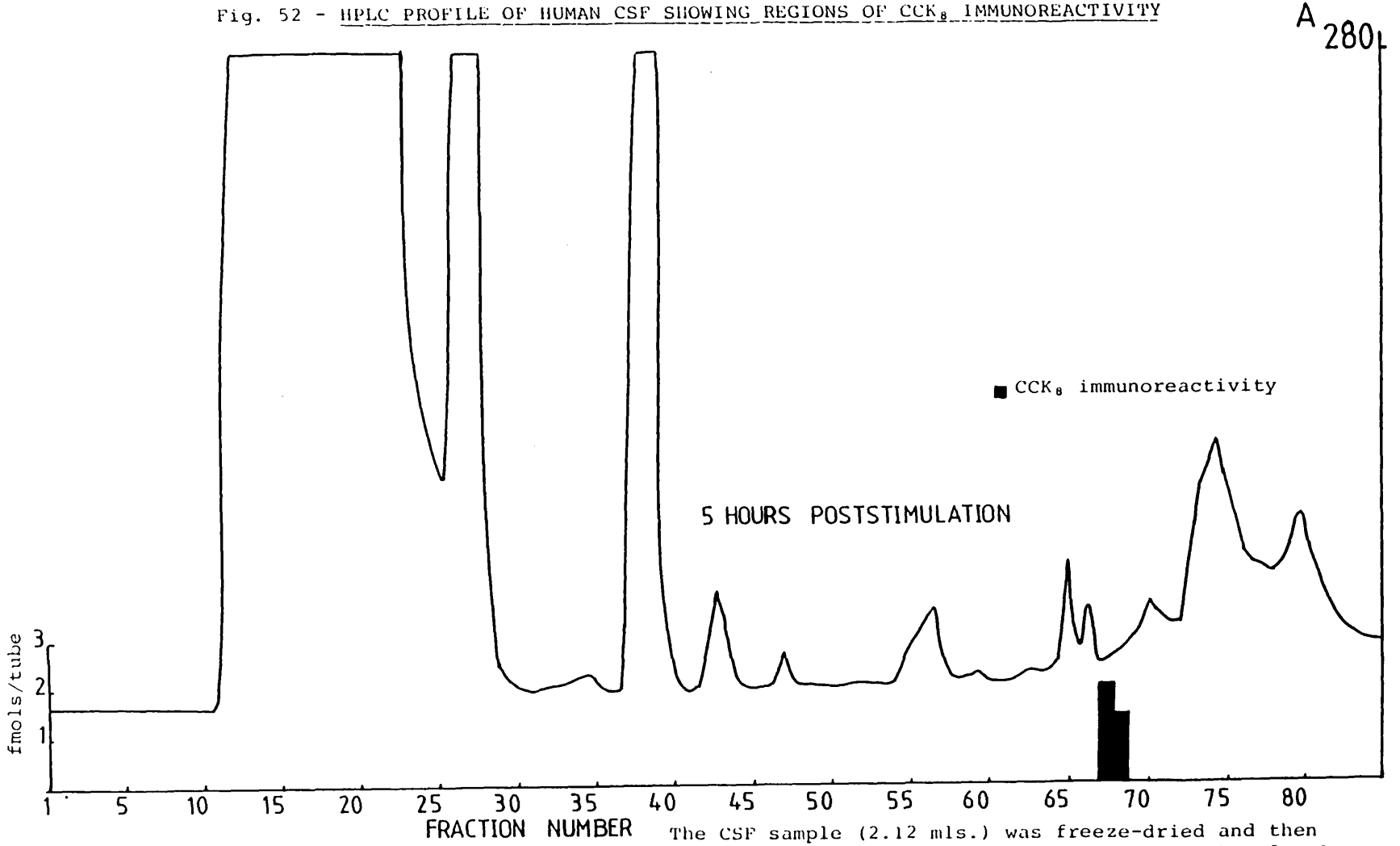
The CSF sample (1.77 mls.) was freeze-dried and then chromatographed on a reverse phase μ Bondapak C18 column. Fractions were then removed and assayed for CCK_8

Fig. 51 - HPLC PROFILE OF HUMAN CSF SHOWING REGIONS OF CCK_8 IMMUNOREACTIVITY



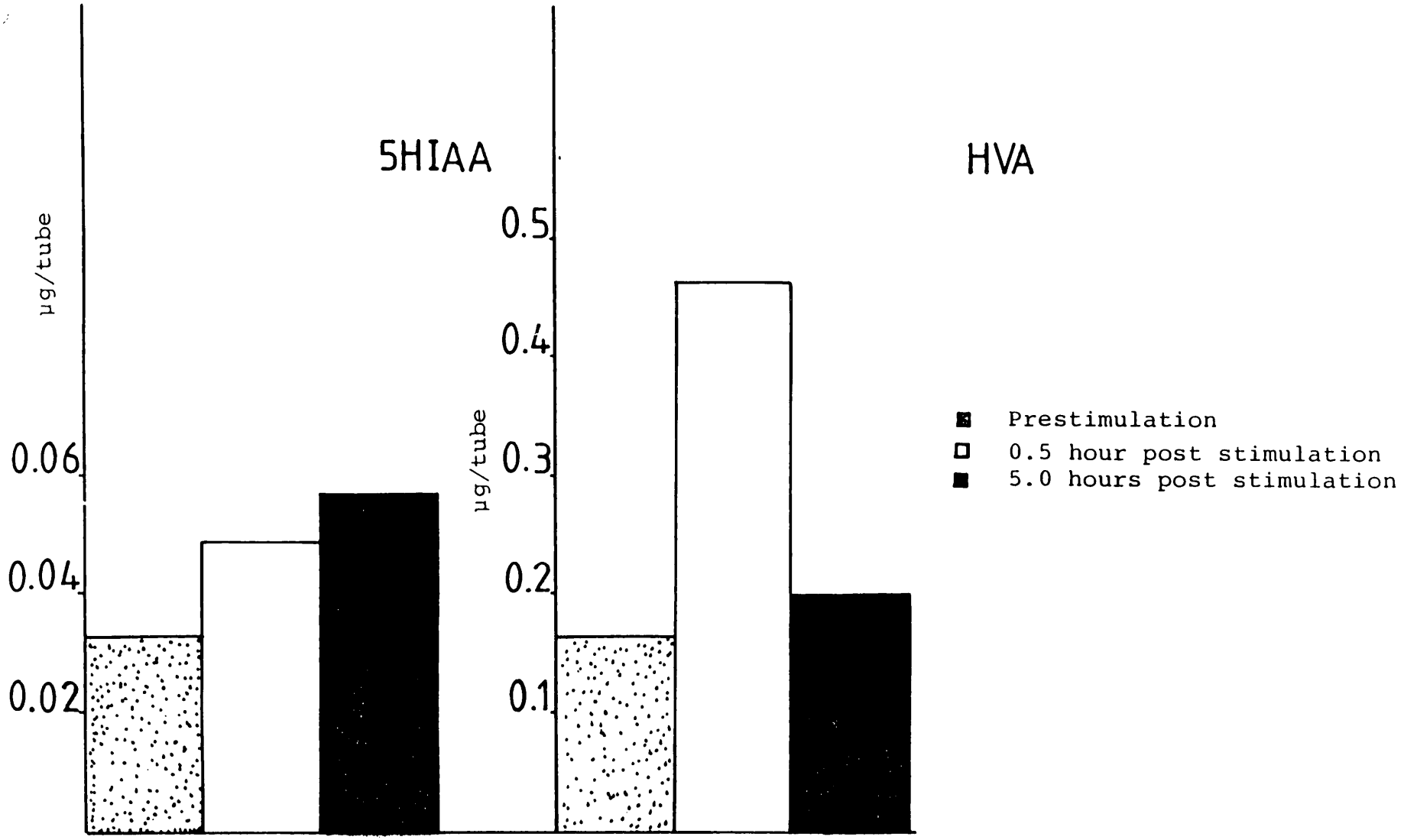
The CSF sample (2.77 mls.) was freeze-dried and then chromatographed on a reverse phase μ Bondapak C18 column. Fractions were then removed and assayed for CCK_8

Fig. 52 - HPLC PROFILE OF HUMAN CSF SHOWING REGIONS OF CCK_8 IMMUNOREACTIVITY



The CSF sample (2.12 mls.) was freeze-dried and then chromatographed on a reverse phase μ Bondapak C18 column. Fractions were then removed and assayed for CCK_8

Fig. 53 - 5HIAA AND HVA DETERMINATIONS IN HUMAN CSF (PAIN PATIENTS)



(5HT and dopamine respectively) in the CNS. Until recently the lack of sensitivity of analytical techniques dictated the use of radioactive tracers in order to measure the small amounts of biogenic amines and their metabolites in reasonable volumes of CSF. The recently developed technique of combining high performance liquid chromatography with electrochemical detection (LCEC) makes possible direct measurement of endogenous amines and their metabolites in CSF.

There is strong evidence implicating 5HT (serotonin) in pain modulating pathways in the CNS. For example, increasing serotonergic neurotransmission in raphe spinal neurones by pharmacological or electrophysiological manipulations reduces sensitivity to noxious stimulation (Messing and Lytle 1977, Berge 1982), and injection of 5HT into the lumbar subarachnoid space produces a behaviourally defined analgesia in rats, cats and rabbits (Yaksh and Wilson 1979). Peripheral application of 5HT is, however, reported to produce pain in humans (Armstrong et al. 1952). Thus it is apparent that central and peripheral administration of 5HT can have different effects on pain sensitivity.

Dopamine is classically associated with motor disorders such as Parkinson's Disease. However, recently other roles have been suggested. Although it is well established that descending serotonergic systems are involved in nociceptive mechanisms, there is also increasing evidence implicating the central catecholamine systems in these mechanisms. For instance, Akil and Liebeskind (1975) reported that by independently manipulating dopamine and noradrenaline activity, reduced brain levels of dopamine were associated with decreased efficacy of stimulation-induced analgesia evoked by PAG

stimulation. They further demonstrated that the effect was reversed by dopamine receptor stimulating agents.

Several groups have recently demonstrated the co-existence of dopamine and CCK_8 in the same neurons (Hökfelt et al. 1980, Studler et al. 1981), and in addition it has been demonstrated that dopamine modulates CCK_8 release in vitro (Meyer and Krauss 1983). The physiological significance of these findings remains to be elucidated. It has been reported that intrathecal (i.t.) infusion of the dopamine agonist, apomorphine, caused analgesia as measured in the tail flick test in spinal rats and that this effect was counteracted stereoselectively by dopamine antagonists (Jensen and Smith 1982). Interestingly it was also shown that i.t. infusion of apomorphine had no analgesic effect in rats with an intact neuraxis. This suggests that descending neuronal pathways inhibit spinal dopaminergic mechanisms. In addition, Jensen and Smith (1983) have recently reported that 5HT mechanisms influence spinal dopamine analgesia.

Our results on the determination of HVA and 5HIAA in the ventricular CSF of a chronic pain patient show that upon electrical stimulation of the PAG, there is an increase in both 5HIAA and HVA compared to prestimulation levels. In addition, at five hours post stimulation the increase of 5HIAA is still evident, yet the levels of HVA have returned almost to prestimulation levels. We can infer from these results that dopamine is released as a direct result of PAG stimulation, but that once released its actions are acute. However, the long term relief of the pain is probably accomplished by the release of 5HT. It is not possible to determine the metabolic or anatomical origin of 5HIAA or HVA from this study, and

obviously further investigations are necessary to clarify dopamine's role in analgesia.

The absence of methionine enkephalin in the CSF samples is rather intriguing since Akil and coworkers (1978) have reported an elevation in "enkephalin-like material" in the ventricular CSF of pain patients after electrical stimulation of periventricular sites. However, the lack of its detection may not be altogether surprising since as mentioned previously the CSF samples used in this study were stored for some months before use. Thus it is possible that in spite of the fact that the samples were stored freeze-dried at -20^0 C during that period, substantial degradation of enkephalin could have occurred. Similarly, the low levels of CCK_8 present in the same samples may be a direct result of its degradation.

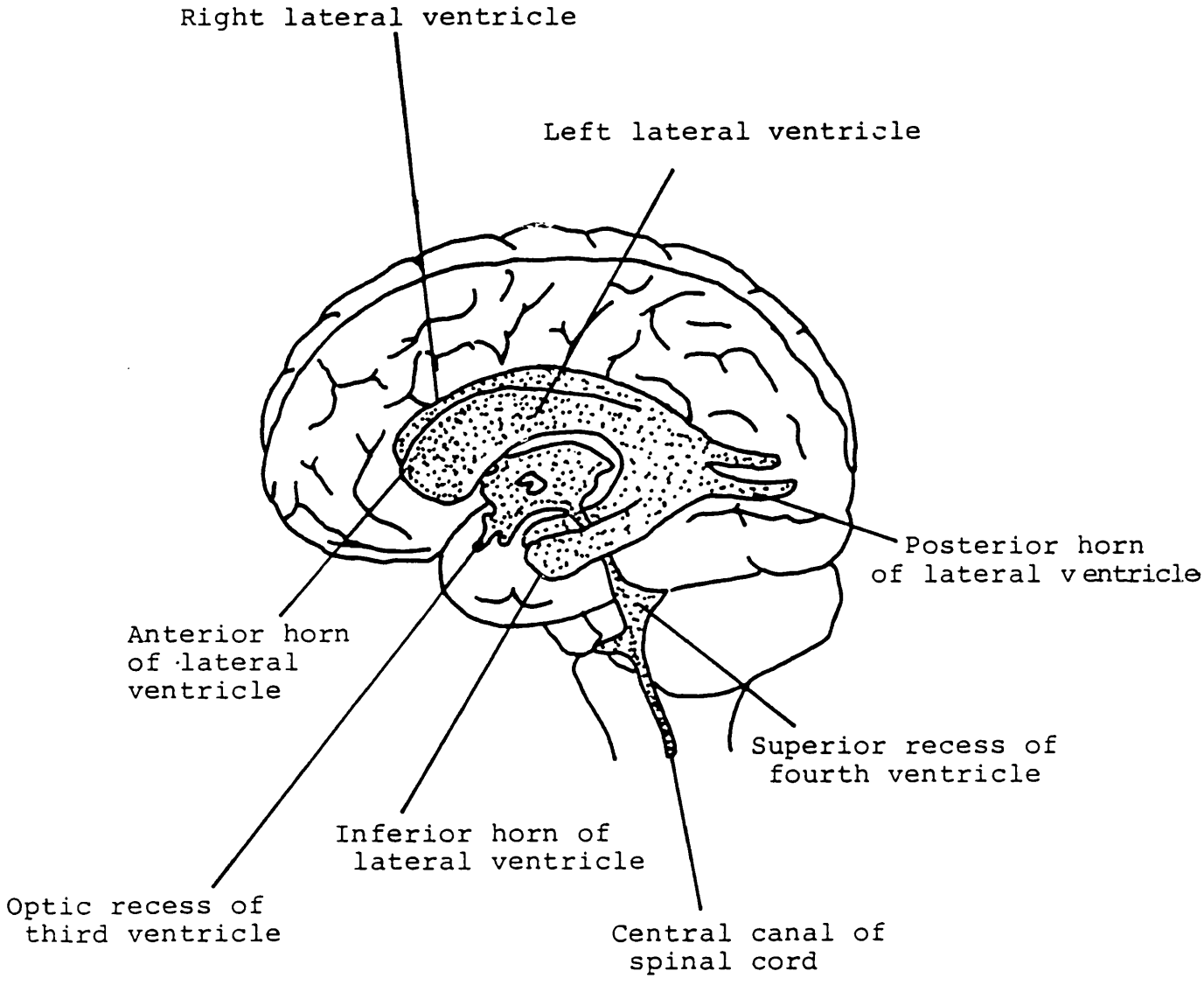
Analysis of metabolites present in CSF has possible clinical application. However, interpretation of such studies remains extremely difficult. For example, caution should be taken when extrapolating data obtained from lumbar CSF to that obtained in ventricular CSF. The significance of, for instance, lumbar CSF content of 5HIAA in terms of central 5HT functions can be questioned. Analysis of Fig. 54 shows clearly the anatomical arrangement of the CSF system in humans. It is evident that cisternal samples of CSF will reflect more closely the events occurring in the CNS compared to lumbar CSF samples.

In summary, it is evident from this preliminary study using human CSF, that

(a) 5HIAA and HVA levels are increased upon electrical stimulation of the PAG;

(b) CCK_8 levels are unaltered;

Fig. 54 - ANATOMY OF HUMAN CSF SYSTEM



(c) methionine enkephalin is not detectable in the CSF.

The exact physiological significance of these findings can only be elucidated by further research. However, to answer the question: "How valid is our animal model in reflecting accurately changes occurring in humans?", we can say that in both the animal and human work our conclusion reached about the neurochemical changes occurring during antinociception remains the same. That is, third ventricular levels of methionine enkephalin is not detectable, and CCK_8 levels are not elevated by PAG stimulation.

CHAPTER 8

GENERAL METHODS

The following design was used in most studies. Any deviation from this design is indicated in the sections dealing with individual experiments.

SUBJECTS

Male Sprague-Dawley rats were used throughout this study. Rats were obtained from Bantin and Kingman, U.K. Ltd. The animals were between 8 and 10 weeks old, and weighed 280-320 g. at the beginning of the experiment. All the animals were kept in individual cages after surgery, and were housed in the experimental room thereafter. They were fed ad libitum and handled frequently throughout the course of the experiments.

SURGICAL PROCEDURES

Rats were anaesthesised with a mixture of halothane (3%), oxygen and nitrous oxide (2:1), and then positioned in a David Kopf stereotaxic apparatus (Schuco Int. London Ltd.) shown in fig. 55, using two steel ear bars. Then halothane, oxygen and nitrous oxide were adjusted to allow a continuous anaesthesia for the whole of the implantation procedure.

One bipolar electrode was aimed stereotaxically at the periaqueductal gray according to the atlas of Pellegrino and Cushman (1979). One concentric push-pull cannula (Plastic Products, virginia, U.S.A.) was aimed stereotaxically at the third ventricle according to the same atlas. The co-ordinates used were AP = -5.4; L = 0.6; D = 6.3 for the electrode, and AP = -2.4; L = 0.0; D = 5.0 mm. for the cannula. Due to geometrical constraints, it was necessary to bend the electrode

Fig. 55

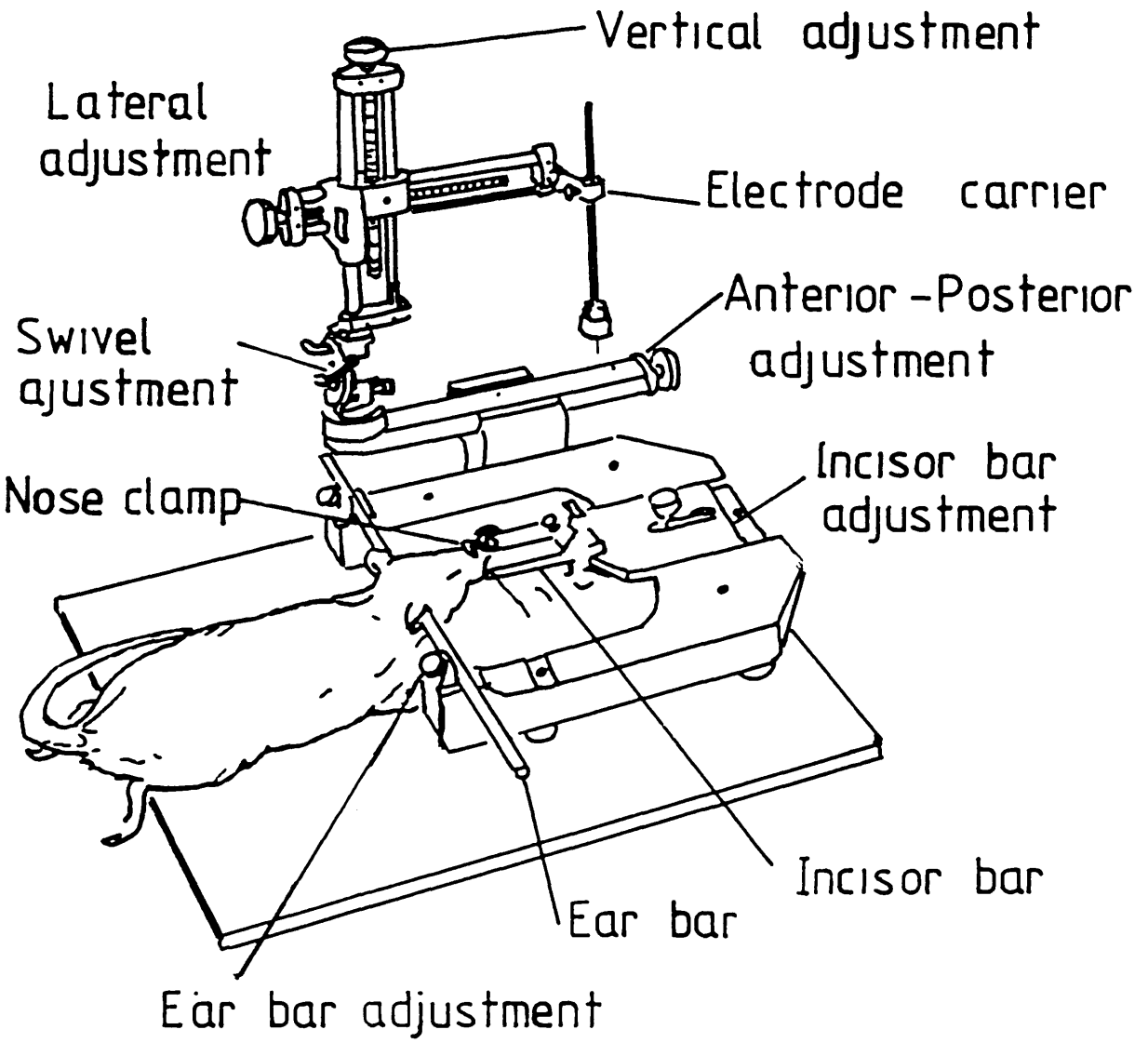


Diagram of a rat properly positioned in the stereotaxic instrument.

caudally. In some experiments, either cannulae or electrodes alone were implanted. Electrodes were constructed of teflon-coated (except at the cross tip) twisted stainless steel wires (Plastïc Products) 0.45 mm. in diameter.

Prior to surgery electrodes and cannulae were sterilised in 70% ethanol. Once in the stereotaxic instrument, the animal's skull was exposed using sterile surgical technique and the skull landmark called, the bregma, (which is the intersection of the coronal and saggital sutures on the surface of the skull) was visualised. This landmark was used as the zero reference point. A 25 gauge needle was then lowered by means of the stereotaxic instrument until it touched the bregma and the zero readings on the anterior-posterior and lateral scales were recorded. Calculations for the predetermined co-ordinates were then made. A small hole was drilled in the skull and a stainless steel screw (8 BA) was threaded into it. Two further holes were drilled at the appropriate points in the skull for the electrode and cannula implantation. The horizontal zero reading was taken when either the cannula and electrode were attached to the stereotaxic frame. Once lowered to their desired co-ordinates, both the electrode and cannula were secured to the skull by first dabbing some superglue around them and then flowing dental cement (Wright Dental Sales, London, Ltd.) around them and the screw. The area around the implant was then sutured. A dust cap (Plastic Products) was then put over the electrode and a stylet (Plastic Products) was placed into the guide cannula. Finally, the operated area was sprayed with an antibiotic mixture (Polybactrin - Calmic Medical Division, the Wellcome Foundation).

PAIN TEST

Analgesia was measured using the hot water tail immersion test; in which one records the latency of the withdrawal of the tail in response to the application of hot water. This test has several advantages. It is easily quantifiable. The dependent variable (response latency) is a continuous measure which allows careful examination of the time course of drug effects. It involves a simple response, a spinal reflex. Howe·er, this response is subjected to descending influences from the brain and narcotic analgesics have been shown to enhance this inhibition (Irwin et al. 1951, Fields and Basbaum 1979).

In the present study, animals were restrained in a cylindrical perspex tube (16.5 cm. X 6.0 cm.) with their tails protruding at one end. There was an opening at the other end which allowed their heads to protrude. Plastic collars were made by cutting a length of plastic (approximately 15 cm. long) and drilling holes along its length. Small copper rings were then soldered through these holes. These collars were put around the neck of the animals on the day prior to testing. The animals were then secured to the perspex tube by attaching a short length of wire through the rings in the collar to holes on the perimeter of the tube. A further piece of perspex (6.5 cm. X 3.5 cm.) was then taped across the rear of the tube allowing space for the tail to protrude. The restraining device was positioned at the end of the bench so that the animal's tail could hang vertically from the edge of the table. Hot water, obtained from a water bath, thermostatically maintained at 59 degrees, was applied to the most distal 3.0 cm. of the tail, and the latency of the withdrawal out of

the water was measured using a stopwatch. At this temperature a baseline latency between 2.0 and 4.0 seconds was obtained. If a response was not obtained in ten seconds (cut off latency) then the hot water was removed from the tail in order to prevent any tissue damage. Once in the restrainers, the dust cap on the electrode was removed, and the electrode plug (Plastic Products) was attached to the electrode. All animals were then left for an adaptation period of one hour with no tail flick testing. An additional advantage of usi.g this procedure for pain testing is that no further handling of the animals was necessary for the rest of the behavioural testing.

TESTING FOR ANALGESIA

After recovery from surgery (at least seven days), all animals were screened in order to determine their baseline responsiveness on the tail flick test as well as the effect on brain stimulation on that baseline. Animals were tested continuously for two hours with an intertrial interval of two minutes. After the first thirty minutes of testing (consisting of fifteen trials), brain stimulation was administered for a total of sixty seconds (30 seconds stimulator on, 15 seconds stimulator off, 30 seconds stimulator on). Immediately after its termination, the hot water was applied to the tail and the tail flick response latency was measured. The animal was tested for a further thirty minutes, at which time a higher intensity of brain stimulation was administered. For one session, three intensities of brain stimulation were tested (4v P-P, 8v P-P and 16v P-P) for each animal with each stimulation period being separated by thirty minutes. At no

time was tail flick testing conducted during brain stimulation. Stimulation parameters were monitored during all stimulation periods with an oscilloscope. Brain stimulation was delivered by a Farnell constant voltage stimulator, and consisted of trains of biphasic rectangular wave pulse pairs. Pulse pairs consisted of two μ second pulse of opposite polarity separated by 100 μ second. Trains of pulses were delivered at four per second. These parameters of brain stimulation are those used by Mayer (1974). The prestimulation baseline (BL) was taken as the mean response time in seconds of the last five trials immediately preceding brain stimulation. The tail flick response latency measured directly after brain stimulation constituted the post stimulation response time (T). The effect of brain stimulation on pain sensitivity was measured in terms of a change from baseline scores to the post stimulation scores. In addition, the fact that there was a cut off point (10 seconds) needed to be taken into account. Therefore, the actual change was compared to the maximum possible change. This measure was termed degree of analgesia (DA) and calculated according to the following formula derived from Harris and Pierson (1964).

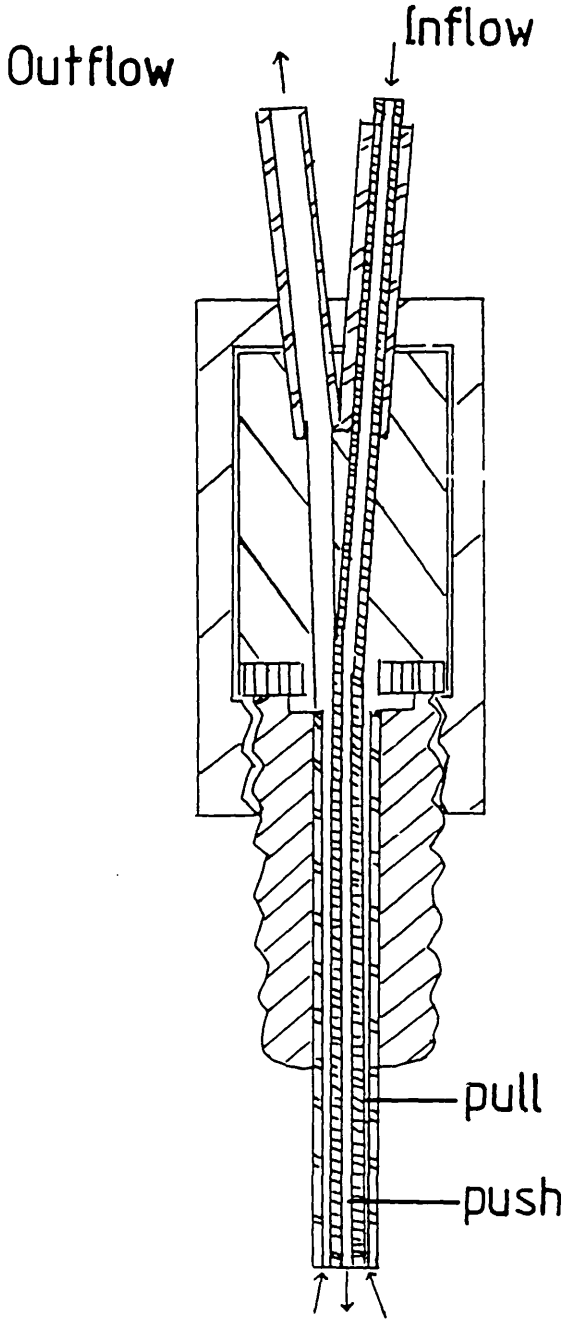
$$DA = \frac{T - BL \times 100}{10 - BL}$$

At the end of the screening session, the animals were removed from their restraining cylinder, and their collars removed. The voltage intensity that was found to produce the maximum degree of analgesia was the intensity used in future experiments.

PERFUSION PROCEDURE

The push-pull cannula (Fig. 56) was first introduced by

Fig. 56



PUSH-PULL CANNULA SYSTEM

Gaddum (1961), and has been used successfully by a number of investigators to study the release of certain neurotransmitters such as acetylcholine (McLenna 1964), norepinephrine (Chase and Kopin 1968), and 5-hydroxytryptamine (Myers and Belesin 1969). It has the advantage of allowing the examination of discrete brain regions, and in addition the animal under study does not have to be killed. The entire principle is based on an exchange of constitutents between perfusate and brain which occurs, presumably, by diffusion. This exchange is two-way. First a drug, transmitter or ion species contained in the perfusion fluid will spread into the neuronal tissue surrounding the tips of the cannula, where a physiological action would be exerted. Conversely, the constitutents released from neural elements at the tips of the cannula can be collected by the perfusion solution as it bathes the surrounding tissue. Seven to ten days after screening the rats for analgesia, rats were perfused in their home cages unrestrained. Prior to a perfusion experiment, the perfusing medium was passed through a 0.22 µm Swinnex millipore filter. On the day of the perfusion experiment the stylet was removed from the guide cannula and the push-pull cannula inserted into the guide. The rat was then connected to the pump (Gilson Minipuls) through polyethylene (PE) tubing. Initial handling of the rat was as brief as possible to allow insertion of the push-pull cannula into the guide cannula and to connect the electrode plug to the electrode. The perfusion medium (either 0.9% saline containing 1.8 mM calcium or artificial CSF mixture) was pumped (10 µl. per minute) through the inflow lines and the inner cannula to the perfusion site. Perfusate was pumped through the outer cannula to the outflow lines. For each perfusion (total perfusion time = 3½ hours)

sequential one hour samples were collected on ice into sterilin tubes. The perfusate samples were collected after a thirty minutes wash period. At the end of the perfusion, the rat's cannula was disconnected from the pump and a stylet replaced in the guide cannula. The volume of the perfusate was measured and the perfusate freeze-dried. Each animal was perfused three times, with each perfusion being separated by seven days. During experimental perfusion, each rat exhibited a full range of normal activities including drinking, feeding, grooming, exploring and sleeping. Also the system was kept patent by perfusing for thirty minutes approximately three days after the last experimental perfusion and the perfusate discarded. In these experiments each animal served as its own control.

HISTOLOGY

At the end of the experiment, the animals were given a lethal dose of pentobarbital sodium (50 mg.). Animals were then rapidly perfused through the heart with 0.9% saline followed by 10% formalin containing 30% sucrose. The brains were removed from the skull and placed in 10% formalin (containing 30% sucrose) for a minimum of five days. The brains were then frozen and sectioned. Tissue was stained with cresyl violet for histological verification of the electrode and cannula tip placement. Fig. 5 shows a typical electrode placement in the PAG of the rat, and Fig. 9 shows a photomicrograph of a representative cannula placement in the third ventricle.

HIGH PRESSURE LIQUID CHROMATOGRAPHY

The large number of small endogenous peptides is becoming increasingly apparent. This renders it difficult to carry out

meaningful physiological/biochemical studies using only radioimmunoassays, and it is difficult to rely exclusively on radioimmunoassays as cross reactivity, even if minimal, can become a significant and misleading factor in interpretation of data. For this reason it is critical to couple radioimmunoassays with reliable chromatographic steps which separate the various peptides.

A high resolution reverse phase high pressure liquid chromatography procedure using an n-propanol:acetic acid elution system had been developed in this laboratory for the separation and characterisation of biologically important peptides (Morris et al. 1980). It offers the following key features:

1. Resolution of a large number of peptides.
2. High reproducibility.
3. High yields.
4. Applicability to fairly crude samples, thereby minimising the number of sample handling steps required.
5. Compatibility with radioimmunoassays.

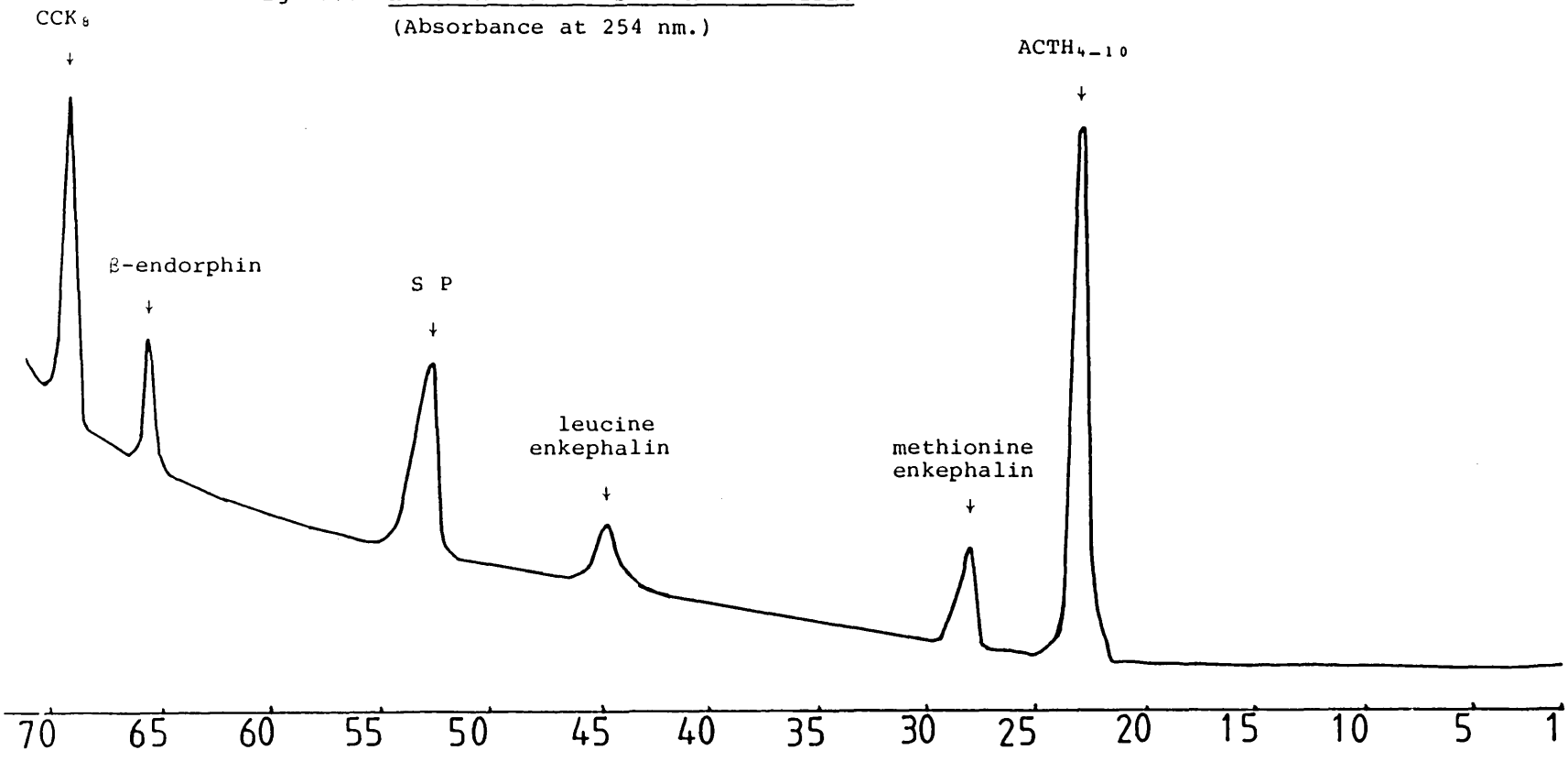
This method at the time had been shown to be viable down to the 5 picomoles level using radiolabelled vasoactive intestinal peptide, VIP (Morris et al. 1980). The work in this thesis demonstrated, for the first time, the applicability of this method at the femtomole level.

The high pressure liquid chromatograph equipped with Universal Injector (U 66 K), Gradient Programmer (Model 660), two solvent Delivery systems (Model 600A), and two fixed wavelength (254 and 280 nm.), u.v. absorbance detectors (model 440) each with a 15 µl. cell of 1 cm. path length was from Waters Associates Instruments, Ltd. (Cheshire). Separations were

carried out on a reverse phase μ-Bondapak column (dimension 0.78 X 30 cm.).

The following gradient was used:
A 60 minute concave gradient (curve No. 7 of Gradient Programmer) from 10:90 (v/v) to 25:75 (v/v) propan -1-ol : 5% acetic acid at a flow rate of 1.0 ml./minute. Then isocratically at 25:75 for 20 minutes followed by 10 minutes from 25:75 to 00:100. One ml. fractions of the eluate were collected in polypropylene tubes and dried on a vacuum pump. In all cases, solvents were of AR quality, propanol was used directly from the bottle, acetic acid was distilled from ninhydrin and the water used to make a 5% solution was purified by the Millipore-Milli Q system. Aqueous solvents were degassed on a water pump before use. All samples were dissolved in propanol:acetic (10:90), spun at approximately 1000 RPM and the supernatant injected directly into the HPLC system. An HPLC chromatogram using the above system is shown in Fig. 57. The recoveries were calculated by chromatographing the peptides using the system described above, and then pooling and reinjecting the same peptides into the HPLC (Table 8). As a direct result of some of the work in this thesis, it became apparent that when a highly sensitive assay method is employed, the danger of carry-over between HPLC runs is evident. Thus, despite the excellent recoveries obtained, the small amount of unrecovered peptides can produce a significant artifact if not completely eluted. For example, if one employs 1 nmole of authentic unlabelled peptide as a standard and recovers 99% of it, the adsorbed 1% still represents 10 picomoles of peptide remaining on the column. This can then be eluted upon later runs, and easily leads to artifactual quantitation in the biological

Fig. 57. HPLC PROFILE OF STANDARD PEPTIDES
(Absorbance at 254 nm.)



Resolution of a mixture of peptides using a 10% to 25% propan-1-ol in 5% acetic acid gradient. See text for details.

TABLE 8. RETENTION TIMES AND THE RECOVERIES OF SIX CNS PEPTIDES.

PEPTIDE	RETENTION TIMES (MINS)	% RECOVERY
$ACTH_{4-10}$	22	86
Methionine Enkephalin	27	89
Leucine Enkephalin	43	84
Substance P	52	93
β-Endorphin	64.5	76
CCK_8	68.5	77

samples.

In some circumstances, particularly when working on individual samples of interest, it was essential to gradient elute the column many times in order to give blanks at the femtomole level. Thus, when standard peptides were chromatographed for calibration, great care was taken to follow these standard runs with numerous blank runs (buffer with no sample added). Additionally, the blank run prior to a sample run was always collected and assayed in an identical manner to the sample run. Once these precautions are taken, it is clear that the reverse phase HPLC/RIA system described in this project, yields good separations between peptides and excellent recoveries in the femtomole range, thus giving both the specificity and sensitivity required.

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