BRAIN DISTRIBUTION AND RELEASE OF CHOLECYSTOKININ OCTAPEPTIDE

Anne Hudson

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To MUM, DAD and RICK

CONTENTS

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ABSTRACT

Cholecystokinin (CCK) comprises a group of related peptides all sharing a common COOH-terminal pentapeptide sequence with gastrin. At least six molecular forms have been described ; a larger component with molecular weight more than 4 000, CCK_{39}, CCK_{33}, CCK_{12}, CCK_8 and CCK_4. The development of radioimmunoassays for CCK, using antisera specific for CCK and not gastrin and radiolabelling using the Bolton-Hunter reagent led to rapid advances in the field.

CCK was found to be present in high concentrations throughout the central nervous system and small bowel as compared to gastrin which was only found in the adeno and neuro-hypophysis and pituitary stalk regions of the brain but with a wide distribution in the gut.

The highest concentration of CCK in the brain occurs in the cortex, in contrast to other hormonal brain peptides where the highest concentration has been found in the hypothalamus. Two related but different forms of CCK_8 account for over 90% of the immunoreactive CCK found in the rat brain; one being identical to CCK_8.

In this thesis the <u>in vitro</u> release of immunoreactive CCK_8 ($iCCK_8$) from rat central nervous system preparations and the regulation of this release have been studied. Rat brain was dissected into the following regions ; hypothalamus, cerebral cortex, striatum and thalamus, according to the method of Brownstein, Arimura, Sato et. al. (1975). CCK_8 was found to be distributed throughout these regions (range of 9 - 300 pmoles), with the highest concentration in cortex (300 pmol). In addition, low levels (range 9 - 30 pmoles) of CCK_8 were found in spinal cord, brain stem and cerebellum, in agreement with other workers.

Immunohistochemical techniques have demonstrated CCK-like immunoreactivity in nerve cell bodies and fibres throughout brain, particularly in the cortex. Subcellular fractionation of rat brain was used to study the subcellular localisation of CCK. Tissue was homogenised to shear

off nerve terminals (synaptosomes) which were purified and used to study the release of the peptide from hypothalamic and extra-hypothalamic nerve endings.

The distribution of CCK_8 from tissue homogenates prepared on continuous sucrose density gradients from four different brain areas was found to coincide closely with the profile of labelled neurotransmitter uptake. The peaks of CCK_8, $[^{14}C]$ choline and $[^{3}H]$ noradrenaline uptake were found in fractions shown by electron microscopy to consist mainly of synaptosomes. The purified synaptosomal pellet obtained by differential centrifugation on a discontinuous sucrose density gradient contained the major concentration of gradient protein, occluded lactate dehydrogenase activity (an enzyme found in high concentrations in the synaptosomal fraction of brain homogenates) and CCK_8 immunoreactivity. CCK_8 was released from synaptosomes prepared from hypothalami, striatum, cortex and thalamus by the depolarising stimuli of 60 mM KCl and 75 μM veratrine in a calcium-dependent manner.

The high concentrations and widespread distribution of CCK_8 in the CNS, the localisation in, and release from, synaptosomes, together with evidence that CCK_8 has central actions on feeding behaviour suggests that the peptide may have a regulatory function at the synaptic level. Thus a new technique was developed to study the release of CCK_8 from synaptosomes.

Instead of the traditional static procedure in which synaptosomes are incubated and the released substance measured in the supernatent (as described above) a new technique was developed involving perifusion of synaptosomes. The highest concentration of CCK_8 in the brain is present in cerebral cortex and thus this region was used for the preparation of synaptosomes in the perifusion study. The method and validation of the perifusion system has been described in detail. Using this technique CCK_8 was found to be released by the depolarising stimuli of 100 mM KCl and 75 μM veratrine in a calcium-dependent manner. Dopamine (at 10^{-6}M and 10^{-8}M) and acetyl choline (at 10^{-6}M) caused a significant release of CCK_8 from perifused synap-

tosomes. Noradrenaline and serotonin at concentrations of 10^{-4} M, 10^{-6}M and 10^{-8}M had no effect.

Immunocytochemical studies have shown the presence of CCK-like immunoreactivity in the dorso medial nucleus of the hypothalamus. In this thesis it has been shown that CCK_8 is localised in, and released from, hypothalamic synaptosomes, suggesting that it may have a role in the regulation of pituitary function. CCK_8 has been shown to release growth hormone (GH) from isolated rat pituitaries. The structurally related peptide, pentagastrin, also stimulates growth hormone secretion in man.

In view of this possible hypophysiotrophic role for this peptide as a regulator of GH, the in vitro incubated rat hypothalamus was used to investigate hypothalamic release of CCK_8. The depolarising stimuli of 60 mM KCl and 75 μM veratrine were found to release CCK_8 in a calcium-dependent manner. Dopamine at 10^{-4}M, 10^{-6}M and 10^{-8}M concentrations caused a significant release of CCK_8. The dopamine stimulated CCK_8 release appeared to be dependent upon noradrenergic modulation as demonstrated by our findings that phentolamine at physiological concentrations inhibited dopamine stimulated release. No inhibition of dopamine stimulated release could be demonstrated by somatostatin (SRIF) or rat growth hormone (rGH). This does not however exclude a GH releasing role of CCK_8 either at a hypothalamic or pituitary level. The lack of effect on CCK_8 release by a number of other neurotransmitters confirmed the specificity of the observed stimulatory responses in this system.

In order to characterise the peptide, extracts of the brain areas and released material from the preparations used were subjected to Sephadex G-50 (sf) and high pressure liquid chromatography and found to coelute with synthetic CCK_8. No material coeluted with gastrin or the larger molecular species of CCK. Serial dilutions of the above samples showed parallelism with synthetic CCK_8 in the radioimmunoassay. In addition, other CCK moieties such as CCK_{33} and CCK_4 crossreact poorly with the antiserum used, and the low concentration of gastrin as compared to CCK in the brain rendered the assay

relatively specific for CCK_8.

In summary these studies show :

1. CCK_8 is present in rat brain with a wide and uneven distribution.

2. CCK_8 is localised to the synaptosome fraction of hypothalamus, striatum, cerebral cortex and thalamus which can be released by depolarising stimuli in a calcium-dependent manner.

3. The establishment of a perifusion system for cortical synaptosomes has proved to have several advantages over the static method.

4. Using this technique dopamine and acetyl choline (10^{-6}M) caused release of CCK_8 while serotonin and noradrenaline had no effect.

5. Incubated hypothalami released CCK_8 in a calcium-dependent manner in response to depolarising stimuli. Dopamine (10^{-4}M, 10^{-6}M and 10^{-8}M) stimulated release of CCK_8 while other neurotransmitters had no effect.

These findings indicate :

1. The distribution of CCK_8 in brain, and its localisation and release from synaptosome fractions, by depolarising stimuli and neurotransmitters suggests a neuromodulatory role for CCK_8.

2. Release of CCK_8 from isolated hypothalami is consistent with the suggested role of CCK_8 in pituitary hormone regulation.

ABBREVIATIONS

Å	Ångström
ACTH	adrenocorticotrophic hormone
Arg	arginine
Asp	aspartate
ATP	adenosine triphosphate
β-LPH	β-Lipotrophin
BSA	bovine serum albumin
C	centigrade
Ca^{++}	calcium
CCK	cholecystokinin
Ci	curie(s)
cm	centimetre(s)
CNS	central nervous system
COOH	carboxy terminal
CO_2	carbon dioxide
cpm	counts per minute
CRF	corticotrophin releasing factor
CSF	cerebro spinal fluid
dpm	disintegrations per minute
FSH	follicle stimulating hormone
g	gram
g	gravity
G	gastrin
GH	growth hormone
GIP	gastric inhibitory peptide
h	hour
iCCK	immunoreactive cholecystokinin
ile	iso-leucine
K^+	potassium
KCl	potassium chloride
Kg	kilogram
l	litre
LDH	lactate dehydrogenase

Leu	leucine
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
M	molar (mole per litre)
met	methionine
mg	milligram
min	minute(s)
ml	millilitre
mm	millimetre
mM	millimolar
mol	mole(s)
MSH	melanocyte stimulating hormone
mRNA	messenger ribonucleic acid
μ	micro
mV	megavolts
n	nano
N	normal (concentration)
n	number of observations
n	nano
Na^+	sodium
NAD	diphosphopyridine nucleotide
NADH	reduced form of diphosphopyridine nucleotide
O_2	oxygen
O.D.	optical density
p	probability
%	percentage
p	pico
PRL	prolactin
r	correlation coefficient
RIA	radioimmunoassay
RGH	rat growth hormone
rpm	revolutions per minute
SD	standard deviation
SEM	standard error of the mean
sf	superfine

SRIF	somatostatin
sq	square
TRH	thyrotrophin releasing hormone
Trp	tryptophan
TSH	thyroid stimulating hormone
u	unit(s)
VIP	vasoactive intestinal poly(peptide)
w/v	weight per volume

CHAPTER ONE

LITERATURE REVIEW

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LITERATURE REVIEW

SECTION I : Cholecystokinin as a Gut and Brain Peptide

A. PEPTIDES OF THE GUT AND BRAIN

Classical endocrinology and the fundamental concept of control of bodily functions by substances produced by distant organs originated after Brown-Séquard's experiments where he showed bodily functions recovered after treatment with extracts of the failing organ.

Further support to this concept was given by the work of Bayliss and Starling (1902), who strongly substantiated the idea that "control of bodily functions was elicited by circulating chemical messengers". They proposed the word 'hormone' be used for these substances.

These same workers (Bayliss and Starling, 1903) suggested that the control mechanisms of gut function were hormonally mediated, being both simple and uniform throughout the vertebrates. A large number of biologically active peptides have since been identified in the gastrointestinal tract and have been designated the 'gut hormones'.

Until recently the gut hormones were thought to be an isolated system more or less exclusively concerned with the regulation of digestion. Von Euler and Gaddum (1931), however, showed that an identical substance was present in both the gut and brain thus disputing the concept of independent endocrine functions of the gut and brain. Rapid advances in the fields of neuroendocrinology and gastrointestinal endocrinology have shown that gut endocrine cells produce peptides found in a wide variety of other tissues including pancreas, pituitary and the central and peripheral nervous system.

The finding that similar peptides are found in both the gut and brain prompted a re-evaluation of their mode of action. There is now strong evidence that the chemical messengers, be they 'neuro-transmitters', 'neurohormones' or 'gut hormones', may reach their targets

by :

i) entering the bloodstream to reach and alter the activity of distant cells (endocrine or hormonal effect)

ii) diffusing locally through interstitial spaces to reach and influence groups of neighbouring cells (paracrine)

iii) crossing a synaptic junction to activate or inhibit synaptic transmission (neurocrine).

At least ten peptides or groups of peptides are known to, or are suspected of mediating regulatory effects via endocrine, paracrine or neurocrine mechanisms in nerve cells of the gut and brain and in mucosal cells of the gut. (Table 1.1)

B EVOLUTION OF NEUROPEPTIDES

This dual distribution and similarities in the amino acid sequences of some of these peptides along with other chemical properties led Pearse (1976) to suggest that these cells may share a common embryological origin in the neuroectoderm. He called these cells the APUD cells because all had the characteristic property of amine precursor uptake and decarboxylation. Recent experiments, however, have failed to demonstrate a neuroectodermal origin for the gut and pancreatic endocrine cells (Andrew, 1976 ; Fontaine and Le Douarin, 1977 ; Pictet, Rall, Phelps et. al. 1976) but indicate an endodermal origin (Dockray, 1979a). Dockray (1979b) has suggested that this dual distribution of peptides in nerves and endocrine cells should be regarded as the capacity of all cells to produce a particular peptide which can be drawn on independently by different cell types so that the same peptide functions in unrelated systems.

The related sequences of gastrin and CCK, and secretin, glucagon, vasoactive intestinal polypeptide (VIP) and gastric inhibitory peptide (GIP), and substance P and neurotensin indicate evolution from common ancestral molecules by gene duplication followed by divergence due to point mutation and amino acid substitution (Barrington and Dockray, 1976 ;

Table 1.1

Peptides Common to Brain and Endocrine Cells of the Gut

Peptide	Author
Sustance P	Pearse and Polak, (1975) ; Nilsson, Larsson, Håkanson et. al. , (1975)
Somatostatin	Polak, Grimelius, Pearse et. al. , (1975) ; Brownstein, Arimura, Sato et. al. , (1975)
LEU and MET Enkephalin	Hughes, Smith, Kosterlitz, et. al. , (1975) ; Polak, Sullivan, Bloom et. al. , (1977b)
Vaso-active Intestinal peptide	Giachetti, Said, Reynolds et. al. , (1977) ; Bryant, Polak, Modlin et. al. , (1976)
Neurotensin	Polak, Sullivan, Bloom et. al. , (1977a)
Bombesin	Polak, Ghatei, Wharton et. al. , (1978)
Cholecystokinin	Rehfeld, (1978a, b)
Gastrin	Rehfeld, (1978c) ; Rehfeld, Stadil, Malmström et. al. , (1975)
Thyrotrophin releasing hormone	Loonen and Soudijn, (1979)
Adrenocorticotrophic hormone	Larsson, (1980)

Dockray, 1979b). Deletion and insertion in the structural gene (Track, 1973) and mutations affecting post-transcriptional splicing of mRNA (Gilbert, 1978) may well account for the molecular diversity of the gut hormones with functionally important residues being conserved. Thus the COOH-pentapeptide common to gastrin and cholecystokinin (CCK) also contains their minimal active fragment.

It is now believed that once an organism has established the capacity to produce a particular peptide, this capacity can be drawn on independently by different cell types by changes in the pattern of gene expression. Thus the same peptide being present in brain and gut is an example of biological conservation. For this view to be correct, a molecular messenger, receptor and degrading pathway must exist that can be used in different systems regulating different functions in the same organism (Dockray, 1979a)

It has been shown that this dual distribution in brain and gut was established early in the vertebrate line ; representatives of the oldest vertebrate group, the cyclostomes, have CCK-like factors in gut endocrine cells and brain (Dockray, 1979a). Larsson and Rehfeld (1977) using antisera specific for different regions of mammalian gastrin and CCK were unable to find separate gastrin and CCK-like peptides in both amphibia and teleosts. These hormones, however, have been localised to different populations of gut endocrine cells in reptiles and birds. (Larsson and Rehfeld, 1977 ; Dockray, 1978). On the basis of this evidence it has been postulated that the evolutionary divergence of these two hormones occured after the amphibian and reptilian lines separated. However, it cannot be excluded that there exists in the amphibia and lower vertebrates gastrin-like peptides that do not cross-react with the mammalian antiserums.

Immunocytochemical studies indicate that in mammals each member of the gastrin family has its own cell of origin with a characteristic distribution (Dockray, 1979b). In lampreys and protochordates both glucagon and gastrin-like immunoreactivities occur in the same cell suggesting that the two main families of gut hormones were originally the

product of a single cell type (Van Noorden and Pearse, 1974 ; Van Noorden and Pearse, 1976).

Many secretory peptides are the product of proteolytic cleavage of large precursor peptides. CCK_{39} is probably the precursor of the 33-residue form, isolated from intestine (Mutt, 1976). CCK_8 has been found in brain (Dockray, Gregory, Hutchison et. al. , 1978) and intestine (Dockray, 1977a) but only relatively small amounts of the larger forms of CCK have been found in brain suggesting that conversion of larger to smaller forms is more or less complete in nerves but incomplete in gut endocrine cells. Recent studies on other peptides (Mains and Eipper, 1978 ; Moody, Jacobson and Sundby, 1978 ; Tager and Steiner, 1973) supports the idea that the same precursor may be produced in different cell types and then processed differently. In addition it has been postulated that distinct peptides can be derived from a single biosynthetic precursor. There is evidence, for example, that β lipotrophin (β LPH) is a product of a larger precursor (31K) which contains the sequence for cortico-trophin (Mains, Eipper and Ling, 1977) and that β LPH is a precursor for β melanotrophin and the endorphins.

The post-translational processing of the precursor peptides directly determines the activity of the final product. During evolution the enzymes involved in these processing pathways must be subject to natural selection. Conservation of the mechanism for proteolytic cleavage of CCK_8 precursor forms is suggested by the fact that peptides are found in lampreys and mammals of similar size to CCK_8.

Lastly there is now evidence supporting the theory that evolutionary changes in target organ receptor mechanisms occurs to match those of their ligands (Holstein, 1975 ; Dockray, 1977b). In the cyclo-stomes the digestive tract is a simple tube and since they possess CCK-like peptides there exists the elements for hormonal control. This might be by local paracrine mechanisms that later give way to endocrine control mechanisms for as Barrington (1971) has pointed out "it is difficult to see how the pancreas could have evolved

without simultaneous development of mechanisms for its control". It has been shown that both teleosts and mammals require a sulphated tyrosine residue to stimulate gallbladder contraction. In mammals, however, the position of the sulphated tyrosine is all important for contraction while in the teleosts there is no precise location required for potency (Vigna and Gorbman, 1977).

Evolution of endocrine systems depends on the action of natural selection on inherited variability. The existence of a pool of variability in receptors provides a mechanism for understanding how structurally related peptides can exert specific actions on different target organs. In particular, an evolutionary approach can help clarify mechanisms governing the function and production of the same peptides in both endocrine cells and neural tissue.

C. CHOLECYSTOKININ

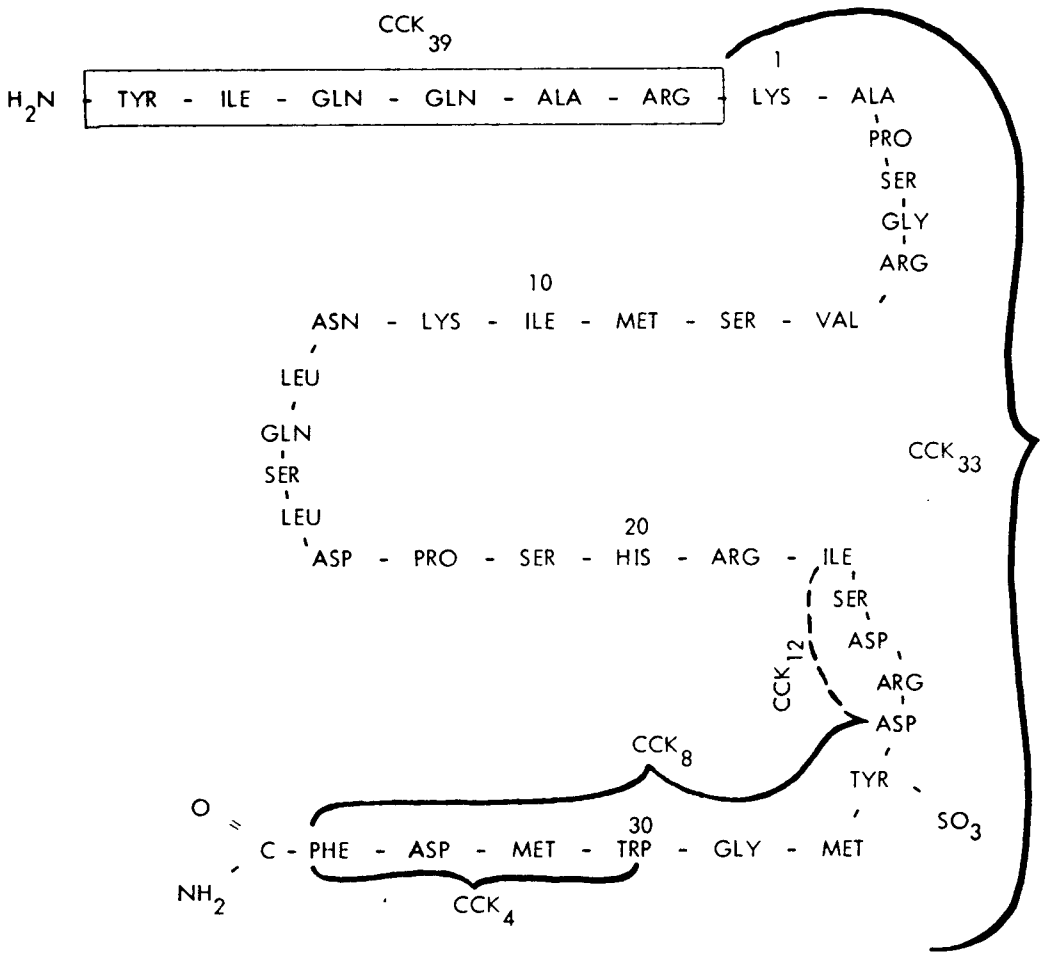
C 1) Historical Aspects

CCK together with gastrin and secretin, comprise the three classical and established gastrointestinal hormones. In 1928, Ivy and Oldberg found a hormone that would stimulate gallbladder contraction and named it cholecystokinin. Later Harper and Raper (1943) found a hormone that would stimulate pancreatic enzyme secretion and called it pancreozymin. It was not until 1968 that Jorpes showed that these two hormones were identical. The hormone is now called cholecystokinin, since stimulation of gallbladder contraction was the first action described.

C 2) Isolation and Structure

CCK was first isolated by Mutt and Jorpes (1967) from hog duodenal extracts and was chemically characterised and shown to contain 33 amino acid residues (Jorpes, 1968 ; Thompson, 1973) (Fig 1.1) the sequence of which is now determined (Mutt and Jorpes, 1971) and confirmed in part by synthesis (Ondetti, Plusec, Sabo et. al., (1970). Subsequently a CCK variant (CCK_{39}) with a further six

Fig 1.1



Structure of CCK_{33} and its variant CCK_{39}. Brackets indicate the smaller molecular weight forms.

amino acid residues linked to the NH_2 terminus of the triacontatria-peptide was described (Mutt, 1976 ; Debas and Grossman, 1973).

A striking feature of both CCK's is that the COOH-terminal penta-peptide sequence, which contains the biologically active region (Mutt and Jorpes, 1967) of the molecule is identical with the COOH-terminal region of the four characterised molecular forms of gastrin (Gregory and Tracy, 1964 ; Rehfeld, 1972 ; Gregory and Tracy, 1975).

The crucial difference between gastrin and CCK is the location of the sulphated tyrosine residue (Fig 1.2). In gastrin it is in position 6 and CCK in position 7 (counting from the COOH-terminus). All known gastrins occur naturally in both the sulphated (G-II) and the unsulphated (G-I) form (Gregory, 1968) whereas CCK occurs naturally in the sulphated form. Desulphation results in loss of effect on the gallbladder and alters it to the gastrin-like pattern of activity in acid secretion (Johnson, Stening and Grossman, 1970).

Smaller molecular forms than CCK_{33}, also having a common penta-peptide sequence with gastrin have been characterised. Vanderhaeghen, Signeau and Gepts (1975) found a peptide in brain that reacted with gastrin antibodies. This was later shown to correspond to CCK_8-like material (Dockray, 1976). Subsequent reports have found :

i) CCK_8-like material in brain and intestine (Dockray, 1976 ; Muller, Straus and Yalow, 1977 ; Rehfeld, 1978b ; Dockray, Gregory, Hutchison, et. al. , 1978 ; Dockray, 1977a).

ii) A closely related but slightly less acidic form than CCK_8 in brain (Dockray, Gregory, Hutichison, et. al. , 1978).

iii) CCK_4-like material in brain and gut (Rehfeld, 1978b ; Rehfeld and Goltermann, 1979 ; Rehfeld and Larsson, 1979).

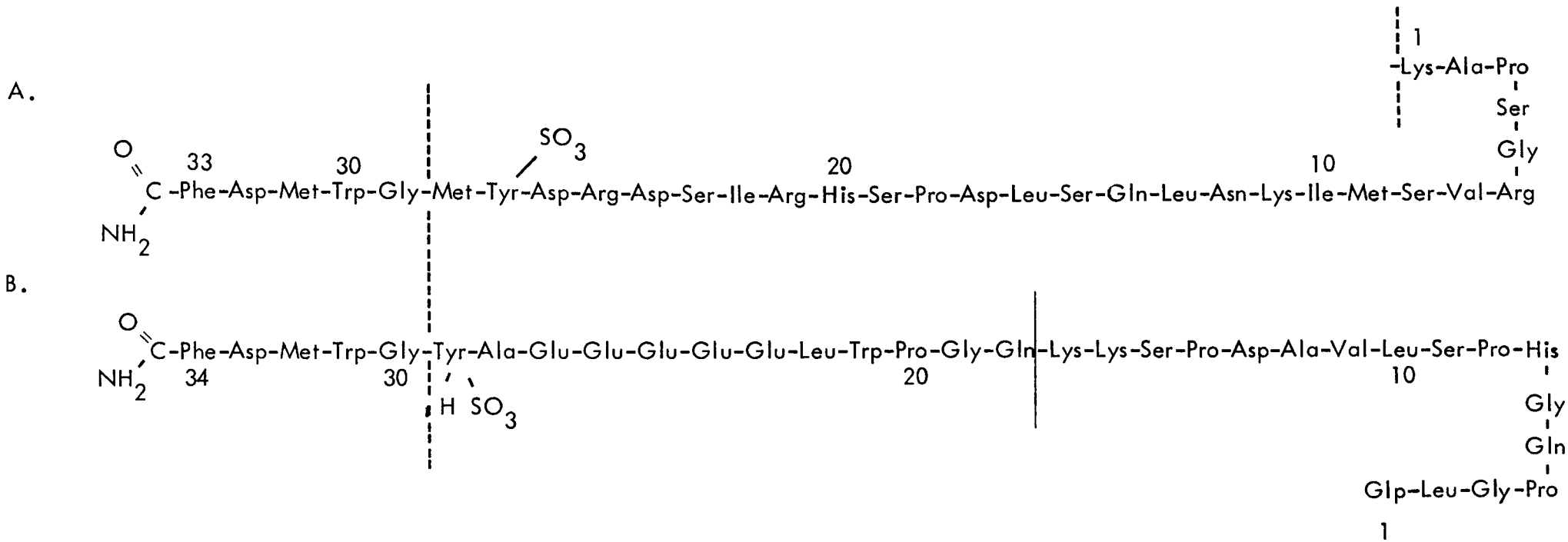
iv) CCK_{12}-like material in the gut (Rehfeld, 1978b).

v) A minor component of 4 - 6 residues in brain (Dockray, 1976).

vi) A larger component than CCK_{39} in gut (Dockray, 1978b ; Rehfeld, 1978b).

Fig 1.2

Comparison of the amino acid structure of CCK_{33} and G_{34}



The amino acid sequence of cholecystokinin - 33 (A) compared to the amino acid sequence of gastrin - 34 (B). Both peptides share the same peptapeptide sequence ; 29 - 33 in CCK and 30 - 34 in gastrin. Gastrin may be sulphated or non-sulphated on Tyr 29 whereas CCK is always sulphated on Tyr 27. Heptadecapeptide gastrin (G-17) is also demarcated (18 - 34 of G-34).

Rehfeld and Larsson (1979) have suggested that CCK_4 predominates in the gut whereas the two CCK_8-like peptides Dockray, Gregory, Hutchison et. al. (1978) have isolated so far, account for the majority of the COOH-terminal immunoreactivity in the brain. CCK_4 has a higher immunochemical potency than CCK_8 and therefore cannot be excluded as the major active form in brain. Since the tetrapeptide amide contains essentially all the biological information present in gastrin and CCK, it has been postulated by Rehfeld and Larsson (1979) to be the principal neurotransmitter of the gastrin/CCK family.

C 3) Distribution in the Gut

Along with gastrin, CCK has been found to have a wide distribution in the gut of all species. High concentrations have been found in the duodenum, jejunum, ileum (Reeder, Becker, Smith et. al. , 1973 ; Bloom and Bryant, 1973), mucosa of the upper small intestine (Jorpes, 1968) and mucosa of the small bowel (Polak, Pearse and Bloom, 1975).

Both CCK and gastrin have been localised to nerve terminals in the gut (Uvnäs-Wallensten, Rehfeld, Larsson et. al., 1977 ; Rehfeld, Larsson, Goltermann et. al. , 1980) and found to be present in distinct endocrine cells, gastrin to the G cell (Solcia, Vassallo and Capella, 1970) and CCK to the I cell (Buchan, Polak, Solcia et. al. , 1978).

C 4) Actions in the Gut

Both CCK and gastrin have a wide range of actions in the gut (Table 1.2) The various molecular forms of CCK and gastrin have different potencies for the actions listed in Table 1.2, for example, CCK_8 has a higher potency than CCK_{33} for gallbladder contraction and CCK_{33} a higher potency than G-17, whereas CCK_4 is more potent than G-17 in insulin release. One must differentiate between physiological and pharmacological actions of these peptides according to the potency and concentration of peptide in each case. Thus the action of G-17 on acid secretion is physiological, whereas its action on gallbladder contraction is probably pharmacological.

Table 1.2

Actions of CCK and Gastrin in the Gut

1. Stimulation of exocrine pancreatic secretion - enzymes and bicarbonate.

2. Stimulation of pancreatic endocrine secretion - insulin, somatostatin, glucagon and pancreatic polypeptide.

3. Stimulation of gastric acid and pepsin secretion.

4. Stimulates secretion of Brunner's glands.

5. Stimulates smooth muscle contraction in gallbladder, stomach and intestine.

6. Inhibition of lower oesophageal sphinctre and sphinctre of Oddi.

7. Stimulates blood flow in superior mesenteric artery.

8. Modulates intestinal absorption of water and electrolytes from jejunum and ileum.

9. Trophic action on the parietal mucosa (gastrin) and pancreas (CCK).

D. CCK IN BRAIN

In 1975, Vanderhaeghen, Signeau and Gepts found a new peptide in the vertebrate central nervous system (CNS) that reacted with antibodies against gastrin. At this time it was unclear what was actually being measured due to the fact that antiserum specific for the COOH-terminus of gastrin cross-reacts with CCK. Dockray, (1976) using extracts of vertebrate CNS tried to clarify the relationship between gastrin-like activity in brain and the characteristic forms of gastrin and CCK. He showed that the chromatographic profile of this material on Sephadex G-25 differed from the previously characterised forms of gastrin and CCK eluting in the same position as the COOH-terminal octapeptide of CCK (CCK_8). The pattern of reactivity with six different COOH-terminal specific gastrin antisera which also cross-reacted with CCK suggested that the brain peptide resembled CCK_8 immunoreactivity more closely than gastrin-like peptides.

In 1977, Muller, Straus and Yalow, using extracts of pig cerebral cortex found two components, one resembling CCK_{33} in size, charge and immunological specificity and the other resembling the COOH-terminal octapeptide. The relative concentrations of each depended on the extraction technique used.

Immunological reactions are not necessarily identical to those responsible for biological activity. Robberecht, Deschodt-Lanckman and Vanderhaeghen (1978), however, showed that water-boiled extracts of this COOH-terminal octapeptide, taken from post mortem human grey matter displayed biological activity of CCK on rat pancreatic preparations.

These biological studies, immunological techniques and fractionation procedures involving size and charge showed that this material was closely related to biologically active CCK_8.

Dockray, Gregory, Hutchison et. al. (1978) detected three components (SBI, II and III) with CCK-like immunoreactivity in sheep brain. Two (II and III) have been isolated in amounts of 150 - 300 μg/100kg fresh brain by gel filtration, ion-exchange chromatography and column

electrophoresis, the third in amounts of 50 µg/100kg fresh brain is not yet homogenous. SBIII was found chemically, biologically and immunochemically to be indistinguishable from synthetic CCK_8 and SBII, present in approximately equal amounts, to be a closely related analogue, the difference between them remains to be established.

Rehfeld (1978b) while studying extracts of tissues from the CNS and small intestine of adult man and hog, extracted at different pH's found seven different molecular forms of CCK. These were found to be similar to a larger peptide than CCK_{39}, CCK_{39}, CCK_{33}, CCK_{12}, CCK_8 and two smaller molecular forms than the octapeptide. These were later found to resemble the NH_2 and the COOH-terminal tetrapeptide fragments of CCK_8 (Rehfeld and Goltermann, 1979). It is unlikely that these two peptides arise artifactually during extraction because CCK_{33} and CCK_8 are both stable during boiling water extraction and also these peptides have since been found in normal unextracted CSF (Rehfeld and Kruse-Larsen, 1978).

The COOH-terminal tetrapeptide-like components appeared to be present in larger amounts than CCK_8 (Rehfeld and Goltermann, 1979). The fact that peptides corresponding to both the NH_2 and COOH-terminal parts of CCK_8 occur in cerebral cortex, suggests that the peptides arise from post translational cleavage of CCK_8 which is abundantly present in cortex. These workers have suggested that CCK_4 is the active neuropeptide in brain.

D 1 Cholecystokinin Converting Enzyme

It has been shown over the last ten years that many peptide hormones are found in their tissues of origin in more than one molecular form. It is generally thought that the larger molecular weight form is a prohormone of the smaller molecular weight forms if it contains an amino acid sequence identical to, or if it is convertible by enzymatic conversion to a smaller, well characterised and more biologically active form, whether or not the biosynthetic precursor relationship has been established (Straus, Malesci and Yalow, 1978). CCK is found in

tissue as a range of molecules of different sizes and biological potencies (Rehfeld, 1978b ; Gregory and Tracy, 1972 ; Rehfeld, 1972). Thus a precursor relationship or prohormone function has been assumed for a) CCK_{39} and CCK_{33} (Mutt and Jorpes, 1968) and b) CCK_{33} and CCK_{12} and CCK_8, the COOH fragments that have greater biological potencies than the presumed precursor CCK_{33}.

The amount of immunoreactivity in components smaller than CCK_{33} is greater in the brain than in the gut and suggested that brain extracts might contain a high potency enzyme that would convert CCK_{33} to the smaller hormonal forms.

Straus, Malesci and Yalow (1978) characterised and partially purified an enzyme from extracts of mammalian brain which converts porcine CCK_{33} to smaller molecular weight forms. This enzyme has since been purified (Malesci, Straus and Yalow, 1980) and shown to consist of at least two separate but closely related enzymes, one cleaving at the Arg-Ile bond to yield CCK_{12} and the other at the Arg-Asp bond to yield CCK_8. These enzymes differ from trypsin in substrate specificity in that they fail to convert G-34 to G-17 and in several physiochemical properties, i.e. molecular size, temperature stability and behaviour in the presence of trypsin inhibitor. There was no evidence that the enzyme was species specific in that both canine and porcine extracts were equally effective in cleaving porcine CCK_{33}. It is not yet known whether the enzyme that must exist in the gut resembles or is different from that found in the brain.

D 2) Distribution and Concentrations of CCK in the Brain

CCK-like peptides have been found in large amounts in the brain and gut of vertebrates and invertebrates; humans (Rehfeld, 1978b), pigs (Muller, Straus and Yalow, 1977), sheep (Dockray, Gregory, Hutchison et. al. , 1978), monkeys, dogs (Straus and Yalow, 1979a), rats, (Lorén, Alumets, Håkanson et. al. , 1979), birds, (Dockray, 1978), fish and amphibia (Larsson and Rehfeld, 1977). Species differences occur in the NH_2 portion of CCK_{33} (Straus and Yalow, 1978).

Rehfeld (1978b) reported differential distribution of CCK in the pig CNS with highest concentrations in the cerebral cortex, 200 - 2,300 p mol/g ; corpus striatum, 158 - 480 p mol/g ; hypothalamus, 24 - 220 p mol/g ; mesencephalon, 13 - 93 p mol/g ; thalamus, 8 - 59 p mol/g and brain stem, 6 - 43 p mol/g. CCK was undetectable in the pineal body, pituitary gland, cerebellum and pons. Rehfeld (1978b) suggested that CCK was unique among the hormonal brain peptides in that :

i) CCK is predominantly located in the cerebral cortex which dominates the CNS in man whereas TRH (Oliver, Eskay, Ben-Jonathan et. al. , 1974 ; Winokur and Utiger, 1974), SRIF (Brownstein, Arimura, Sato et. al. , 1975), substance P (Brownstein, Mroz, Kizer et. al. , 1976), VIP (Larsson, Fahrenkrug, Schaffalitzky De Muckadell et. al. , 1976 ; Said and Rosenberg, 1976), neurotensin (Loonen and Soudijn, 1979) and LHRH (Winters, Eskay and Porter, 1974) are all located primarily in the hypothalamus with low concentrations in cortex.

ii) CCK is present in the hypothalamus in similar concentrations to the other hormonal brain peptides but is approximately 10^3 times more concentrated in cortex than any other brain peptides on a molar basis. Thus, human brains contain 1 to 2mg of CCK_8 in total whereas other hormonal peptides are present in microgram amounts.

Discrepancies in results exist between different workers in the concentrations and hormonal forms of CCK-like peptides in the brain. Rehfeld (1978b) showed that CCK_{33} accounted for 2 - 5% of the total immunoreactivity in man and hog brain while Muller, Straus and Yalow (1977) and Straus and Yalow (1979a) found CCK_{33} to be prominant in pig brain. Dockray (1980) reported that over 90% of immunoreactive CCK in rat cerebral cortex was due to a CCK_8-like peptide and a closely related, slightly less acidic, peptide. Only minimal amounts were CCK_{33}-like. Straus and Yalow (1979a) have accounted for these

discrepancies by the existence, in the brain, of converting enzymes which if not inactivated early in the extraction procedure will result in a larger amount of CCK_8 being found, than would normally be present in vivo while Dockray (1980), suggests that there are species differences in the distribution of CCK_{33}-like material. This discrepancy, however, may reflect the disparity that exists between different workers on the conditions required for extracting CCK, since analysis of the distribution of hormonal forms of CCK in brain tissue requires quantitative recovery of the peptide.

D 3) Distribution of Gastrin Compared to CCK in Nervous Tissue

Gastrin has always been considered to be stored exclusively in specialised endocrine (G) cells of the upper gastrointestinal mucosa. Uvnäs-Wallensten, Rehfeld, Larsson et. al. , (1977), however, showed that G-17 was present in the vagal nerves of cats, dogs and humans whereas CCK-like peptides were totally absent. Gastrin was found to be present in nerves of the intestinal wall, predominantly in the large and distal small intestine in amounts ranging from 16 - 273 p mol/g of nerve tissue. These findings suggested that G-17 may be a new vagal neurotransmitter. In contrast Dockray, Gregory and Tracy (1980) have shown CCK_8 to be the predominant immunoreactive form of G-CCK in the dog vagus, G-17 being of minor importance. Further study is needed to clarify this discrepancy.

Rehfeld (1978c) showed that molecular forms of gastrin closely resembling those found in antrum (Rehfeld, Stadil, Malmström et. al. , 1975) were localised to the neuro and adenohypophysis and pituitary stalk whereas CCK was only present in the pituitary stalk in very small amounts. Traces of gastrin were found in the hypothalamus but were undetectable by chromatography and absent from the rest of the brain.

These results add a new hormone to the list of peptides localised to the CNS and gut. The differential distribution of gastrin in relation to that of CCK is of interest in that it occurs in regions free of CCK. How and why the common ancestor of

gastrin and CCK in antrum and hypophysis develops exclusively to gastrin but in jejunum and the remaining brain to CCK is an intrigu ing question.

D 4) Gastrin and CCK in Cerebrospinal Fluid

Gastrin has been found in the CSF from neurologically normal people at concentrations of 1.5 - 8.0 p mol as compared to CCK which ranges from 4 - 55 p mol (Rehfeld and Kruse-Larsen, 1978). Using sequence specific antisera and gel chromatography, gastrin was found to be present in molecular forms corresponding to G-34 and G-17 and CCK to CCK_8 and a fragment corresponding to the sequence 25 - 29 of CCK_{33}. The similarity of molecular forms of gastrin and CCK in brain extracts and CSF supports the concept that gastrin and CCK in CSF originate from brain tissue and is further supported by the fact that serum gastrin showed no correlation with CSF gastrin in the same patient (Rehfeld and Kruse-Larsen, 1978).

D 5) Cellular Localisation of CCK and Gastrin

Immunocytochemical and immunohistochemical studies, using sequence-specific antisera have shown CCK and gastrin (G) to be located in neurones of the central and peripheral nervous system in a specific and differential manner (Table 1.3). The peptides are present in both the neuronal cell bodies and in terminal processes. It is difficult to differentiate between CCK and gastrin though evidence suggests that this immunoreactive material is CCK_8 (Lóren, Alumets, Håkanson et. al., 1979; Innis, Corrêa, Uhl et. al., 1979) other forms may also contribute. The distibution observed of these peptides :

i) suggests important functions of G-CCK in cortex, limbic structures, including the mesencephalon, the posterior horn of the spinal cord and the hypothalamohypophyseal system.

ii) closely resembles that found by radioimmunochemical studies

Table 1.3

Immunohistochemical localisation of G - CCK

Area	Cell Fibre	Cell Body	Author
Cortex	*	*	1, 2, 4, 5
Amygdala	*	*	1, 3, 4
Olfactory bulb	*	*	1
Hypothalamus	*		3, 4
Hippocampus	*	*	1, 4, 5
Paraventricular hypothalamic magnocellular nuclei	*	*	1, 5
Supraoptic hypothalamic magnocellular nuclei	*	*	1
Circularis hypothalamic magnocellular nuclei	*	*	1, 5
Preoptic hypothalamic nuclei	*	*	1
Hypothalamic dorso-medial nucleus		*	6
Nucleus tractus solitarii	*		1
Posterior horn	*		1
Brain stem nuclei		*	6
Posterior hypophysis	*		5
Spinal cord	*		4, 5
Spinal ganglia	*		4
Caudate putamen	*		1
Accumbens or tuberculum olfactorium	*		1
Limbic structures	*		1
Medial forebrain bundle	*		1
A-10, A-9, A-8 region of Dahlström and Fuxe		*	1
Substantia nigra		*	1
Mesencephalon central grey		*	5
Medulla oblongata	*	*	5
Interstitial nucleus of the ventral tegmental decussation		*	6
Ventral periqueductal grey		*	6
Dorsal raphe		*	6

Authors

1. Vanderhaeghen, Lotstra, De Mey et. al. (1980)

2. Strauss, Muller, Choi et. al. (1977)

3. Hökfelt, Elde, Fuxe et. al. (1978)

4. Larsson and Rehfeld (1979)

5. Lóren, Alumets, Hǿkanson et. al. (1979)

6. Innis, Corrêa, Uhl et. al. (1979)

though it is difficult to compare these procedures in detail because immunocytochemical and immunohistochemical procedures are not quantitative (Innis, Corrêa, Uhl et. al. 1979).

D 6) Subcellular Localisation and Release of CCK

CCK and its COOH-terminal fragments have been localised to synaptosome rich subcellular fractions of rat cortex (Pinget, Straus and Yalow, 1978). The recovery in this pellet of 40% of the total immunoreactivity in the initial cortical extract is comparable to the recovery of other peptides i.e. VIP (Besson, Rotsztejn, Laburthe et. al. , 1979 ; Giachetti, Said, Reynolds et. al. , 1977), and SRIF (Epelbaum, Brazeau, Tsang et. al. , 1977), which are also located in synaptosomes. Pinget, Straus and Yalow, (1979) have shown that the release of immunoreactive CCK is increased 200% by depolarisation of the plasma membrane with K^+ and that this release is Ca^{++} dependent. Rehfeld, Goltermann, Larsson et. al., (1979) showed high K^+ evoked a significant Ca^{++}-dependent release of CCK from superfused cortical slices from rat brain.

Release of CCK can be effected under the conditions used to release other synaptosomal materials which are known to affect synaptic function and it has therefore been suggested that CCK may have a physiological role as a neurotransmitter and/or modulator in the CNS.

It has been impossible to study the subcellular localisation and release of gastrin from neurones in the CNS due to the low levels of gastrin in nerves. Evidence for gastrin being a neurotransmitter awaits the development of a more sensitive gastrin assay.

D 7) Synthesis of CCK in the Brain

In order for CCK to be a neurotransmitter it must be synthesised in neuronal tissue. Rehfeld, Goltermann, Larsson et. al. (1979) have shown :

i) an extensive and rapid in vivo biosynthesis of CCK in rat cerebral cortex.

ii) that a number of molecular forms of CCK are synthesised corresponding to a larger component than CCK_{39}, CCK_{39}, CCK_{33}, CCK_8 and CCK_4.

iii) that the large molecular forms are precursors of the smaller molecular fragments.

and thus have shown that CCK like other neuropeptides, is synthesised within nerve cells.

E. FUNCTION OF CCK IN THE BRAIN

E 1) Inhibition of Feeding

Over the past few years it has been suggested that CCK may serve as a satiety signal within the CNS and in the periphery, perhaps through some type of negative signal from the gastrointestinal tract (Schally, Redding, Lucien et. al. , 1967 ; Gibbs, Young and Smith, 1973 a,b ; Gibbs, Falasco and McHugh, 1976). It has been shown that exogenously administered CCK_{33} or CCK_8 could induce cessation of eating as well as the full sequence of "behavioural satiety" in rats (Gibbs, Young and Smith, 1973 a,b ; Antin, Gibbs, Holt et. al. , 1975 ; Gibbs and Smith, 1977).

Blass, Beardsley and Hall (1979) found that CCK exogenously administered (10 - 40 u/kg body weight) to neonatal rat sucklings lowered milk intake at the age when the established specific inhibitors of ingestion first became effective and did not reduce intake at a time when other feeding inhibitors were not effective.

CCK_8 specifically decreased food intake in a dose-related manner when injected continuously into the lateral cerebral ventricles of sheep that had been deprived of food for 2, 4, 8, and 24 hours. This effect was not related to water deprivation (Della-Ferra and Baile, 1979). Pentagastrin was found to decrease feeding in the 2 hour group but only at a much higher dose and resulted in abnormal behaviour. This could be explained by the similarity in the COOH-terminal pentapeptide sequence of the two peptides. Secretin had no effect on food intake. Rats appear to be less

sensitive than sheep to the CNS-mediated inhibition of feeding by CCK_8 or pentagastrin. Only in rats deprived of food for 4 hours before intraventricular injection at a higher dose range 200 - 300 p mol/kg was food intake found to be decreased (Della-Ferra and Baile, 1979).

Mueller and Hsiao (1979) working with rats deprived of food for 0, 5, 19, 48 and 92 hours showed that synthetic CCK_8 produced a dose-related suppression of liquid diet, lasting 105 min, far beyond the 15 min half life of CCK and that this onset of satiety was dose-related. The effect of CCK on food-sated rats was observed by administering hypertonic saline before presentation of a liquid diet. They will drink the liquid diet for the water content. 40 u/kg CCK suppressed intake by 76% which was shown not to be due to thirst.

CCK, thus seems to fulfill one of the criteria for a short-term satiety signal and that is that the satiety inducing effect of CCK should interact in a consistent and adaptive manner with the state of nutritional deficit of the organism (Mueller and Hsiao, 1978).

CCK_{33} and CCK_8 have been shown by Lorenz, Kreielsheimer and Smith (1979) to cause satiety in sham fed rats when slowly infused intravenously. Thus satiety did not depend upon high circulating levels of CCK that can occur after intravenous or intraperitoneal injections. Gastrin, secretin and GIP also released by ingested food stimuli had no effect on sham feeding thus demonstrating the specificity of CCK for satiety. It has been reported, in conflict to earlier reports (Anika, Houpt and Houpt, 1977 ; Houpt, Anika and Wolff, 1978) that bilateral subdiaphragmatic vagotomy markedly reduces or abolishes the satiety effect of CCK_{33} and CCK_8 (Lorenz and Goldman, 1978 ; Smith and Cushin, 1978) suggesting that the vagus nerve forms the link between the visceral site of action and the brain.

E 2) Affect of CCK on Different Nutritional States

Straus and Yalow (1979b) have demonstrated that obese (ob/ob) mice with hyperphagia have significantly lower cerebral cortical concentrations

of CCK than their lean litter-mates and other normal mice. Schneider, Monahan and Hirsch (1979) in contrast, using the same extraction procedure, assay system and source of mice found no difference between ob/ob mice and their lean litter-mates.

Conflicting data also exists between these two groups on the levels of CCK in the brain in fasted and fed mice. Schneider, Monahan and Hirsch (1979) showed that extremes in nutritional status and feeding behaviour had no apparent effect on the concentrations of CCK in the major anatomic regions of the brains of rats and mice. Different groups of rats were subjected to severe early malnutrition, deprivation of food for 72 hours, highly palatable diets causing hyperphagia and surgical or chemical lesioning of the ventromedial hypothalamus causing hyperphagia. All groups showed no alterations in levels, molecular forms or regional distribution of brain CCK from those found in control animals. In contrast Straus and Yalow (1980) found that iCCK was accutely responsive to changes in feeding patterns. Mice, fasted for 2 - 5 days showed a reduction in iCCK from 274 ± 2ng/g to 133 ± 6ng/g and concluded that acute fasting markedly suppresses cortical iCCK.

Straus and Yalow (1979b ; 1980) found no difference in brain weights between ob/ob mice and their lean litter-mates and fasted and fed mice. Schneider, Monahan and Hirsch (1979), however, found that at every age studied the ob/ob mice had significantly lower brain weights than their lean litter-mates. This is in agreement with other workers (Margules, Moisset, Lewis et. al., 1978 ; Van der Kroon and Speijers, 1979 ; Rossier, Rogers, Shibasaki et. al. , 1979). The method of analysis employed permits reproducible quantitative determinations of the amount of peptide per gram of tissue. Such a system, however, depends on the existence of significant changes in levels of peptides in an entire region of brain, although a more refined dissection technique may show local changes. In the circumstances where experimental animals had lower brain weights

(undernourished large-litter rats, Zucker fatty rats and ob/ob mice) than controls, the total brain content of CCK would have been correspondingly reduced whereas the amount per gram of tissue was not. Whether concentration of brain CCK is more physiologically relevant than total brain content of CCK remains to be determined. It is also possible that CCK is unchanged as a result of parallel changes in the synthetic and degradative rates of the peptide (Schneider, Monahan and Hirsch, 1979). In addition both groups were looking at static levels rather than dynamic alterations in function. If the ob/ob genotype has reduced levels of CCK it suggests that CCK_8 may play a role in the genesis of obesity (Straus and Yalow, 1979b).

E 3) Affect of CCK on Pituitary Hormone Release

Intraventricular injections of CCK_8 into conscious ovarectomised rats was found to suppress thyroid stimulating hormone (TSH) and leuteinizing hormone (LH) but to elevate prolactin (PRL) and growth hormone (GH). Plasma follicle stimulating hormone (FSH) was not altered. Intravenous injections of CCK_8 caused no significant changes in GH, TSH and FSH. PRL was elevated by the low dose but this was thought to be a stress effect; PRL being rapidly elevated by stress. Control injections of saline intravenously or intraventricularly did not modify plasma hormone levels (Vijayan, Samson and McCann, 1979). These results indicate that CCK can alter pituitary hormone release via a hypothalamic action and suggests that it may act as a neurotransmitter or neuromodulator controlling the release of hypothalamic releasing and/or inhibiting hormone. Support for this concept came from Morley Melmed, Briggs et. al., (1979). They showed that CCK_8 released GH from incubated rat anterior pituitary quarters and from cultured GH_3 pituitary tumor cells and that in the GH_3 cell line CCK_8 reversed the inhibitory effect of SRIF on GH release. Thus it was suggested that CCK_8 was an endogenous GH releasing factor.

E 4) The Target - the CCK Receptor

If CCK is involved in the regulation of brain functions, then brain cells should possess CCK receptors analagous to those for other polypeptides. Saito, Sankaran, Goldfine et. al., (1980) using ^{125}I-Bolton-Hunter CCK, a CCK analogue which has full biological activity, have measured specific CCK binding sites in particulate fractions of rat brain, known to contain iCCK. The brain CCK receptor was shown to interact with CCK_{33}, CCK_8 and CCK_4, three of the different molecular forms of CCK found in the brain. The concentration and distribution of the receptor sites closely resembled that of iCCK determined by RIA (Table 1.3).

It has been postulated that CCK is involved in satiety. McLaughlin and Baile (1979) found that obese Zucker rats were less sensitive to intraperitoneal injections of CCK (20 u/kg), than their lean littermates, to inhibition of feeding after a six hour fast. This suggests that absolute levels of CCK may be unimportant as opposed to differences in sensitivity at the receptor sites.

Zetler (1979) showed that the intestinal actions of CCK_8 could be competitively antagonised by morphine, β-endorphin and the enkephalins. The receptors for these two groups of peptides are thought to be different, antagonism occurring by a specific physiological interaction. This and their distribution in gut and brain suggests that these two groups of peptides interact on both myenteric and CNS receptors and are involved in the regulation of intestinal motility. Evidence showing that genetically obese mice have elevated levels of β-endorphin (Margules, Moisset, Lewis et. al., 1978) and low levels of CCK (Straus and Yalow, 1979b) suggests that these two groups of peptides interact to bring about satiety regulation. In addition the amino acid sequence of (Trp^4, Met^5) an enkephalin analogue resembles the NH_2-terminal sequence of CCK_7. Schiller, Lipton, Horrobin et. al., (1978) have shown that unsulphated CCK_7 has an affinity for the opiate receptor in the micromolar concentration range though as yet this has no known physiological significance as unsulphated CCK has not been found in brain (Dockray, 1980). Further studies on the

relations between brain and CCK levels, CCK receptors and CCK action will help explain the nature of polypeptide regulation of brain function.

SECTION II : Measurement of Cholecystokinin

A. DEVELOPMENT OF A RADIOIMMUNOASSAY FOR CCK

Three factors made the development of a sensitive and reliable radioimmunoassay for CCK difficult :

1. Conventional isotope labelling techniques involve oxidation. This results in oxidation of the three methionine residues of CCK in positions 9, 28 and 31 which are crucial for the preservation of bioactivity and immunoreactivity (Mutt and Jorpes, 1971 ; Mutt, 1964).

2. The common COOH-terminus of gastrin and CCK renders many antisera raised against CCK unspecific.

3. Pure CCK is not readily available.

Go, Ryan and Summerskill (1971) ; Harvey, Dowsett, Hartog et. al. , (1973) used ^{125}I-G-17 as radioactive tracer for measuring CCK by radioimmunoassay. Rehfeld (1978a) used ^{125}I hydroxyphenyl propionic-acid succinimide ester (Bolton and Hunter, 1973) which resulted in a tracer which displayed minimal preparation damage, an immuno-reactivity indistinguishable from that of native labelled CCK with all the antisera employed and a high specific radioactivity. The quality of the tracer compared well with high quality monoiodinated tracers in other peptide radioimmunoassay systems (Stadil and Rehfeld, 1972 ; Damkjaer, Jørgensen and Giese, 1971).

A modified method of iodination of CCK to ^{125}I Bolton-Hunter (BH) reagent resulted in the production of a stable, high specific activity radio-iodinated CCK. The tracer thus prepared was biologically active; ^{125}I-BH-CCK binding to specific receptor sites on isolated pancreatic acini (Sankaran, Deveney, Goldfine et. al. , 1979). Thus using this tracer it is now feasible to study CCK by bioassay, immuno-assay and radioreceptor assay (Gorden, Gavin, Kahn et. al. , 1973).

Antisera of high titres and avidities, raised toward partially purified porcine CCK_{33} (either free or coupled to bovine serum albumin),

were found to react with different regions along the chain of CCK_{33} (Rehfeld, 1978a). Thus it has been shown that CCK displays a high immunogenicity generating antisera of high titers and binding affinities and that sensitive radioimmunoassays for CCK can be established (Rehfeld, 1978a). Postulates of CCK being a poor immunogen are thus incorrect.

B. FRACTIONATION OF iCCK

Straus, Malesci, Pinget et. al. , (1979) have shown that it is possible to distinguish between the basic precursor peptides CCK_{33} and its variant CCK_{39} and their COOH-terminal 12 and 8 amino acid fragments in plasma and tissue extracts by adsorption of the peptides to silica and talc. CCK_{39} and CCK_{33} are quantitively adsorbed whereas CCK_{12} and CCK_{8} are not detectably adsorbed by either.

Prior to this discovery intact CCK and CCK fragments have been distinguished either by radioimmunoassay or separation on Sephadex G-50 (sf).

C. RADIOIMMUNOCHEMICAL QUANTITATION OF GASTRIN AND CCK

Radioimmunoassays for gastrin and CCK have mainly been developed for the smaller molecular forms, CCK_{8} and G-17 (Rehfeld, 1978a). The lack of knowledge about the extent to which each assay measures the larger molecular precursors and the fact that pure CCK's from different species are not readily available as radio-immunoassay standards hinders the quantitative determination of the different forms. De Magistris and Rehfeld (1980) have developed an enzymatic procedure for determining the extent of binding of large precursor forms to antisera raised against the small hormonal forms and allows accurate quantitation of the different molecular forms of gastrin and CCK in biological fluids.

CHAPTER TWO

MATERIALS AND METHODS

INTRODUCTION

This section covers all general methodology and more specific techniques used in the study. Details of experimental procedures will be described in full in the relevant chapter.

In this chapter the tissue dissection of the rat brain is described along with the technique for extracting $iCCK_8$ from the regional areas of the brain relevant to the study ; cerebral cortex, hypothalamus, thalamus, striatum, brain stem, cerebellum and spinal cord.

Using the same extraction technique $iCCK_8$ was found to be present in synaptosomes prepared from rat cerebral cortex, hypothalamus, thalamus and striatum. The preparation of these synaptosomes, which were subsequently used for *in vitro* incubation experiments, will be described in detail here. In addition, techniques used for determining the viability and purity of the *in vitro* tissue preparations, measurement of protein, oxygen consumption, lactate dehydrogenase activity and electron microscopy will be described. And finally the radioimmunoassay used to measure the amount of CCK_8 and the criteria used to characterise the material will be outlined.

A. ANIMALS

Adult male Long-Evans rats (200 - 250g) were housed under conditions of temperature (18 - 20°C) and humidity (40 - 60%) with alternating 12 hour light-dark cycles and allowed free access to commercial rat pellets (Epol Foods, Cape Town) and water, were used in all studies. Rats were decapitated between 09h00 and 10h00, the brains removed to an ice tray and dissected.

B. DISSECTION OF BRAIN AREAS

The brain was dissected into cerebral cortex, hypothalamus, thalamus, striatum, spinal cord, cerebellum and brain stem as described by Brownstein, Arimura, Sato et. al. (1975). The vertebral canal was dissected open, the spinal cord exposed and removed.

i) The first cut was at the level of the optic chiasm through the brain in the coronal plane.

ii) The second cut 3mm anterior to the first (rostral).

iii) The third cut 3mm posterior to the first (caudal).

iv) The rostral slice was dissected into striatum and cortex. The cortex was defined by the corpus collosum and the striatum by the corpus collosum and the lateral ventricles. (Fig. 2.1)

v) The caudal slice was dissected into hypothalamus, thalamus and cortex. The hypothalamus being defined laterally by the amygdala and extended dorsally from the top of the brain to the top of the third ventricle. The hippocampus and internal capsule defined the thalamus which was removed from the slice. (Fig. 2.2)

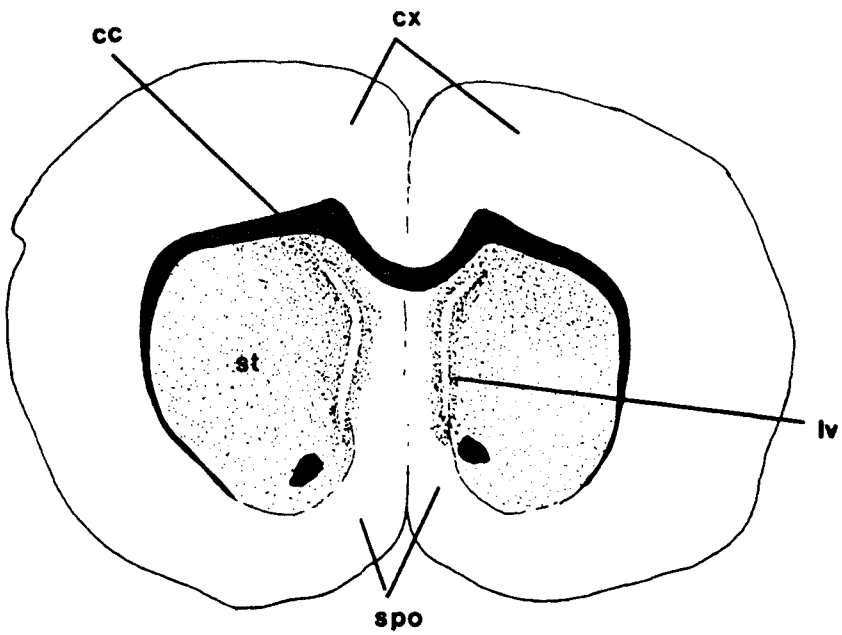
vi) The cerebral cortex from both slices were pooled.

C. TISSUE EXTRACTION

C 1) In boiling water

Immediately after dissection the tissue was put into 25ml glass scintillation vials and weighed on a top loading balance (Sartorius 1205 MP, readability 0.001g) and snap frozen in liquid nitrogen (-196°C). The tissue was transferred from the liquid nitrogen into 3ml of boiling distilled water and placed in a boiling water bath for 20 min. After boiling the liquid was decanted, (the tissue kept for protein estimation) made up to 5ml with distilled water and centrifuged at $1.7 \times 10^3 g$ for

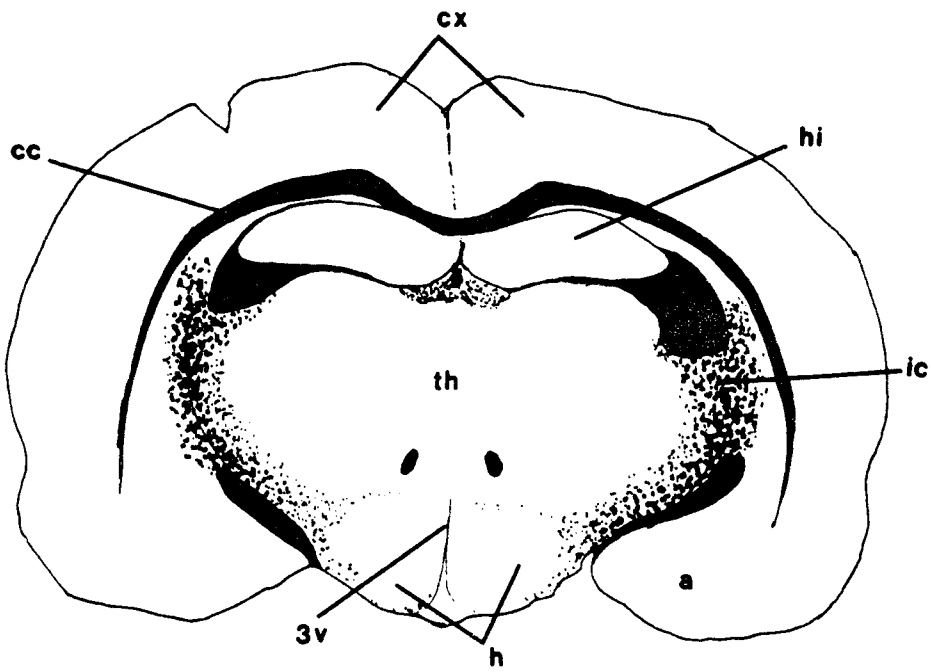
Fig 2.1



Rostral slice of rat brain

spo	septum and preoptic area
lv	lateral ventricle
st	striatum
cc	corpus callosum
cx	cerebral cortex

Fig 2.2



Caudal slice of rat brain

h	hypothalamus
a	amygdala
ic	internal capsule
cc	corpus callosum
3v	third ventricle
th	thalamus
hi	hippocampus
cx	cerebral cortex

30 min at 4°C to remove any extraneous pieces of tissue. Following centrifugation the supernatent was decanted, frozen, lyophilised and stored at -20°C until assayed. The capped vials were reweighed and the tissue weights recorded.

C 2) In acetic acid

Exactly the same procedure was used except after boiling in water for 20 min, 3ml of 0.5 M acetic acid was added to the 3ml of boiling distilled water in the tubes to give a final concentration of 0.25 M acetic acid and boiled for a further 10 min.

The lyophilised samples were resuspended in sodium barbital buffer pH 8.4 and appropriately diluted on the day of assay. All samples were assayed with an antigastrin G-2 antiserum which had 7% cross-reactivity with CCK_8. (Chapter Two)

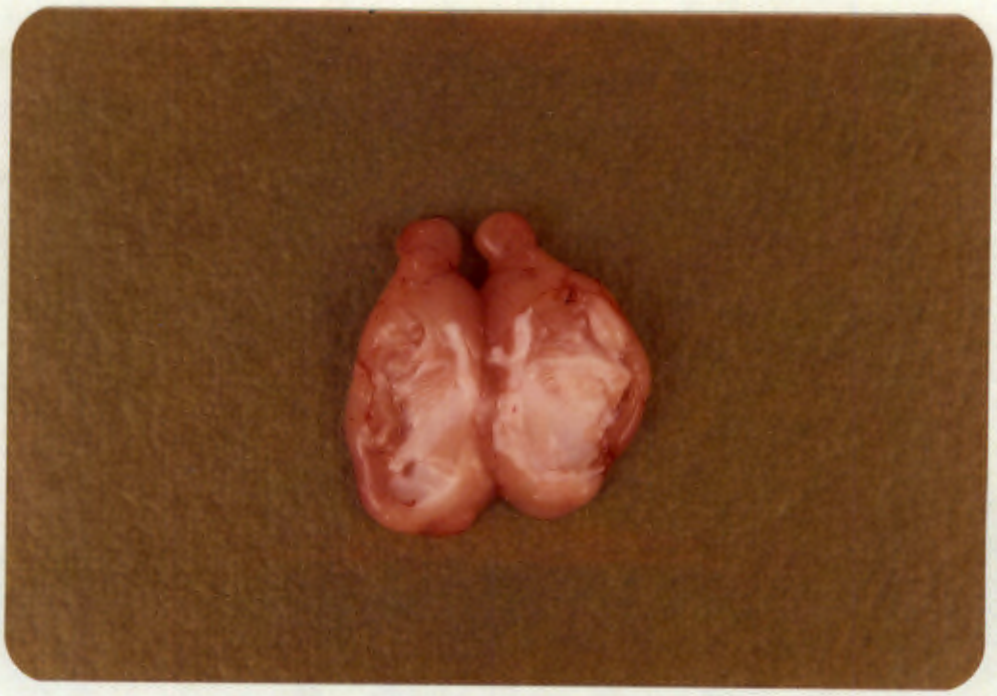
The tissue samples were solubilised by homogenising in 2ml of 0.5% w/v sodium deoxycholate. The homogenates were appropriately diluted in 0.5% w/v sodium deoxycholate and protein estimation was performed according to the method of Lowry, Rosebrough, Farr et. al. (1951).

D. PREPARATION OF SYNAPTOSOMES

Immediately after decapitation the brains of male Long-Evans rats were transferred to an icy tray, 4°C. Synaptosomes were prepared from cortex, hypothalamus, thalamus and striatum according to the method of Gray and Whittaker (1962) as modified by Bennett and Edwardson (1975). Thalamus and striatum were dissected as previously described, the cerebral hemisphere was removed as one piece (Fig. 2.3) and the hypothalamus was removed as a block 1mm deep bounded by the optic chiasm, lateral hypothalamic fissures and mamillary bodies.

In each experiment brain areas from 5 - 10 rats were pooled and homogenised in 10 volumes (w/v) ice-cold 0.32 M sucrose (Miles Laboratories). A glass homogeniser with a perspex pestle and a clearance of 0.25mm was used for 13 passes at a speed of 900 revolutions per min

Fig 2.3



Cortex of the rat brain

(Bennett and Edwardson, 1975) (Fig 2.4). The homogenate was centrifuged for 10 min at 1×10^3 g in a Sorvall refrigerated centrifuge (Du Pont Instruments). A pellet containing nuclei and debris (P1) was discarded and the supernatent (S1) aspirated using a wide-bore pipette and centrifuged for 20 min at 2×10^4 g. The pellet (P2) consisting of myelin, synaptosomes and mitochondria was resuspended in $\pm$ 5 ml 0.32 M sucrose, carefully applied to a discontinuous sucrose density gradient consisting of 10ml of 1.2 M sucrose and 12ml of 0.8 M sucrose and centrifuged for 90 min at 7.5×10^4 g (Beckman ultracentrifuge model L5-65, SW 25.1 rotor). Myelin sedimented at the 0.32 M - 0.8 M interface, synaptosomes at the 0.8 M - 1.2 M interface and mitochondria at the bottom of the tube, confirmed by electron microscopy, enzyme activities and oxygen consumption. The myelin and synaptosome layers were removed separately using a wide-bore pipette and the mitochondrial pellet resuspended in 0.32 M sucrose (Fig 2.5).

Samples from each of these layers were extracted for CCK_8 as previously described.

In order to obtain a synaptosomal pellet for <u>in vitro</u> incubation studies the 0.8 M - 1.2 M layer was slowly diluted 2.2 x volume with ice-cold triple distilled deionised water to give approximately a 0.45 M sucrose synaptosomal suspension. This was centrifuged for 20 min at 4×10^4 g at $4^{\circ}C$. The synaptosomal pellet was resuspended and used according to the protocol of the experiment.

E. MEASUREMENT OF PROTEIN CONCENTRATION

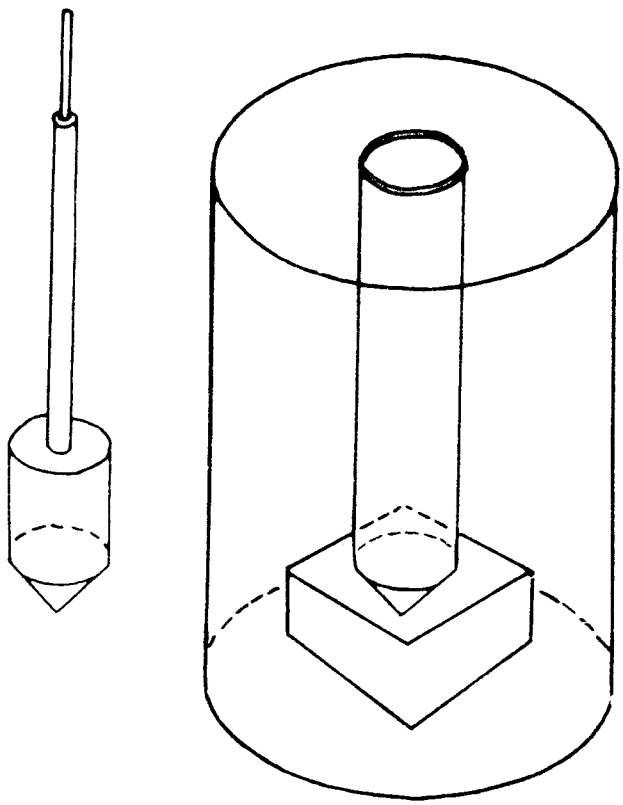
Protein estimation was performed according to the method of Lowry, Rosebrough, Farr et. al. , (1951).

Reagents :

A 2% w/v Na_2CO_3 in 0.1N NaOH.

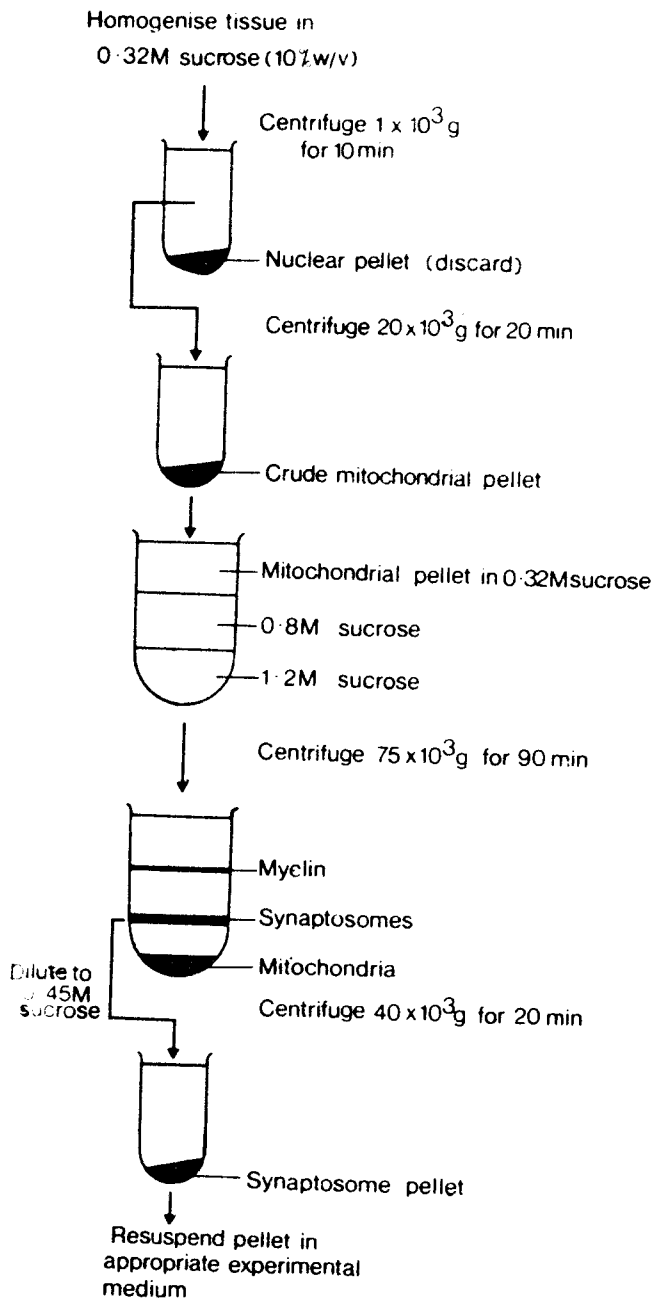
B 0.5% w/v $CuSO_4 \cdot 5H_2O$ in 1% w/v potassium sodium tartrate.

Fig 2.4



Tissue homogeniser. A perspex pestle rotates in a smooth precision-bore glass tube which is surrounded by ice to maintain temperature of homogenate close to 4°C.

Fig 2.5



Preparation of purified synaptosome fraction from homogenates of rat brain

C Before use 50ml of reagent A was mixed with 1ml of reagent B.

D Folin-Ciocalteu reagent (Merck, Darmstadt) diluted 1 : 1 with distilled water.

E Standards : purified crystalline bovine serum albumin (BDH) dissolved in 0.154 M NaCl, containing 0.5% w/v sodium deoxycholate (Merck), to give solutions containing 360, 240, 160, 120, 80 and 40 µg/ml.

Method : All assays were carried out in triplicate as follows :

1 0.2ml of standard or sample.

2 1.0ml of reagent C, mixed and allowed to stand at room temperature.

3 After 10 min 0.1ml of the Folin-Ciocalteu reagent (solution D) was added and mixed.

4 After 30 min at room temperature absorbance was read at 750nm by means of a Zeiss PMZD spectrophotometer.

Results : Absorbance was linear over the working range (40 - 360 µg/ml).

Reproducibility : Interassay coefficient of variation was 2.2% (n = 9).

F. MEASUREMENT OF LACTATE DEHYDROGENASE ACTIVITY

Johnson, 1960

L - lactate : NAD oxidoreductase, EC 1.1.1. 27

The synaptosome fraction of the brain is rich in occluded lactate dehydrogenase (LDH) activity (Marchbanks, 1967). The plasma membrane of the synaptosome is destroyed when treated with a non-ionic detergent (Triton X - 100) thus releasing the LDH occluded within the synaptosome. The amount of LDH released was measured using the following technique :

Principle : Pyruvate + NADH $\rightleftharpoons$ lactate + NAD
The oxidation of NADH is observed at 340nm and in the absence of interfering reactions is a direct measure of the reduction of pyruvate to lactate (Kornberg, 1955).

Reagents :
0.01 M Na Pyruvate (Hopkins and Williams Ltd)
0.002 M NADH (Sigma) in 1% $NaHCO_3$
0.15 M Triethanolamine HCl (Sigma) adjusted to pH 7.4
10% w/v Triton X - 100 (BDH)

Procedure : Reagents were added in the following sequence :

1 5ml Na Pyruvate
2 5ml NADH (freshly prepared)
3 50ml Triethanolamine buffer
4 90ml distilled water

3ml of this mixture was added to 4ml glass cuvettes (Beckman) with a 1cm lightpath. Small volumes of sample (5, 10 or 20 µl) were added to the cuvette and contents mixed. The change in optical density (O.D.) was determined at 340nm (change in E340) over approximately 1 min at 25°C by means of a Unicam SP1700 unltraviolet spectrophotometer (A). 300µl of Triton X - 100 was now added, the cuvette contents mixed, and optical density changes noted over a further 1 min (B).

Results :

i) A represents LDH activity not occluded within synaptosomes.
ii) B is the total LDH activity in the sample after disruption of the synaptosome by detergent.
iii) B - A is occluded LDH activity.

Definition of unit and specific activity : one unit of enzyme is defined as that amount which causes an initial rate of oxidation of 1µmol of NADH per min. Specific activity is expressed as units per mg protein (Kornberg, 1955).

$$\text{specific activity} = \frac{\Delta \text{ O.D.} \;/\; \text{min}}{6.22} \times \frac{1}{\text{mg protein}}$$

where 6.22×10^6 sq cm x mole^{-1} is the extinction coefficient of the pyridine nucleotide at 340nm (Horecker and Kornberg, 1948).

G. MEASUREMENT OF OXYGEN CONSUMPTION USING THE WARBURG TECHNIQUE

Umbreit, 1949 ; Laser, 1961.

Principle : At constant temperature and constant gas volume any changes in the amount of a gas can be measured by changes in its pressure.

The apparatus consists of a detachable flask, containing the tissue suspension, attached to a manometer containing a liquid of a known density. The flask is immersed in a water bath at 37°C and shaken between readings to promote a rapid gas exchange between the fluid and gas phase. The temperature of the manometer is assumed to be constant. A reference point on the closed side of the manometer (usually 150 or 250mm) is chosen and the liquid in the closed arm of the manometer is always adjusted to this point before recording pressure changes. The principle of the method is to hold the gas and fluid volumes constant and to measure the decrease or increase in pressure when one gas alters its amount.

If the gas volume of the flask, the volume of fluid in the flask, the temperature of operation, the gas being exchanged and the density of the fluid in the manometer are known it is possible to derive a flask constant with which one can convert mm pressure change into µl gas consumed.

i) Calulation of flask constant (k)

$$k = \frac{Vg \; \frac{273}{T} + V_f \alpha}{P_o}$$

where V_g = volume of gas phase in flask including connecting tubes down to the manometer reference point,

V_f = volume of fluid in vessel,

P_o = standard pressure (760mm Hg or 10 000mm Kreb's fluid),

T = temperature of water bath in absolute degrees (= 273 + temperature in 0^oC),

α = solubility in reaction liquid of gas involved.

ii) Calculation of amount of gas exchanged (x)

$x = hK$

where x = amount of gas exchanged (µl) at 0^oC and 760mm Hg pressure,

h = alteration in reading on open arm manometer (mm),

k = flask constant

G 1) Use of the Warburg instrument for the measurement of respiration of living cells

In most living cells the biproduct of O_2 consumption is release of CO_2 which can be absorbed by alkali rendering the CO_2 pressure in the air to zero within the limits of measurement. Thus the change noted on the manometer is a measure of O_2 utilisation.

The uptake of O_2 in mm is calculated by subtracting the initial reading from all subsequent readings (total uptake method). A thermobarometer, consisting of a flask containing only water, is used as a measure of the changes in atmospheric pressure and temperature of the water bath. The readings are corrected for any changes occuring during the experiment. The product of the uptake in mm (x) and the flask constant (k) for the conditions employed gives the µl O_2 consumed.

Method :

i) All the manometer joints were greased with inert silicone vacuum grease.

ii) 3ml of Kreb's Ringer's Phosphate buffer (as descibed in chapter four) was added to the flask.

iii) 2 pellets of KOH were added to the central well.

iv) Hypothalami or a suspension of cortical synaptosomes were added to the flasks. The flasks attached to the manometer and placed in a shaking water bath at 37°C.

v) 10 - 15 min was allowed for equilibration of the manometers before the fluid was adjusted to the reference point (150mm) on the closed side of the manometer with the stop-cock open.

vi) Stop-cocks were closed and readings begun.

G 2) Calculation of O_2 consumption

$$\mu\text{mol } O_2 \text{ consumed / hour g tissue} = \frac{hk}{22.4 \times \text{wet weight (g)}}$$

H. ELECTRON MICROSCOPIC EXAMINATION OF SUBCELLULAR FRACTIONS FROM THE SYNAPTOSOME PREPARATION

Myelin, mitochondria and synaptosomes prepared by differential centrifugation, as previously described, were gently resuspended in 3.0ml of ice-cold 4% w/v glutraldehyde and allowed to fix for 60 min at 4°C. The fractions were then washed 3 times in 0.1 M phosphate buffer, pH 7.2 and stored for 24 hours at 4°C in 0.1 M phosphate buffer. Pellets were subsequently fixed in 1% w/v osmium tetroxide for 15 min, dehydrated in acetone and embedded in standard Spurr's resin at 70°C for 24 hours. The tissue was sectioned at 500 Å intervals on an unltramicrotome and stained with uranyl acetate (saturated solution) and 0.2% w/v lead citrate. The resultant sections were examined with the use of a Siemen's transmission electron microscope.

Electron microscopy was performed by J Horne.

I. CHROMATOGRAPHY

Extracts from brain areas, discontinuous gradient fractions, released material from incubated synaptosomes and incubated hypothalami were lyophilised, diluted in 1ml of sodium barbital buffer 0.05 M pH 8.4 and applied to a Sephadex G-50 superfine (sf) column (150 x 1.5cm). The column was equilibrated in and eluted with 50 mM sodium barbital buffer pH 8.4, containing 0.01% sodium azide, at a flow rate of 16ml/h at 4°C. 1.6ml fractions were collected. The column was equilibrated with blue dextran, cyanocobalamine, SHG-17-I, CCK_{33}, CCK_8 pentagastrin and potassium iodide.

J. RADIOIMMUNOASSAY OF CCK_8

Immunoreactive CCK_8 was measured by radioimmunoassay using two different antisera, either CP6 or G-2. The reference standard used for both radioimmunoassays was synthetic CCK_8 (Squibb, batch no. NNO14NB) which was reconstituted in ammonium hydrogen carbonate (0.25 M) and stored at 1mg/ml concentration. The protocol for each assay is described below and the antiserum used to measure the amount of immunoreactive CCK_8 will be specified in each chapter.

J 1) Radioimmunoassay using the G-2 Antiserum

CCK_8 was measured by radioimmunoassay using the G-2 antiserum which was raised by the immunization of a rabbit with human gastrin (SHG) 2-17. The label used was ^{125}I-SHG-17-1 purchased from CEA Sorin, France, with a specific activity of 1000 - 1300 μCi/μg. The assay procedure was as follows : To each tube was added 700 μl sodium barbital buffer (0.05 M), pH 8.4, 100 μl unknown sample or CCK_8 standard, 100 μl antiserum (diluted 1 : 500 000) and 100 μl label (approximately 10 000 cpm). The tubes were allowed to incubate for 24 hours at 4°C and free antigen

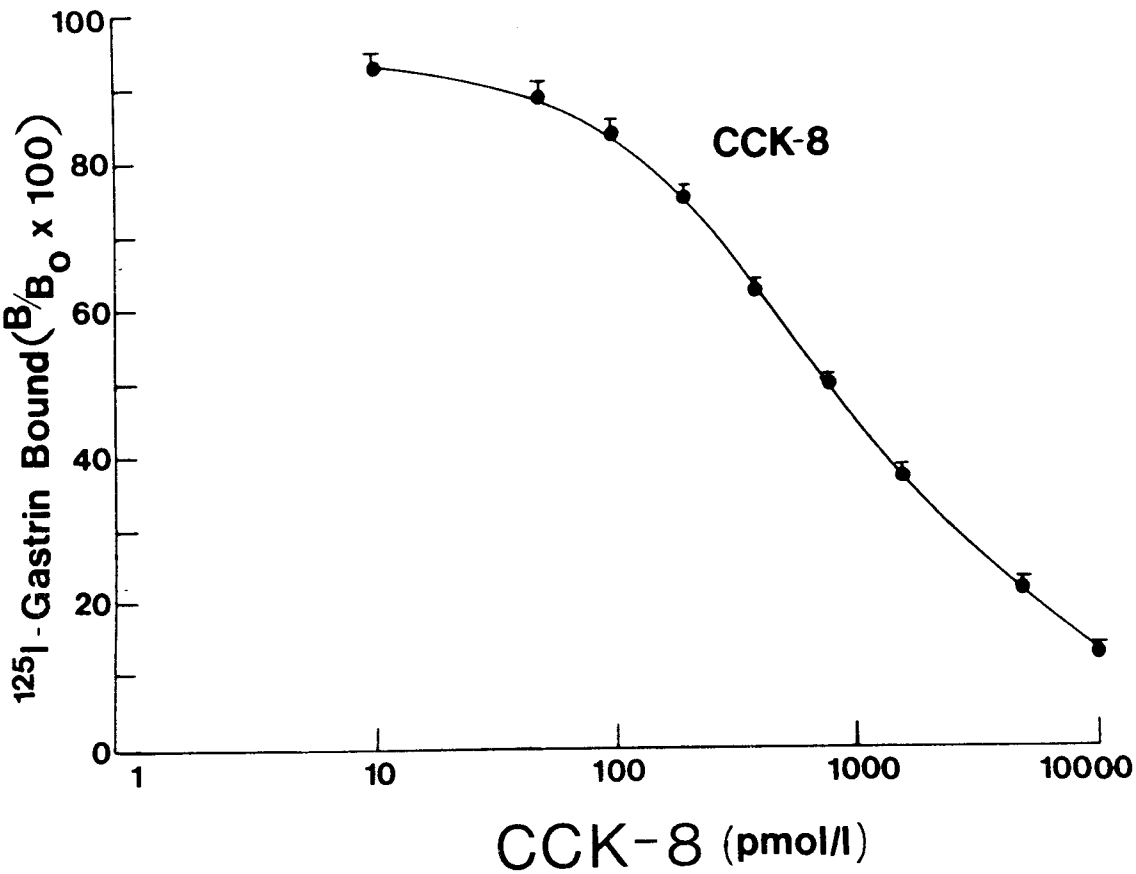
was separated from antibody bound antigen by precipitation with 500 µl of 4% charcoal treated with dextran (0.4%) and centrifugation. The precipitates were counted for 2 min or 10 000 counts. In 6 consecutive assays the mean binding in the absence of added unlabelled CCK_8 was 50.1% ± 0.73% (mean ± SEM) and the non-specific binding was 1.8% ± 0.2% (mean ± SEM).

The ID_{50} for the CCK_8 dose response curve was 836 ± 37 pmol/l (mean ± SEM) n = 6 (Fig 2.6). The interassay coefficient of variation was 17.2% (n = 6) at the mid-point of the curve and the intra-assay coefficient of variation was 6.8% (n = 10). The lowest detectable dose of CCK_8 was 50 pmol/l. The antiserum crossreacted with CCK_8 7% (courtesy of Mr Luciano, Squibb Laboratories) ; CCK_{33} 0.7% (courtesy of Dr V Mutt, Karolinska Institute, Stockholm) and CCK_4 0.4% (courtesy of Dr J Morley, Imperial Chemical Industries, London) when compared with non-sulphated heptadecapeptide gastrin (G-17-1) (courtesy of Dr Morley).

J 2) Radioimmunoassay using the CP6 Antiserum

CCK_8 was measured by radioimmunoassay using the CP6 antiserum which was raised by the immunization of a rabbit with CCK_8 (courtesy of Mr Luciano, Squibb, Princeton, N.J., U.S.A.) conjugated to whelk haemacyanin (Sigma). The label was ^{125}I-SHG-17-I purchased from CEA Sorin, France, with a specific activity of 1000-1300 µCi/µg. The assay procedure was as follows : To each tube was added 700 µl sodium barbital buffer (0.05 M), pH 8.4, 100 µl unknown sample or CCK_8 standard, 100 µl antiserum (diluted 1 : 1500) and 100 µl label (approximately 10 000 cpm). The tubes were allowed to incubate for 48 hours at 4°C and free antigen was separated from antibody-bound antigen by precipitation with 500 µl 4% charcoal treated with dextran (0.4%) and centrifugation. The tubes were counted for 2 min or 10 000 counts. In 10 consecutive assays the mean binding in the absence of added unlabelled

Fig 2.6



Displacement of ^{125}-I-SHG-17-1 bound to the G-2 antiserum by increasing amounts of unlabelled CCK_8 expressed as % bound. The mean $\pm$ SEM of 6 consecutive immunoassays are shown.

CCK_8 was 45.2 ± 1.1% (mean ± SEM) and the non-specific binding was 9% ± 0.1% (mean ± SEM).

The ID_{50} for CCK_8 was 307 ± 14.7 pmol/l (n = 6) (Fig 2.7) whilst the lowest detectable level was 30 ± 0.5 pmol/l (n = 6). The interassay coefficient of variation at 360 pmol/l was 13.7% and at 1250 pmol/l was 22.1% (7 assays) and the intra-assay coefficient of variation was 8.3% (n = 8). The antiserum cross-reacted with synthetic human gastrin (SHG) 17-I 270% (courtesy of Dr J Morley, Imperial Chemical Industries, England) ; natural human gastrin (NHG) 17-II 305% (courtesy of Dr J Walsch, Cure, Los Angeles) ; CCK_4 0.1% (Dr Morley) and CCK_{33} ·79% (courtesy of Dr V Mutt, Karolinska Institute, Stockholm) as compared to CCK_8.

Crossreactivity with VIP, met and leu enkephalin, substance P, neurotensin, TRH, insulin, glucagon, bombesin, SRIF, LHRH and PRL was less than 0.001% with both the G-2 and CP6 antisera. In addition the test substances in the concentrations used, did not inhibit binding in the assays.

Thus these hybrid assay systems, although crossreacting with other gastrin peptides, were relatively specific for CCK_8 in view of their low crossreactivity with other CCK-like peptides and the low concentrations of gastrin as compared to CCK_8 in the brain (Rehfeld, 1978 b,c).

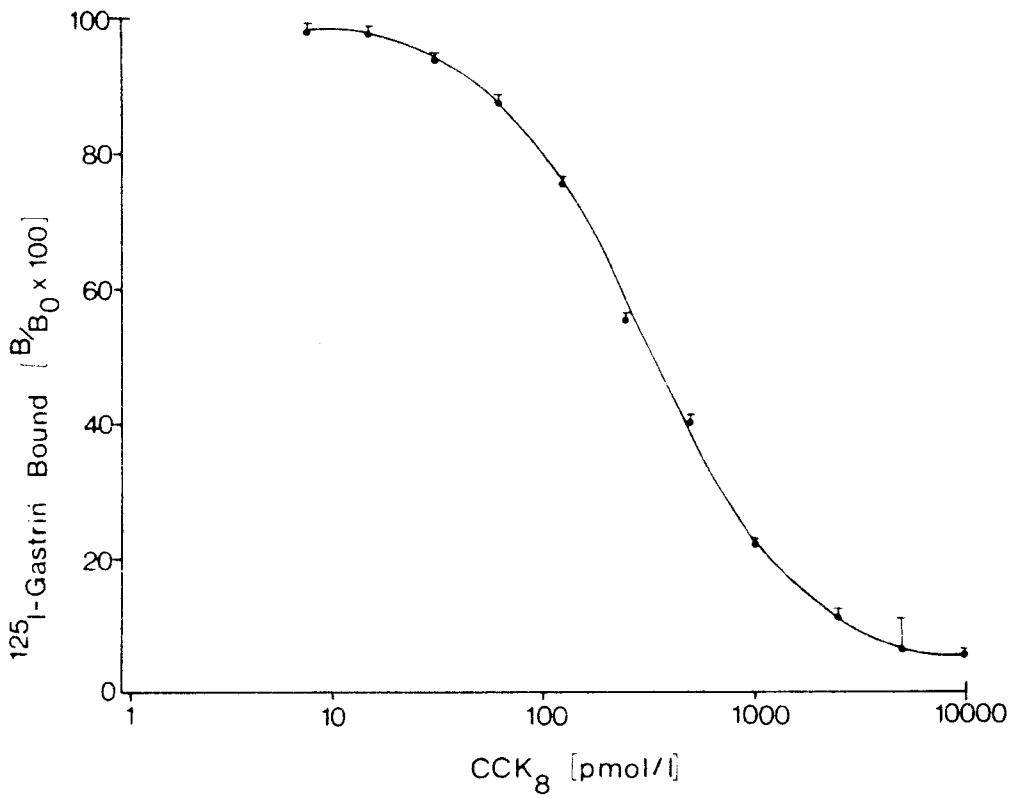
K. PARALLELISM

If the amount of immunoreactivity in an unknown sample falls linearly with dilution and is parallel to the synthetic standard inhibition curve it suggests identity between the unknown sample and the synthetic standard.

Method :

Brain extracts and released material from incubated hypothalami and synaptosomes were serially diluted in assay buffer and compared to the inhibition curves of G-17 and CCK_8 standards. The results were analysed by linear transformation of the inhibition curves (Feldman

Fig 2.7



Displacement of ^{125}I-SHG-17-1 bound to the CP6 antiserum by increasing amounts of unlabelled CCK_8 expressed as % bound. The mean $\pm$ SEM of 10 consecutive immunoassays are shown.

and Rodbard, 1971) to enable comparison of slopes. ^{125}IG-17 binding to antibody (B) was expressed as logit $^{B}/_{Bo}$ where :

Bo = ^{125}IG-17 binding to antibody in the absence of un-labelled CCK_8.

$$\text{logit } {}^{B}/_{Bo} = \log_e \left[\frac{{}^{B}/_{Bo}}{1 - {}^{B}/_{Bo}} \right]$$

Logit $^{B}/_{Bo}$ was plotted on the ordinate against log dose or dilution on the abscissa and the data subjected to linear regression analysis, allowing the regression coefficient (b) and correlation coefficient (r) of each line to be calculated.

Statistical Methods

1 The Mean

$$\bar{x} = \frac{1}{n} \Sigma x$$

where x = the observations

Σ = the sum of

n = the number of observations

2 Standard Deviation

$$S.D. = \Sigma \sqrt{\frac{(x - \bar{x})^2}{n - 1}}$$

where $n < 30$

3 Standard Error of the Mean

$$SEM = \frac{S.D.}{\sqrt{n}}$$

4 Coefficient of Variation

$$= \frac{S.D.}{\bar{x}} \times 100$$

5 Least Square Regression Line

A line of the form $y = a + bx$ was fitted to pairs of figures $(x_1, y_1), (x_2, y_2) \ldots\ldots\ldots (x_n, y_n)$

when $$a = \frac{(\Sigma y)(\Sigma x^2) - (\Sigma x)(\Sigma xy)}{n\Sigma x^2 - (\Sigma x)^2}$$

$$b = \frac{n\Sigma y - (\Sigma x)(\Sigma y)}{n\Sigma x^2 - (\Sigma x)^2}$$

6 Correlation Coefficient

$$r = \frac{n\Sigma xy - (\Sigma x)(\Sigma y)}{\sqrt{\left[n\Sigma x^2 - (\Sigma x)^2\right]\left[n\Sigma y^2 - (\Sigma y)^2\right]}}$$

7 Comparison of the slope of the regression lines was by the analysis of covariance (Snedecor and Cochran, 1967).

8 Mean Integrated Response (Richardson, Walsch, Hicks et. al. , 1976).

Numbers 7 and 8 were calculated using programmes developed for the specific purpose by Dr L J Klaff, Department of Medicine, Medical School, Cape Town.

CHAPTER THREE

CENTRAL NERVOUS SYSTEM DISTRIBUTION OF CHOLECYSTOKININ OCTAPEPTIDE

INTRODUCTION

The discovery of several hypothalamic-releasing peptides, in extra hypothalamic brain areas has led to speculation as to the role of peptides in neurosecretion and their possible function as neurotransmitters or neuromodulators within the central nervous system (Guillemin, 1978).

A number of criteria however, must be fulfilled before a substance can be defined as a neurotransmitter (Frederickson, 1977), the first of which is that it must be present throughout the central nervous system usually with a wide and uneven distribution.

CCK immunoreactivity has been found in most regions of the brain of several vertebrate species and has been shown to be attributable largely to the COOH-terminal octapeptide although CCK_{33} is also present in the brain (Robberecht, Deschodt-Lanckman and Vanderhaeghen, 1978 ; Muller, Straus and Yalow, 1977). In contrast heptadeca-peptide gastrin was found only in pituitary stalk and anterior and posterior pituitary (Rehfeld, 1978 a,b), whilst CCK was absent in these areas.

Tissue extraction of CCK with boiling water was compared with 0.25 M acetic acid extraction since Dockray (1977a) ; Muller, Straus and Yalow (1977) and Rehfeld (1978 a,b) have shown that the amounts of structurally related CCK components that can be extracted from pigs depends upon the pH of the extraction. Dockray (1980) however, has shown subsequent to the completion of this work that the large molecular forms of CCK were obtained in similar yields in acid and neutral extracts in the rat. Thus the optimum extraction

conditions for the larger molecular forms of CCK in the rat may differ from that in pigs.

The first aim of the study was to investigate the regional distribution of CCK in the CNS of the rat.

MATERIALS AND METHODS

The tissue dissection of the rat brain along with the extraction procedure for tissue CCK has been described in detail in Chapter Two.

Using these techniques the tissue content of CCK_8 from cortex, hypothalamus, thalamus, striatum, cerebellum, brain stem and spinal cord was measured by radioimmunoassay (chapter two), using the G-2 antiserum.

Known amounts of CCK_8 were added to the tissue samples prior to extraction, to assess the efficiency of the extraction procedures. The lyophilised extracts were reconstituted in assay buffer, serially diluted and assayed for CCK_8 immunoreactivity. The elution volumes of boiling water and acetic acid extracted CCK immunoreactivity was compared on Sephadex G-50 (sf) chromatography. Synthetic CCK_8 and synthetic CCK_{33} were also subjected to gel filtration chromatography for identification purposes.

RESULTS

A The recovery of CCK_8 added to the brain areas prior to extraction in boiling water and acetic acid were 72% - 99%. Tissue extracts had no effect on tracer or antibody integrity.

B CCK/gastrin-like immunoreactivity was extractable in the highest concentrations from the cortex, but was also present in lower amounts in the hypothalamus, thalamus, striatum, brain stem and spinal cord. Lowest concentrations were found in the cerebellum (Table 3.1). Results were expressed as nanogram equivalent CCK_8 per milligram of protein.

C In the hybrid assay system, using ^{125}I-G-17-1 as tracer and G-2

antiserum to G-17-I and CCK_8, serial dilutions of the extracted material caused displacement of tracer parallel to CCK_8 standards but not to the SHG-17-I standards (Fig 3.1).

D The immunoreactive gastrin-like material extracted by boiling water from all areas tested eluted as a single peak in the identical region to the synthetic CCK_8 on gel chromatography and no material coeluted with G-17 or CCK_{33} (Fig 3.2). Acetic acid extracts did not differ.

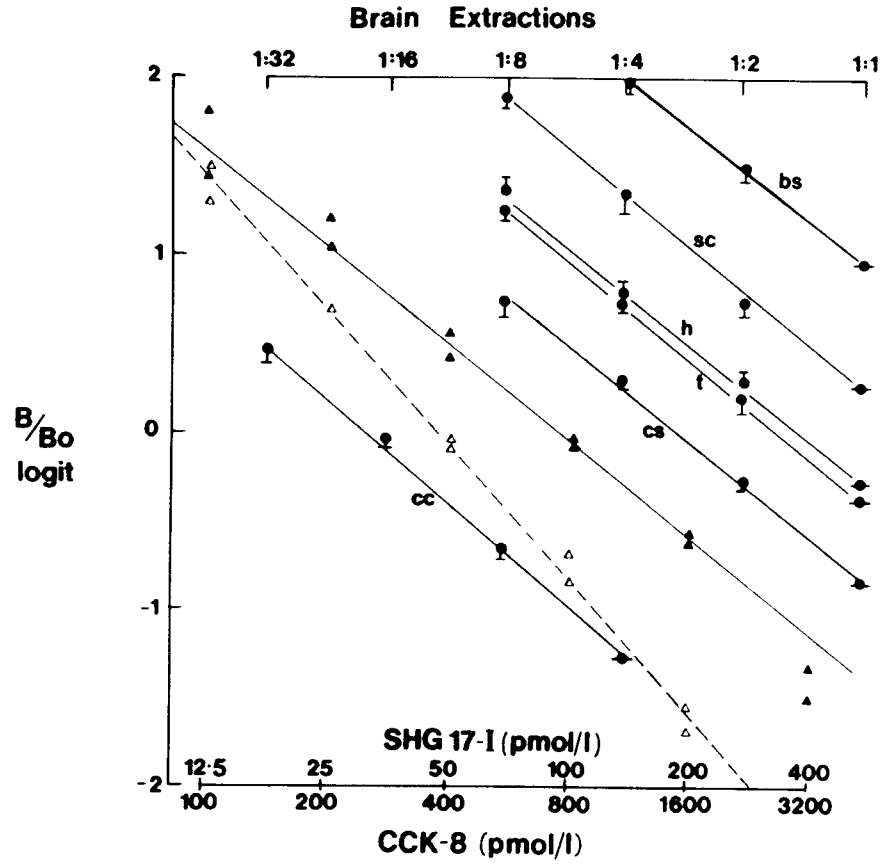
Table 3.1

Regional Distribution of CCK_8 Immunoreactivity in Rat Central Nervous System

	CCK_8 Concentrations	
	pmoles/g Wet Weight	nmoles/g Protein
Cortex	290 ± 13.5	5.82 ± 0.27
Corpus Striatum	189 ± 7.4	2.62 ± 0.10
Thalamus	105 ± 3.5	1.30 ± 0.04
Hypothalamus	161 ± 11.0	2.04 ± 0.14
Cerebellum	9 ± 3.8	0.13 ± 0.05
Brain Stem	27 ± 2.2	0.42 ± 0.03
Spinal Cord	32 ± 3.5	0.46 ± 0.05

Figures denote mean ± SEM (n = 6)

Fig 3.1

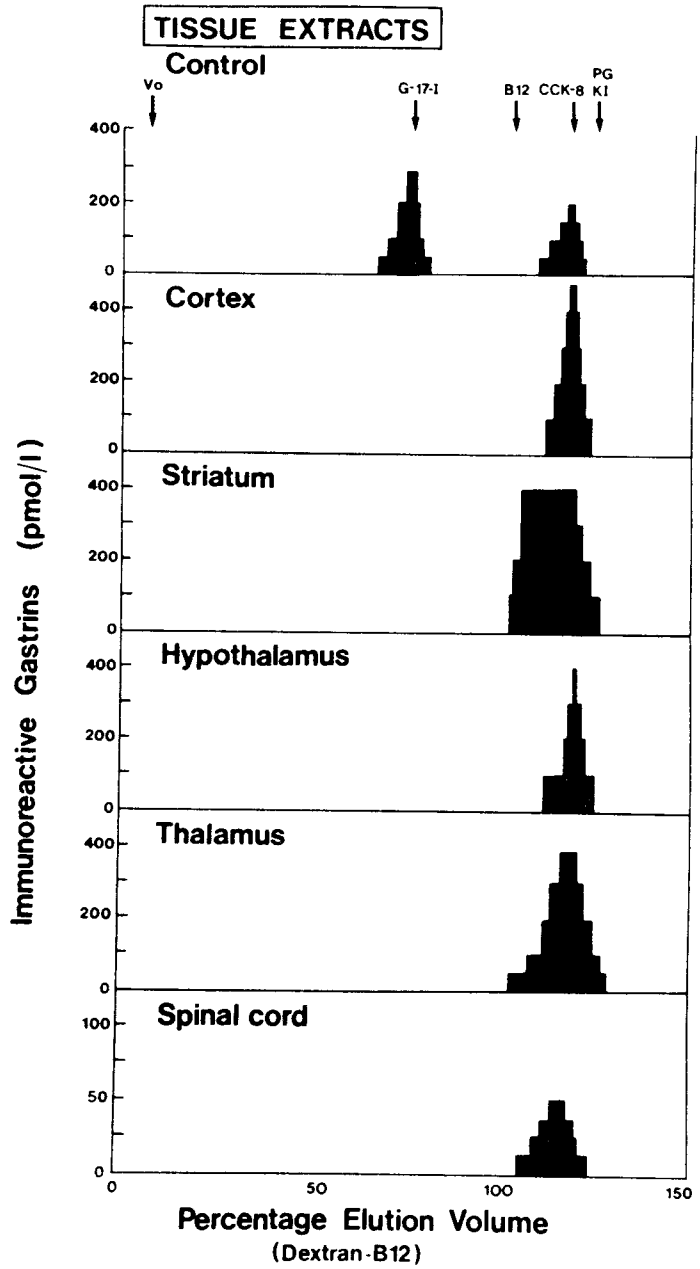


$^{B}/_{B_o}$ (logit) v $\log_e$ of gastrin (SHG-17-1) and cholecystokinin octapeptide (CCK_8) standards compared with displacement of ^{125}I-G-17-1 by serial dilutions of extracts of synaptosome fractions from various areas of rat CNS.

Standard	Key	Slope
CCK_8	▲——▲	- 0.79
SHG-17-1	△- - △	- 1.09
cortex	cc	- 0.82
hypothalamus	h	- 0.78
thalamus	t	- 0.77
straitum	cs	- 0.77
spinal cord	sc	- 0.79
brain stem	bs	- 0.74

All brain area extracts diluted in parallel with CCK_8 standards ($p > 0.05$) but not with SHG-17-1 ($p < 0.01$)

Fig 3.2



Gel chromatography of boiling water extracts from different areas of rat brain on Sephadex G-50 (sf). The column was equilibrated with blue dextran (Vo), synthetic human heptadecapeptide gastrin (SHG-17-1), cyanocobalamin (B12), cholecystokinin octapeptide (CCK_8), pentagastrin (PG) and potassium iodide (KI). The abscissa is expressed as the percentage of the column volume eluted between Vo and B12.

DISCUSSION

The immunoreactive material extracted from the different regions of the rat brain was found to be CCK_8-like. The G-2 antiserum used in this study was more specific for unsulphated gastrins than for CCK_8 but crossreacted 7% with CCK_8 and showed no crossreactivity with a wide range of hormones, peptides and neurotransmitters (Chapter Two). None of the immunoreactivity was found to correspond to gastrin-like material, all the brain extracts caused parallel displacement of tracer parallel to CCK_8 standards and coeluted on Sephadex G-50 (sf) chromatography with synthetic CCK_8. This is in agreement with Dockray (1980) who has shown that two CCK_8-like peptides are the principal immunoreactive forms of CCK in rat cerebral cortex, accounting for over 90% of the total CCK-like activity.

Dockray (1980) showed that only minimal amounts of CCK immunoreactivity corresponded to CCK_{33} in rat brain. No large molecular weight material was detected in any of the brain areas extracted here though if it was present in only very low concentrations it is unlikely that the antiserum used, having a poor crossreactivity with CCK_{33}, would detect such low levels. Thus the findings of Muller, Straus and Yalow (1977) who found CCK_{33} to be prominant in pig brain and Rehfeld (1978 a,b) who found CCK_{33} accounted for 2 – 5% of the total immunoreactivity in human and pig brain can be explained by species differences in the tissue distribution of CCK_{33} (Dockray, 1980).

We were however, unable to differentiate between CCK_8 and the COOH-terminal hexapeptide (CCK_6) and tetrapeptide (CCK_4) which might be present in rat brain (Deschodt-Lanckman, Robberecht, Vanderhaeghen et. al. , 1978) and shown to be present in hog brain (Rehfeld, 1978b ; Rehfeld and Goltermann, 1979). Dockray (1980) has been unable to find any forms smaller than CCK_8 in the rat brain but this may have been overlooked due to the immunochemical potencies of the different molecular forms. Thus the possibility still exists that CCK_4 may exist in rat brain.

The concentrations of immunoreactive CCK_8 material described here are similar to those reported by Schneider, Monahan and Hirsch (1979) in rats, Vanderhaeghen, Signeau and Gepts (1975) in humans and Muller, Straus and Yalow (1977) in pigs. The highest concentration of CCK_8-like material is found in cortex but it is also present with a wide and uneven distribution throughout the rest of the rat brain. This differs from that of other brain peptides in that the cortical amounts of CCK in vertebrates exceeds the amounts of other brain peptides reported so far by 10- to 100-fold (Rehfeld, 1978b). Thus it is reasonable to expect that alterations in the levels of brain CCK might have profound effects on cerebral function.

CHAPTER FOUR

SUBCELLULAR DISTRIBUTION OF CHOLECYSTOKININ OCTAPEPTIDE IN BRAIN REGIONS

INTRODUCTION

Using appropriate homogenisation procedures, the nerve endings of neurones can be sheared from their axon and reseal to form intact nerve-endings (Synaptosomes) in which all the main structural features of the nerve-ending are preserved (Whittaker, Michaelson and Kirkland, 1964).

The synaptosome thus shows a wide range of metabolic activities characteristic of the nerve endings of neurones. These include synthesis of lactate and amino acids, generation of ATP and phosphocreatinine, accumulation of potassium ions and extrusion of sodium ions against a concentration gradient and high linear respiration (Ling and Abdel-Latif, 1968 ; Bradford, 1969 ; Bradford and Thomas, 1969).

A wide variety of hypothalamic hormones and neuropeptides have been shown to be present in nerve terminals of the CNS using immunohistochemical techniques, radioimmunoassay and bioassay. These include TRH and LHRH (Barnea, Ben-Jonathon, Colston et. al. , 1975), VIP (Giachetti, Rosenberg and Said, 1976), vasopressin and CRF (Mulder, Geuze and de Wied, 1970), SRIF (Epelbaum, Brazeau, Tsang et. al. , 1977), enkephalins (Frederickson, 1977), prolactin (Fuxe, Hökfelt, Eneroth et. al. , 1977), substance P (Duffy, Mulhall and Powell, 1975) and α-MSH (Barnea, Oliver and Porter, 1977).

Straus, Muller, Choi et. al. (1977) showed by immunohistochemistry that CCK_8 was present in cell bodies of the cortical grey matter of rabbits and Pinget, Straus and Yalow (1978) demonstrated CCK-like immunoreactivity in isolated nerve terminals (synaptosomes) prepared

from rat cerebral cortex. The recovery in this pellet of 40% of the initial cerebral cortical content of CCK_8 is comparable to that of VIP (Giachetti, Said, Reynolds et. al. , 1977 ; Besson, Rotszteun, Laburthe et. al. , 1979) and SRIF (Epelbaum, Brazeau, Tsang et. al. , 1977).

Depolarising stimuli, high extracellular K^+ or electrical stimulation, have been shown to stimulate the release of transmitter substances in a calcium-dependent manner. VIP (Giachetti, Said, Reynolds et. al. , 1977), substance P (Schenker, Mroz and Leeman, 1976), LHRH and TRH (Warberg, Eskay, Barnea et. al. , 1977) have been shown to be released from synaptosomes by K^+ in the presence of Ca^{++}.

Pinget, Straus and Yalow (1979) showed release of immunoreactive CCK from rat cortical synaptosomes increased by 200% in solutions containing K^+ and Ca^{++}. Using Quso adsorption Straus, Malesci, Pinget et. al. (1979) showed that CCK_{33} was not released under non-stimulatory conditions but under stimulatory conditions both CCK_{33} and COOH-terminal fragments were released.

These data together with other evidence that CCK has an effect on satiety suggests that CCK may have an important regulatory function at synaptic level in at least the long term regulation of appetite (Straus and Yalow, 1979b).

In this chapter, localisation of CCK_8 immunoreactivity to the synaptosome fraction of rat brain, in vitro incubation experiments showing the Ca^{++} dependent release of CCK_8 by depolarising stimuli and characterisation of the released material will be described. Immunoreactive CCK_8 was measured by radioimmunoassay using the G-2 antiserum.

MATERIALS AND METHODS

Dissection of the rat brain into cortex, hypothalamus, striatum and thalamus and the preparation of purified intact syanptosomes from these areas has been described in Chapter Two.

A All fractions from the gradient were measured for protein estimation after solubilisation in 0.5% w/v sodium deoxycholate according to the method of Lowry, Rosebrough, Farr et. al. (1951) (Chapter Two). Occluded lactate dehydrogenase (L-Lactate : NAD oxido reductase) EC 1.1.1.27, an enzyme found in high concentrations in the synaptosome fraction of brain homogenates, was measured in all fractions before and after disruption of the membrane with Triton X-100. This is generally used as a marker for intact synaptosomes (Marchbanks, 1967) (Chapter Two).

All samples were extracted for $iCCK_8$ as described in Chapter Two. The lyophilised samples were reconstituted in assay buffer immediately prior to the measurement of CCK_8 by radioimmunoassay (Chapter Two).

Proteins, occluded LDH and $iCCK_8$ from the myelin, mitochondrial and synaptosome layers were expressed as a percentage of total gradient concentration.

Samples from the myelin, mitochondria and synaptosome layers were fixed in glutaraldehyde and electron microscopy performed on them (Chapter Two).

B To determine the localisation of CCK to synaptosomes, the hormone concentration in fractions from continuous sucrose density gradients was compared with the incorporation of labelled neurotransmitters during a preincubation prior to gradient centrifugation. The crude homogenates from hypothalamus, cortex, striatum and thalamus were obtained as described in Chapter Two and allowed to warm slowly to room temperature. One ml was then added to 5ml of KRB as described in Section C containing, in addition

glucose	10 mM
adenosine triphosphate	0.45 mM
sodium pyruvate	10 mM
ascorbic acid	0.03 % w/v

either

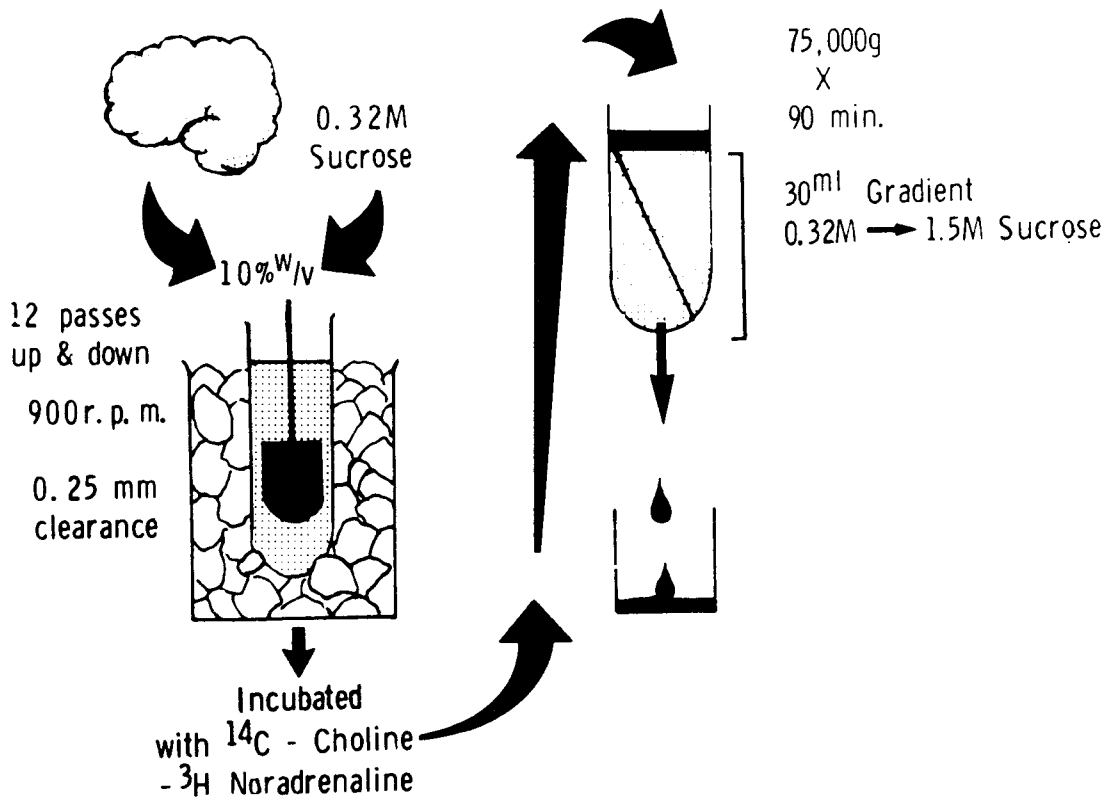
physostigmine hydrochloride 50 mM (Esserine) with $[^{14}C]$ Choline 5 µl (methyl $[^{14}C]$ Choline Chloride, 52 mCi/mmol, Amersham)

or

phenelzine sulphate 100 mM (Nardil) with [^{3}H] Noradrenaline 25 µl (L - 7 - [^{3}H] Noradrenaline, 5,8 Ci/mmol, Amersham) pregassed with 95% O_2, 5% CO_2. The mixture was incubated in a gently shaking water bath at 30°C for 30 min. As a control, synaptosomes were incubated at 4°C. Uptake was terminated by rapid cooling in an ice bath and unincorporated tracer was removed by centrifuging the mixture at 10 000g for 5 min at 4°C. The resultant pellet, resuspended by gentle homogenisation (Thomas homogeniser, No 54, Thomas Co, Philadelphia, U.S.A.) in 0.32 M sucrose, was then applied to a linear continuous density gradient of 0.32 M - 1.5 M sucrose prepared in 30ml cellulose nitrate centrifuge tubes (Beckman Instruments) and centrifuged at 75 000g for 90 min in an SW 25.1 rotor (spinco). The tubes were punctured and 1ml fractions of the gradient collected (Fig 4.0). 100 µl was solubilised in 500 µl soluene (Packard Instruments), 5ml of scintillation fluid, Instagel (Packard) was added to all samples and the radioactivity determined by counting in a Packard Automatic Liquid Scintillation Counter, appropriately set for [^{3}H] and [^{14}C]. The remainder was snap frozen and stored at -20°C until assayed in dilution for CCK. The same homogenisation and density gradient centrifugation procedure was followed on three occasions for each area, without prior incubation with labelled neurotransmitters, to ensure that the CCK profile was unchanged.

C To study the release of CCK-like immunoreactivity in vitro the synaptosome pellet obtained at the end of the preparation (as described in Chapter Two) was resuspended by homogenisation (Thomas homogeniser, No 54, Thomas Co., Philadelphia, U.S.A.) in Krebs-Ringer Bicarbonate medium (KRB), (composition mmol/l^{-1} ; NaCl 118.5 ; $NaHCO_3$ 25 ; KCL 4.75 ; $Mg\ So_4 \cdot 7H_2O$ 1.18 ; KH_2PO_4 1.18 ; $CaCl_2 \cdot 2H_2O$ 2.52, pH 7.4, containing 10 mM D-glucose and pregassed with 95% O_2. 5% CO_2) in final concentrations equivalent to 1 thalamus, 1 hypothalamus, ½ striatum and 1/6 of a cortex per ml. These concentrations were strictly adhered to throughout all experiments.

Fig 4.0



To determine the localisation of CCK_8 tissue homogenates were incubated with $\left[^{14}C\right]$ choline and $\left[^{3}H\right]$ noradrenaline and separated into subcellular fractions on a continuous density gradient of 0.32 M - 1.5 M sucrose. 1ml fractions of the gradient were collected.

Incubations were carried out in stoppered glass vials (10ml) containing 0.5ml aliquots of synaptosome suspensions, placed in a gently shaking water bath at 37°C. The synaptosomes were preincubated for 25 min before the addition of test substances and thereafter for a further 30 min in an atmosphere of 95% O_2 and 5% CO_2. To study the release of immunoreactive CCK in response to depolarising stimuli and the calcium dependence of this release the following test substances were added to give final concentrations of :

KCl	60 mM
Veratrine	75 μM (Sigma)
KCl 60 mM + Ethyleneglycol - bis - (β-aminoethyl ether) (NN'-tetra-acetic acid (EGTA, Sigma)	1 mM
Veratrine 75 μM + tetrodotoxin	1 μM (Calbiochem)

An equal volume of medium was added to the control incubations. After the 30 min period of incubation the suspension was centrifuged for 5 min at 2×10^3g at room temperature, the supernatent removed, boiled, snap frozen and stored at -20°C for measurement of immunoreactive CCK (Chapter Two). Differences between test and control release of $iCCK_8$ were evaluated with the use of Wilcoxon unpaired ranking tests (Mann-Whitney) for non-parametric data.

D In a further set of experiments 0.5ml suspensions of rat cortical synaptosomes were incubated for 15, 30 or 45 min periods with and without the addition of 60 mM KCl to determine the time course of the release of $iCCK_8$.

E Oxygen consumption, as measured by the Warburg technique, (Bradford, 1969) was used as an estimation of metabolic activity on a suspension of cortical synaptosomes (Chapter Two). Na_2HPO_4 25 mM replaced $NaHCO_3$ in the incubation medium which was gassed with 100% O_2. To assess membrane integrity of the incubating synaptosomes, occluded LDH was measured at 0, 15, 30 and 45 min periods of incubation from hypothalamic and cortical synaptosomes.

F To establish identity of the $iCCK_8$ the synaptosome pellet was extracted as previously described in Chapter Two and serial dilutions were compared for parallelism to the immunoassay dose response curve. In addition the elution profile of the extract was compared after gel chromatography, Sephadex G-50 (sf) with that of synthetic CCK_8 (Chapter Two).

RESULTS

A. Continuous sucrose density gradient unltracentrifugation of homogenised hypothalamus, thalamus, striatum and cortex revealed a peak of CCK_8 immunoreactivity, coinciding with the peaks of specific $[^{14}C]$ choline and $[^{3}H]$ noradrenaline uptake (Fig 4.1). The CCK peak retained the same sedimentation characteristics when studied without prece ding incubation. Another peak of CCK immunoreactivity in high concentration occurred in the 0.32 M area of the gradient coinciding with unincorporated $[^{14}C]$ choline or $[^{3}H]$ noradrenaline. The addition of sucrose in equivalent concentrations to that in the gradient fractions (1.5 M, 1.2 M, 0.8 M, 0.32 M) did not cause displacement of the CCK_8 dose response curve.

B. The purified synaptosomal fraction obtained following differential centrifugation and ultracentrifugation on a discontinuous sucrose density gradient contained more than 60% of the CCK immunoreactivity recovered from all four areas. This immunoreactivity coincided with the major concentration of occluded LDH activity and gradient protein (Fig 4.2). Electron microscopy i.e. examination of glutaraldehyde fixed samples of all gradient fractions from hypothalamus and cortex confirmed the major concentration of intact synaptosomes to coincide with CCK immunoreactivity, protein and occluded LDH activity (Fig 4.3, 4.4, 4.5). Fig 4.6 shows a synaptosome particle packed with secretory granules.

C. Both 60 mM KCl and 75 μM veratrine stimulated a significant release of $iCCK_8$ from the synaptosomes from all four areas of the brain studied. This release was calcium-dependent as shown by the inhibition of release by the calcium chelating agent

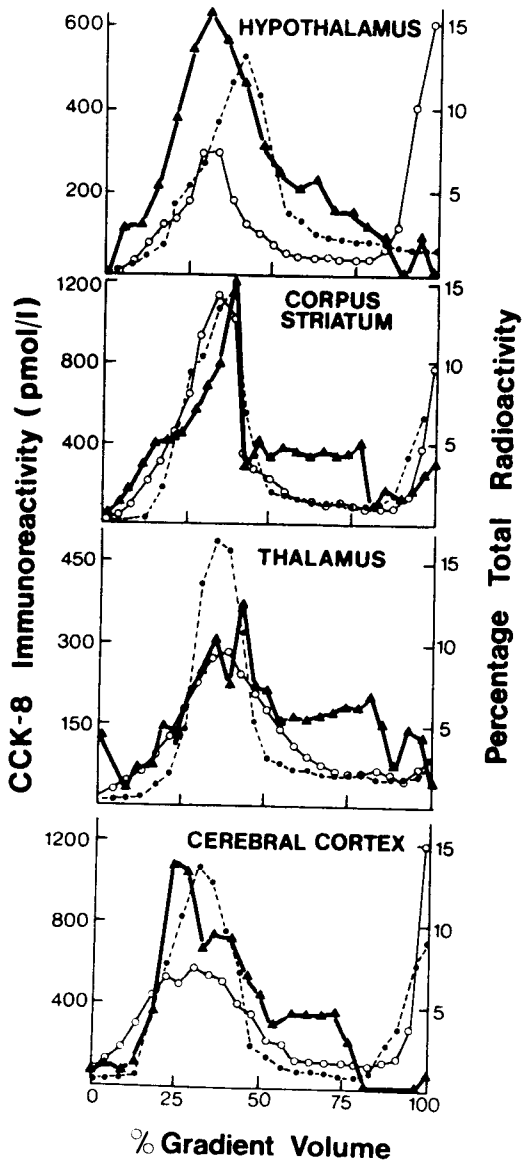
EGTA. Furthermore, veratrine-stimulated release, which occurs by the opening of sodium channels was inhibited by its specific antagonist tetrodotoxin (Table 4.1). Test substances did not crossreact with the G-2 antiserum.

D. A time course study showed that both basal and stimulated release of $iCCK_8$ increased over the full 30 min period, whereafter the rate of release decreased (Fig 4.7).

E. The synaptosomes prepared from hypothalamus and cortex were found to be intact over the 45 min of incubation. Results (Table 4.2) demonstrate occluded LDH expressed as a percentage of the total LDH activity, remains constant throughout the 45 min period whereas the supernatents from each of the time periods (i.e. for measurement of released $iCCK_8$) contained no LDH activity. Suspensions of cortical synaptosomes remained metabolically active over a 55 min period of study as shown by linear oxygen consumption over that time. Mean oxygen consumption was 66.0 ∓ 7.1 µmol O_2 /100mg synaptosome protein hour (n = 6) (Fig 4.8).

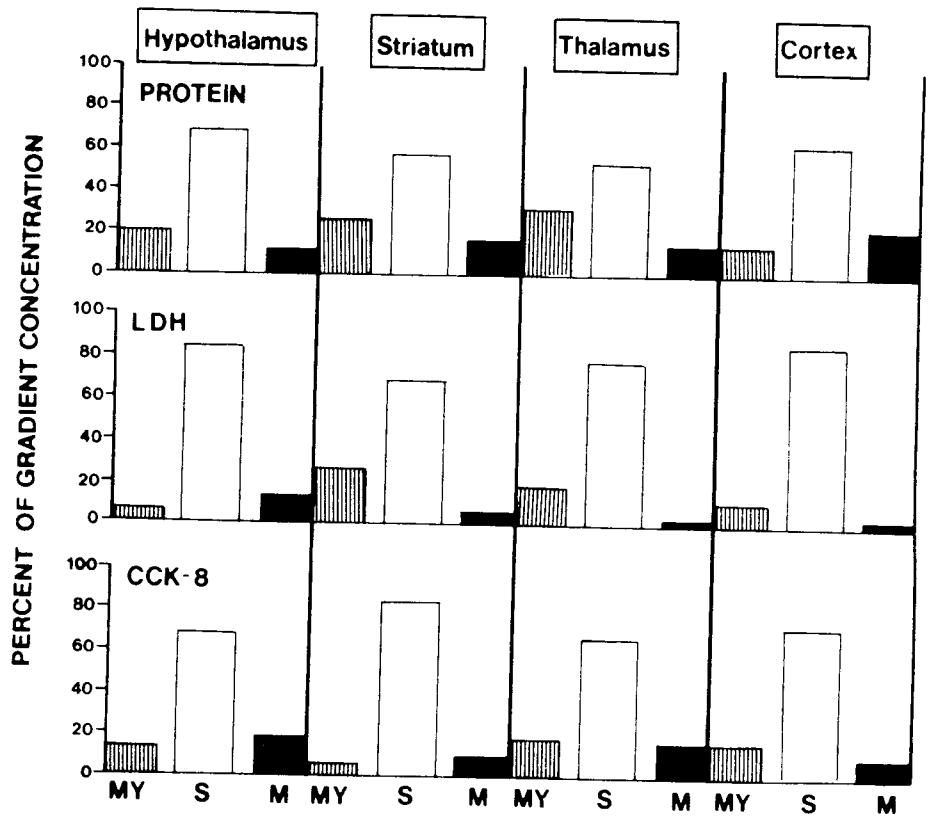
F. Extracts from cortical synaptosomes diluted out in parallel to CCK_8 but not to G-17-I and coeluted with synthetic CCK_8 on gel chromatography (Fig 4.9).

Fig 4.1



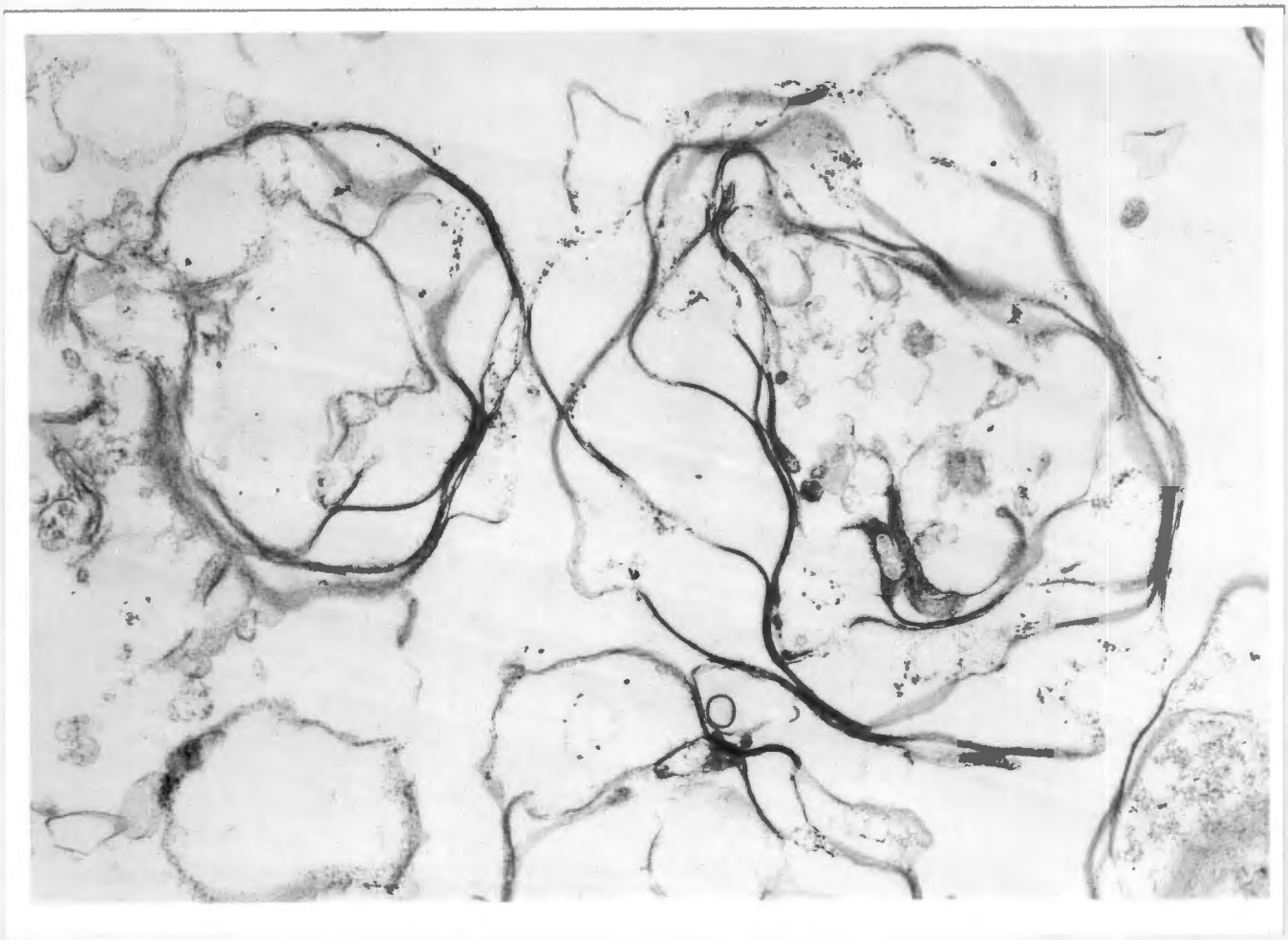
Profiles of CCK_8 immunoreactivity in ng/ml [▲——▲] [^{14}C] choline [○——○] and [^{3}H] noradrenaline [●- - -●] specific uptakes, expressed as percentages of total radioactivity, after continuous sucrose density gradient ultracentrifugation of homogenates from different areas of rat brain.

Fig 4.2



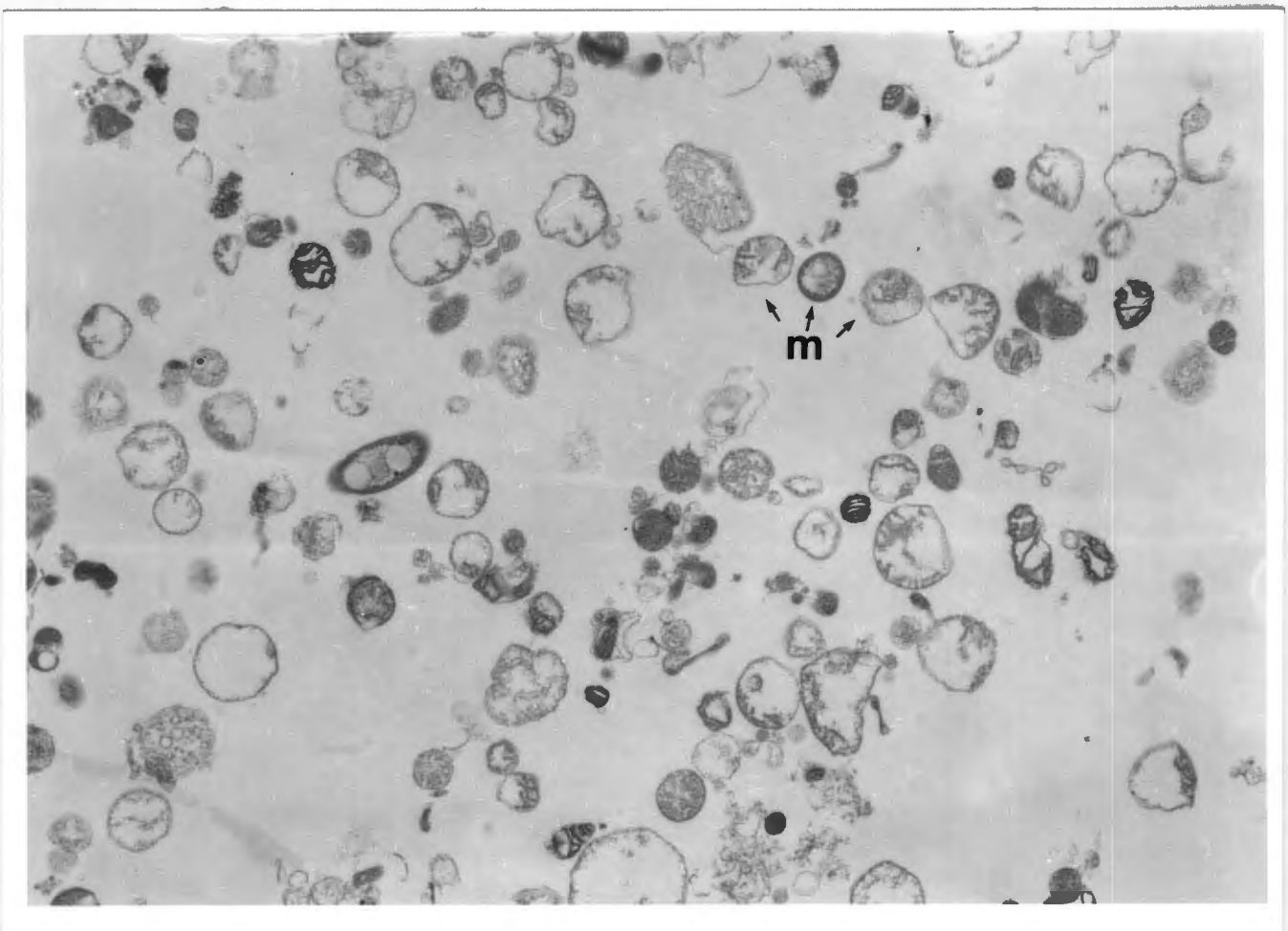
Distribution of protein, occluded lactate dehydrogenase activity (LDH) and cholecystokinin octapeptide (CCK_8) immunoreactivity on discontinuous sucrose gradients. MY represents 0.32M - 0.8M (myelin), S is 0.8M – 1.2M interface (synaptosomes) and M represents the pellet (mitochondria). Results are expressed as percentage of total gradient concentration.

Fig 4.3



Myelin fraction x 12 000

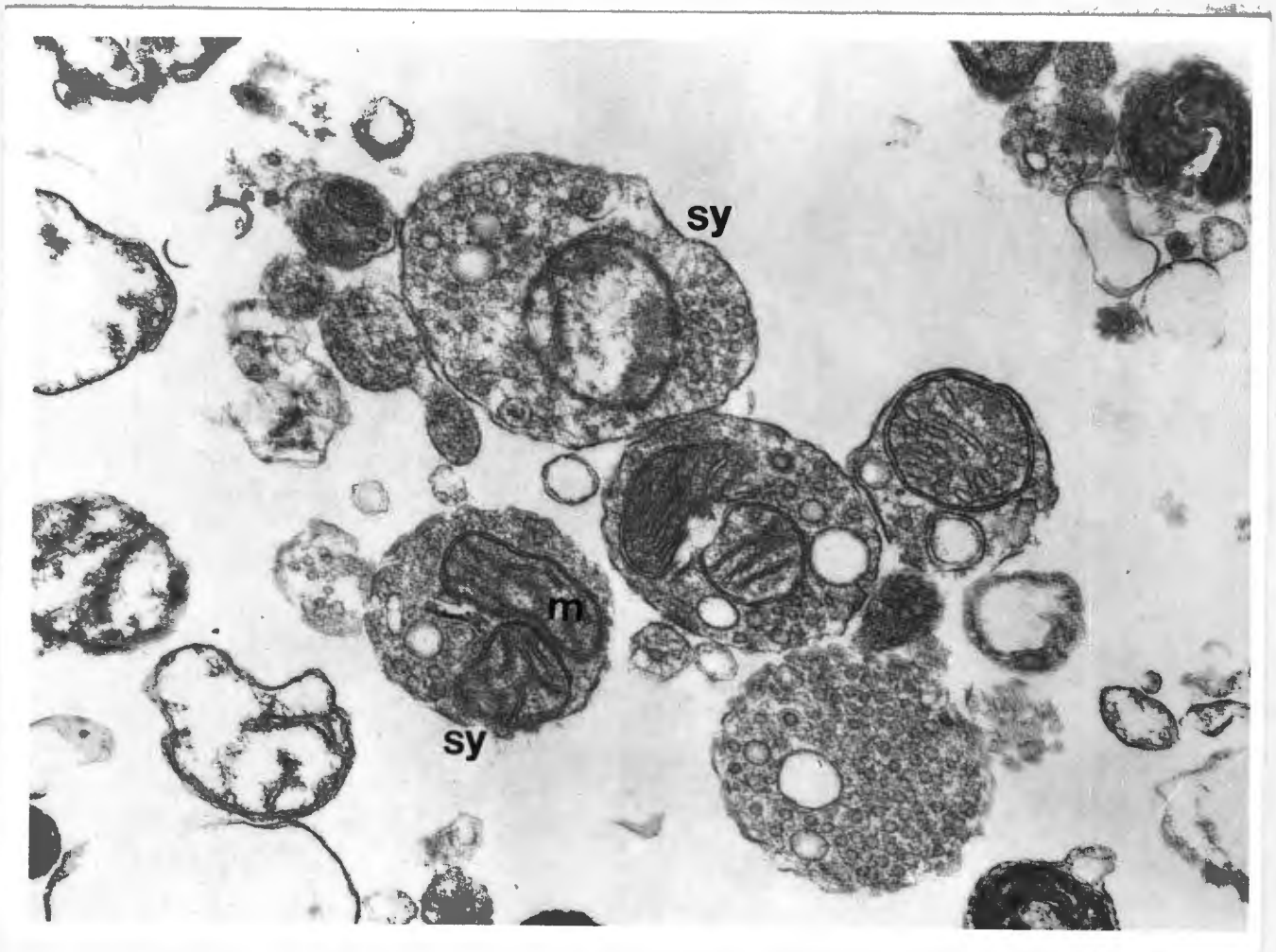
Fig 4.4



Mitochondrial fraction x 5 000

m mitochondria

Fig 4.5

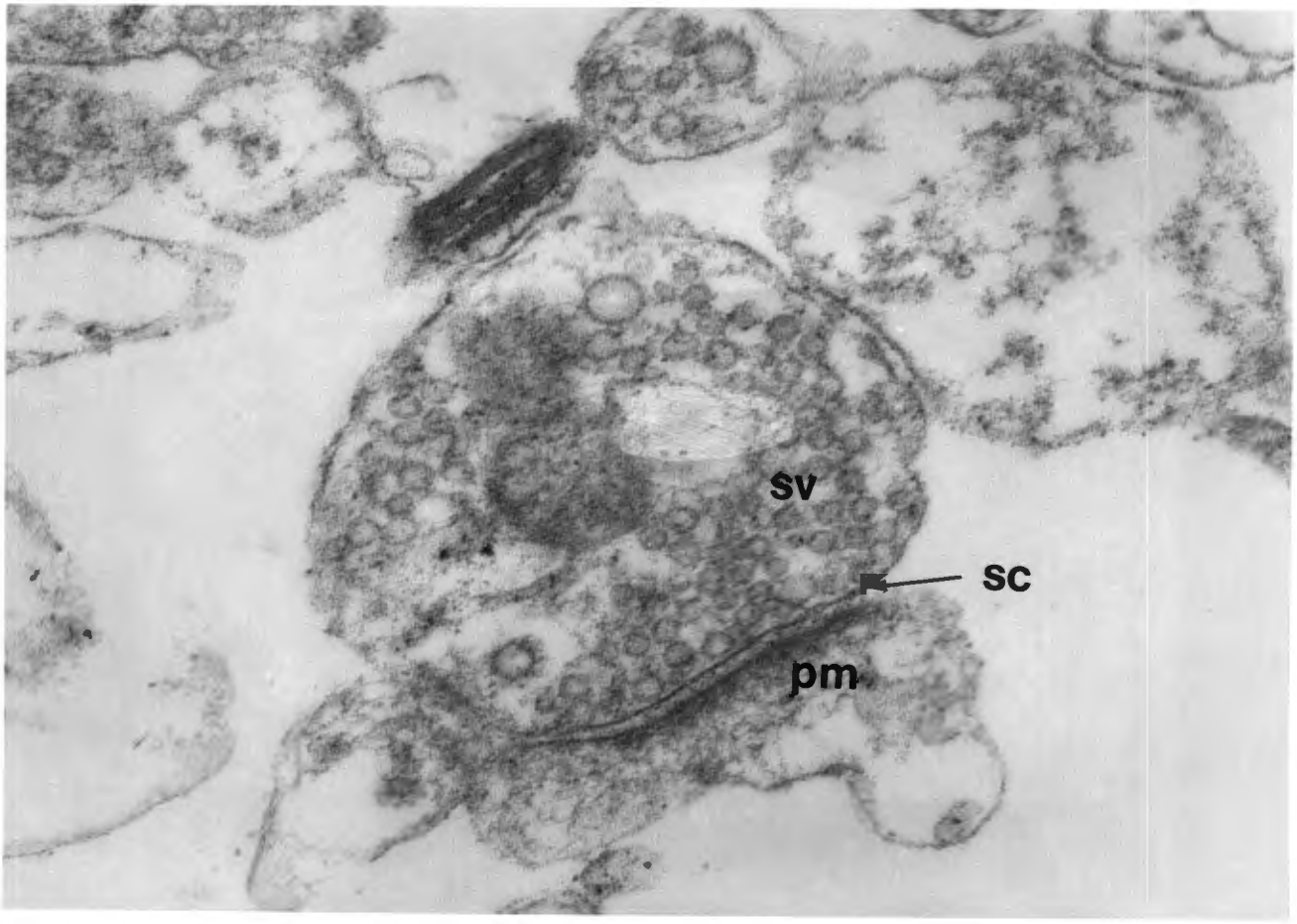


Synaptosomal fraction x 20 000

Sy synaptosomes

m mitochondria

Fig 4.6



Synaptosome x 40 000

sv synaptic vesicles

sc synaptic cleft

pm post synaptic membrane

Triton X-100, to disrupt the synaptic membranes and centrifuged for 10 min at 1.7×10^3g at 4°C. 500 µl of supernatent was transferred to 25ml scintillation vial (Packard), 5ml of scintillation fluid, Dimilume (Packard) was added to all samples and the radioactivity determined by liquid scintillation counting with a Packard Tricarb Z650 appropriately set for $[^3H]$ and $[^{14}C]$.

C 2) Potassium-stimulated release in relation to time of basal perifusion.

To show that 100 mM K^+-stimulated release occurred at all times equally throughout the perifusion, K^+-stimulated release was compared in three simultaneous parallel channels at times 0, 35 and 70 min following the equilibration period of 45 min and basal period of 30 min. In addition, in all experiments after an initial test substance stimulus 100 mM K^+ was used in a second stimulus at time $\pm$ 65 min (i.e. $\pm$ 130 min after the perifusion was started) to check on the functional viability of that particular column of synaptosomes. If K^+ given at this time did not cause CCK_8 release from a column which had not previously released CCK_8 at first stimulation, the preparation was considered non-functional.

C 3) $[^{14}C]$ choline uptake after exposure to a test substance.

In a number of experiments $[^{14}C]$-c and eserine 100 mM were perifused following the 20 min period of stimulation to assess the viability of the synaptosomes immediately after stimulation.

D. The ability of synaptosomes to release CCK_8 in response to a depolarising stimulus in a calcium-dependent manner was tested by parallel perifusion with 100 mM KCl in KRBGG with and without 1 mM EGTA. Veratrine (75 µM) was employed as an alternative depolarising agent in another experiment and the specificity of this response investigated by the addition of 1 µM tetrodotoxin along with veratrine in a parallel channel. In these experiments a smaller final volume of 4ml was used and distributed between two perifusion columns.

E. Neurotransmitters used to study the modulation of CCK release *in vitro* were :

Dopamine	(Sigma)
Noradrenaline - HCl	(Sigma)
Serotonin	(Sigma)
Acetyl Choline perchlorate	(BDH)

All were used at the concentrations of 10^{-4}M, 10^{-6}M and 10^{-8}M dissolved in KRB containing 0.01% ascorbic acid.

F. The synaptosome suspension was extracted before and after perifusing with 100 mM KCl to determine how much of the total CCK immunoreactivity was released in response to a test substance.

G. Enzymic degradation of released $iCCK_8$ was assessed by perifusing 10ng of synthetic CCK_8 through the synaptosomes. A pooled basal release was obtained and subtracted from the amount recovered after perifusion with synthetic CCK_8.

H. To establish identity of the released material synthetic CCK_8 and CCK_8 immunoreactivity extracted from cortex by boiling in neutral distilled water were fractionated on HPLC using a reverse phase system (Spectraphysics RP 8 10) 25cm x 0.46cm. Solvent A was 10% methanol, 0.01 M ammonium acetate pH 4.1, and solvent B was 90% methanol, 0.01 M ammonium acetate pH 4.1. A linear gradient of 30% B to 90% B was run over 15 min at a flow rate of 1.6ml/min. In addition boiling water extracts of cortex and the material released after KCl depolarisation was applied to a Sephadex G-50 (sf) column (Chapter Two).

I. Stimulated release was calculated as the integrated response above basal by the formula described by Richardson, Walsh, Hicks et. al. (1976) and expressed as n mol, min, ml^{-1}. Differences between basal and stimulated responses were compared by Wilcoxon signed rank test for paired samples.

RESULTS

A. During perifusion with KRBGG the synaptosomes initially showed a high rate of release of CCK_8 of ($\pm$ 5ng/ml) falling to a steady basal state of ($\pm$ 15pg/ml) by 30 min. This basal state remained constant over 30 - 140 min (Fig 5.3 inset). An equilibration time of 45 min was therefore chosen for all further experiments prior to the collection of any samples.

B. The iCCK content in the synaptosome pellets, from each column, after extraction was :

column	pg/mg protein
1	562.9
2	577
3	652

Thus it appears that the syanptosomes were equally distributed between the columns. This was also confirmed by the protein estimation of the column contents over a number of experiments.

C. C 1) The synaptosomes were found to incorporate $[^3H]$ noradrenaline and $[^{14}C]$ choline over the 135 min of incubation. Incorporation of the neurotransmitters over the 0 - 45 min period was lower than at the 45 - 90 or 90 - 135 min periods which were similar. This process could be inhibited by the addition of the ATPase inhibitor ouabain (10^{-4} M) or by inactivating the metabolic processes by perifusing at $4^{o}C$. Diminution of uptake of $[^3H]$ noradrenaline and $[^{14}C]$ choline occurred regardless of whether 0 - 45 min, 45 - 90 min or 90 - 135 min was chosen as the control time period (Fig 5.4).

C 2) Peaks of CCK immunoreactivity released in response to the depolarising stimuli 100 mM KCl occurred over the 135 min of incubation, a peak being associated with each time period of 0 - 35, 35 - 70, 70 - 105 (Fig 5.5). A second peak of released iCCK in response to the depolarising stimuli 100 mM KCl after 45 min at basal conditions could be demonstrated after previous exposure to a

test substance. This second peak was lower than the first peak if the test substance had caused release of CCK. If the test substance had failed to cause any release of CCK it could be shown that the addition of 100 mM KCl caused a greater release of CCK than in the channel that had previously been stimulated (Fig 5.7).

C 3) When $\left[^{14}C\right]$ choline and eserine were added immediately after stimulation, a second peak of released iCCK could be demonstrated which was greater than the initial peak in response to the test stimulus (Fig 5.9). Uptake of $\left[^{14}C\right]$ choline was similar to the 45 - 90 and 90 - 135 min periods in experiment C 1).

D. The depolarising stimuli, high extracellular K^+ and veratrine caused a significant increase in the release of CCK. The K^+ induced release could be inhibited by the addition of the Ca^{++} chelating agent, EGTA, thus demonstrating the Ca^{++} dependence of this release (Fig 5.3). The veratrine induced release could be inhibited by the addition of its specific antagonist, tetrodotoxin (Fig 5.6). The integrated release of CCK_8 in these experiments was greater than those reported below because of the higher concentration of tissue used per column. Test substances had no cross-reactivity with the G-2 antiserum.

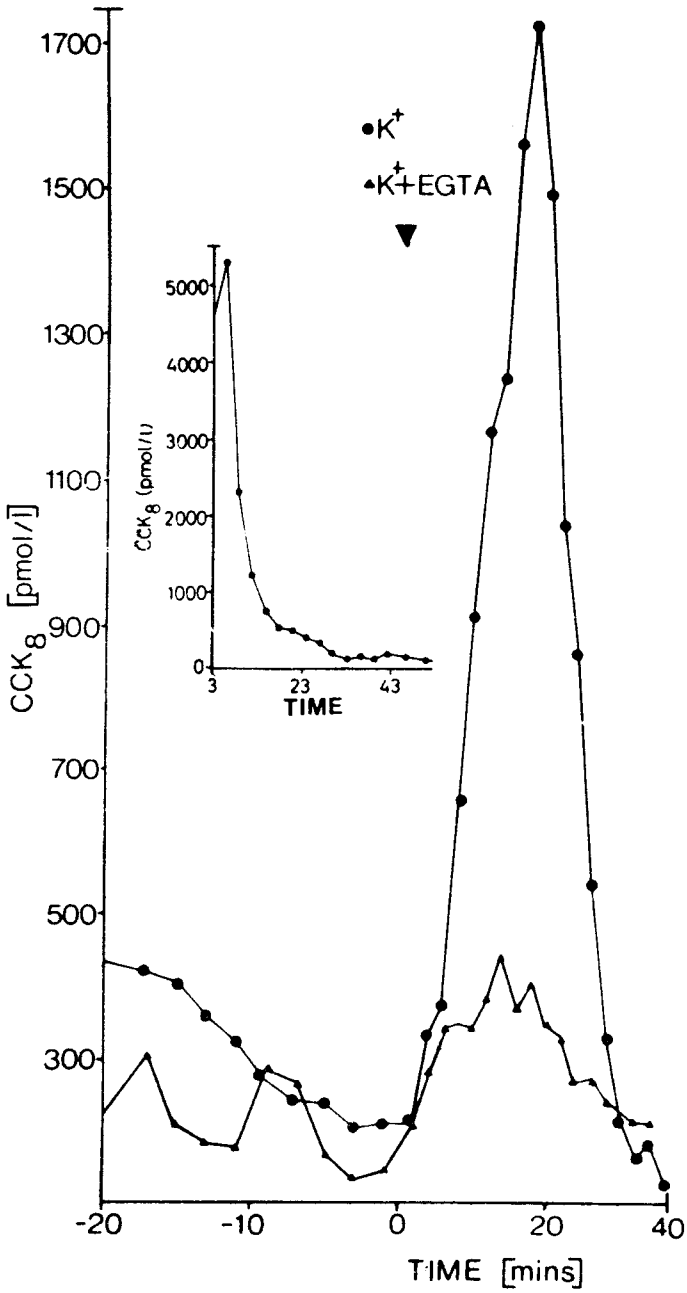
E. Perifusion with the neurotransmitters, serotonin and noradrenaline at 10^{-4}M, 10^{-6}M and 10^{-8}M concentrations had no effect on the release of CCK (Fig 5.7) (Table 5.1). Dopamine at 10^{-4}M concentration had no effect while 10^{-6}M caused a significant increase in the release of CCK (Fig 5.8). Dopamine at 10^{-8}M concentration caused release in four out of five cases but this did not reach statistical significance (Table 5.1). Perifusion with acetyl choline at 10^{-4}M and 10^{-8}M concentrations had no effect while 10^{-6}M concentration significantly increased the release of CCK (Fig 5.9) (Table 5.1). There was no crossreactivity with the G-2 antiserum with any of the test substances used.

F. 18.9% $\pm$ 3.77% (n = 3) of the total amount of iCCK present in the synaptosomes was released in response to the depolarising stimulus of 100 mM KCl.

G. Recovery of synthetic CCK_8 perifused through the incubating synaptosomes was 90.1 $\pm$ 7.8%.

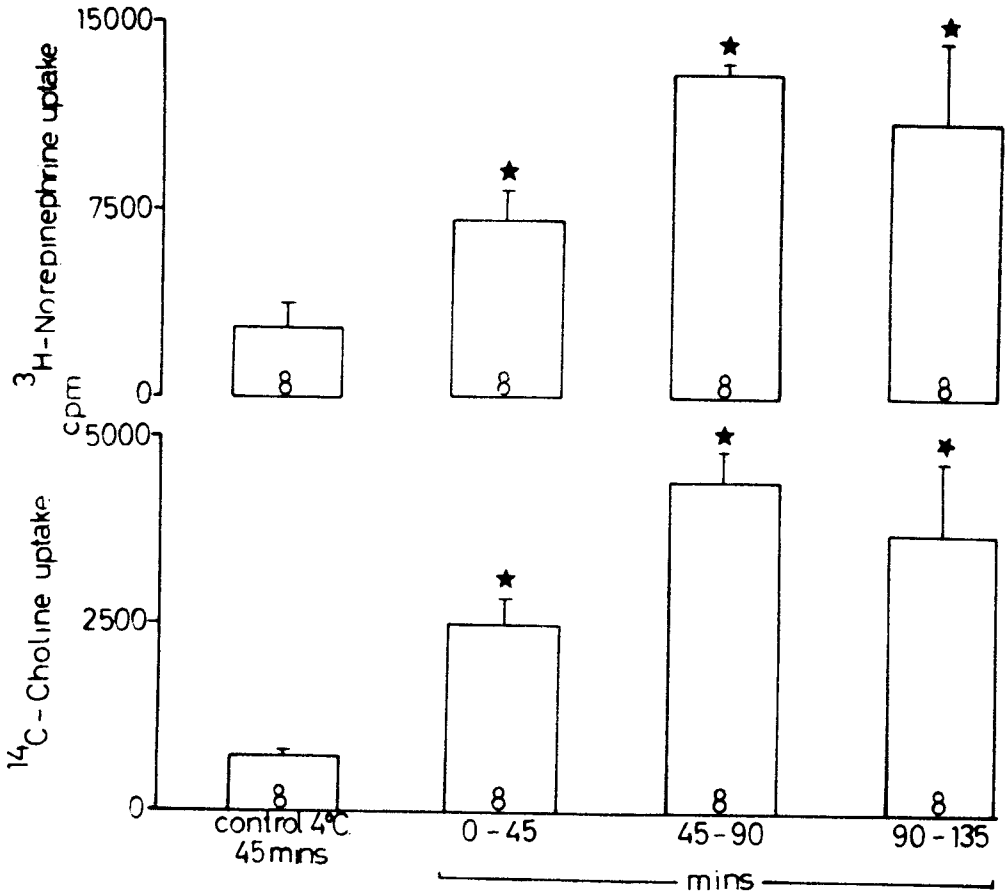
H. Boiling water extracts of cortex yielded radioimmunoassay displacement curves parallel to those of synthetic CCK_8 standards (Fig 3.1) and coeluted with synthetic CCK_8 with both the HPLC (Fig 5.10) and Sephadex G-50 (sf) chromatography (Fig 5.11). In addition the synaptosome release material coeluted with both the cortical extracts and the synthetic standards. This does not however, exclude the possibility of other CCK-like peptides being released but rather demonstrates the relative specificity of this antiserum for CCK_8.

Fig 5.3



CCK_8 release from parallel synaptosome/Bio-gel columns in response to 100 mM KCl and 100 mM KCl + EGTA.
The inset shows release of CCK_8 immediately following the commencement of perifusion. Stable release occurs after 30 min.

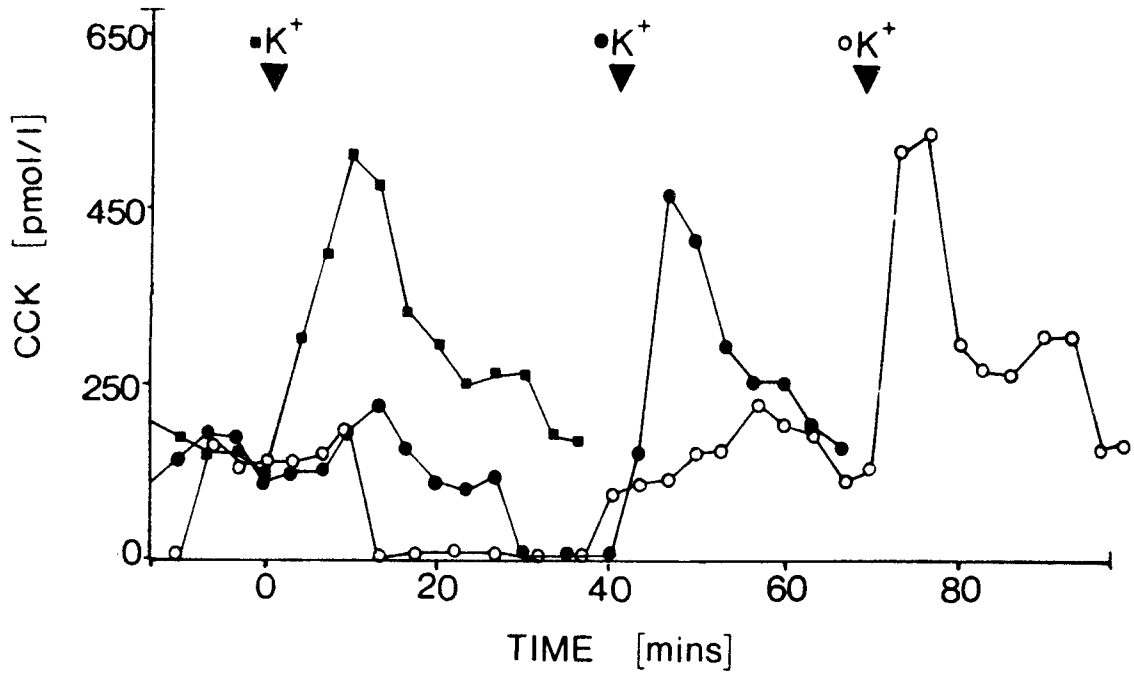
Fig 5.4



(mean ± SEM) uptake, at 37°C, of $[^3H]$ noradrenaline and $[^{14}C]$ choline during incubation periods 0 - 45, 45 - 90 and 90 - 135 min. The control was perifused for 45 min, at 4°C, at one of the above time periods.

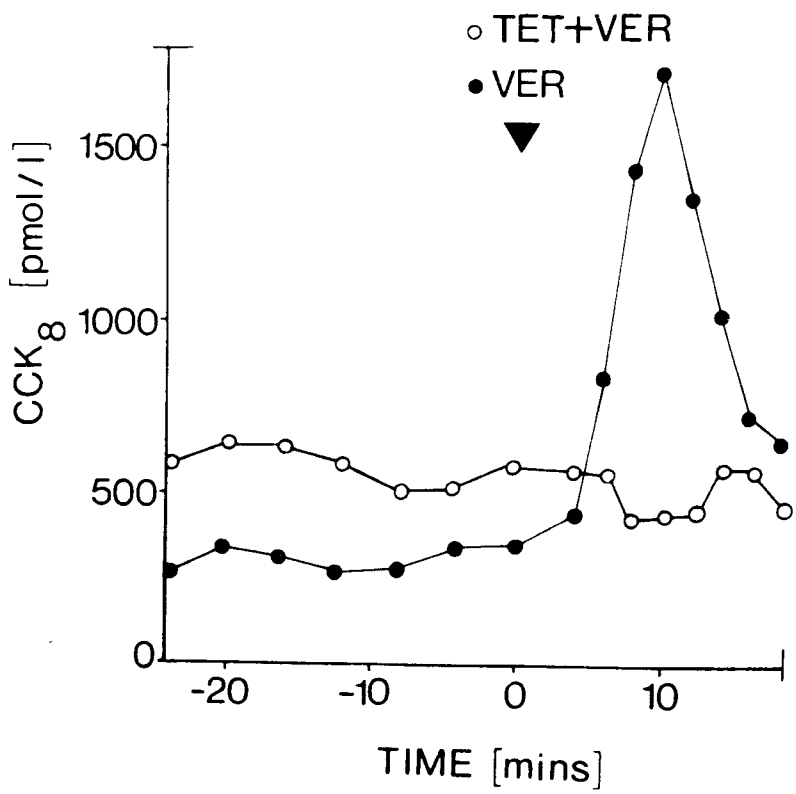
* $p < 0.05$ above control. The number of experiments is indicated

Fig 5.5



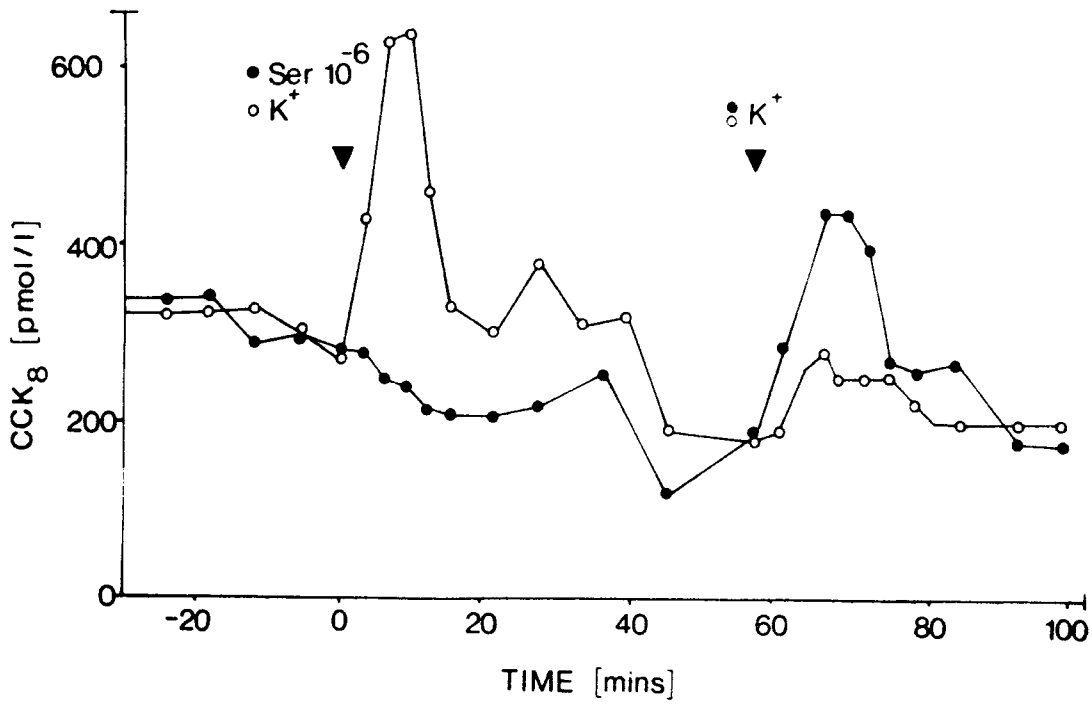
Release of CCK_8 from synaptosomes in response to 100 mM KCl at varying times over 135 min after the commencement of perifusion. The figure does not indicate the 45 min equilibration and 30 min basal periods preceeding the first stimulus.

Fig 5.6



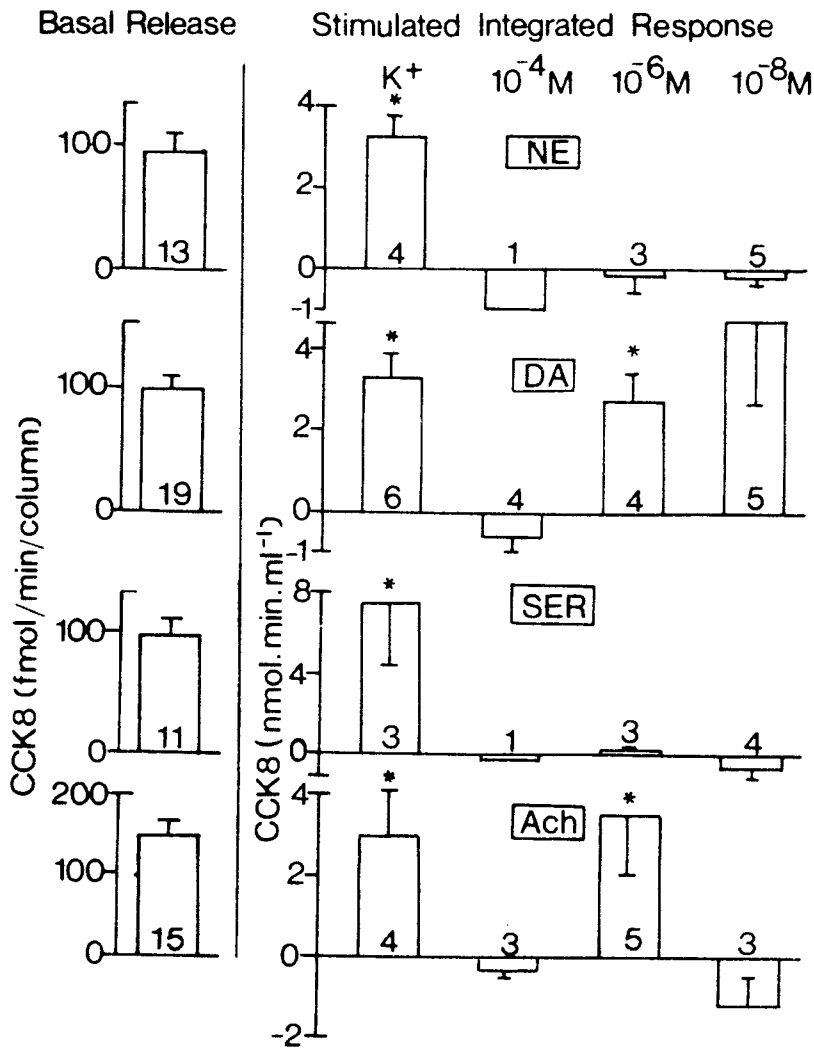
CCK_8 release from parallel synaptosome/Bio-gel columns is response to 75 μM veratrine and 75 μM veratrine + 1 μM tetrodotoxin.

Fig 5.7



Perifusion with 100 mM KCl caused significant release of CCK_8 whereas perifusion with serotonin (10^{-6}M) in a parallel column had no effect. At 60 min 100 mM KCl stimulated release in both columns showing viability of the synaptosomes.

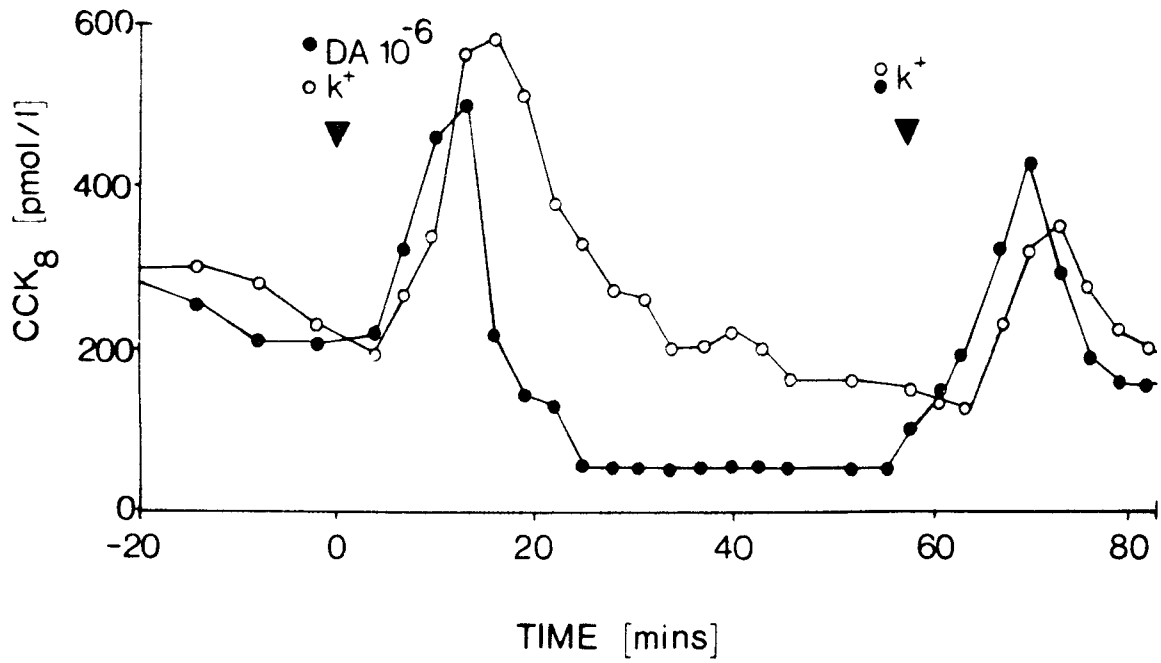
Table 5.1



Effect of 100 mM KCl and biogenic amines (10^{-4}M, 10^{-6}M and 10^{-8}M) on CCK_8 release from perifused cortical synaptosomes. Basal release (f mol/min/column) did not differ significantly between groups of experiments. Results are expressed as mean $\pm$ SEM where the number of experiments are indicated.

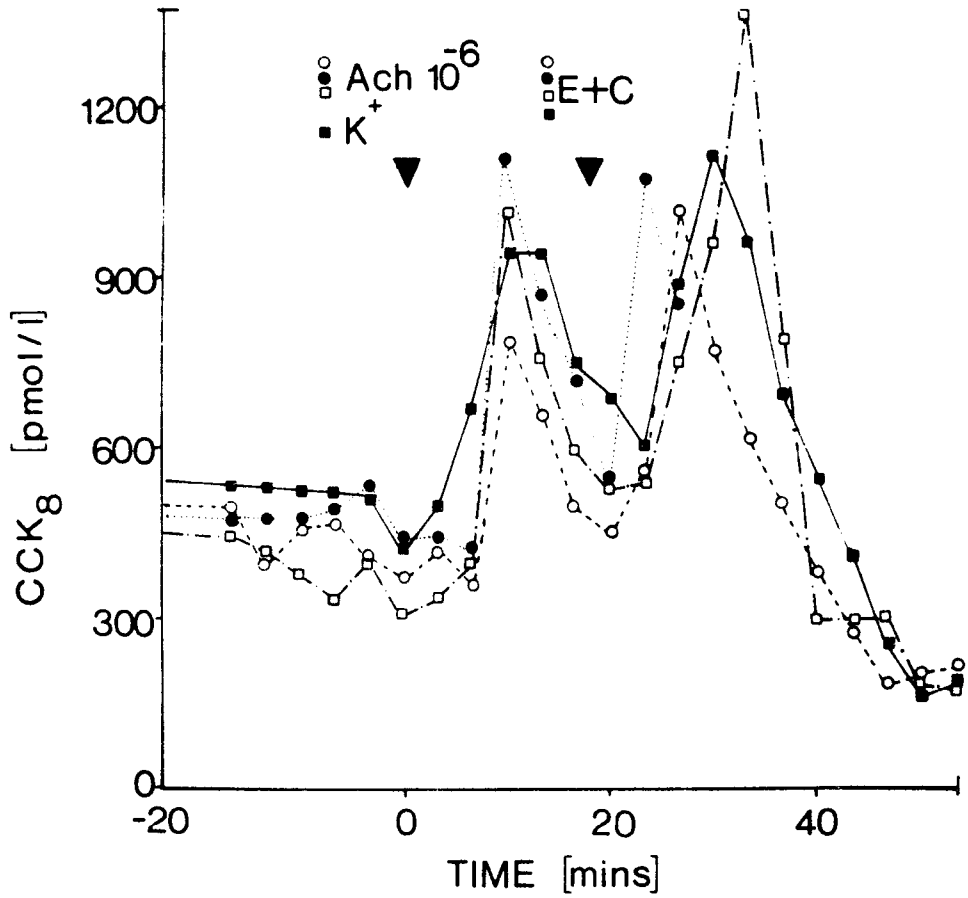
* $p < 0.05$ significant response above basal

Fig 5.8



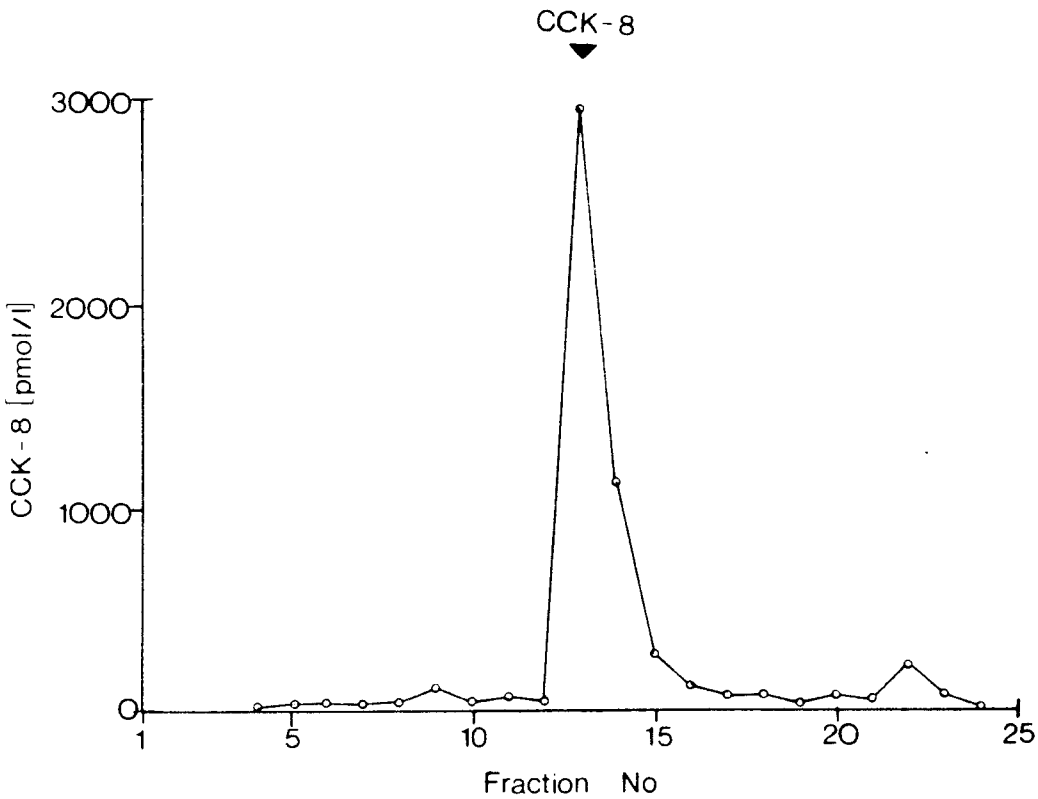
Perifusion with 100 mM KCl in the control column and dopamine (10^{-6}M) in a parallel column caused significant release of CCK_8 from the synaptosomes. 100 mMKCl stimulated release in both columns 58 min after initial stimulation.

Fig 5.9



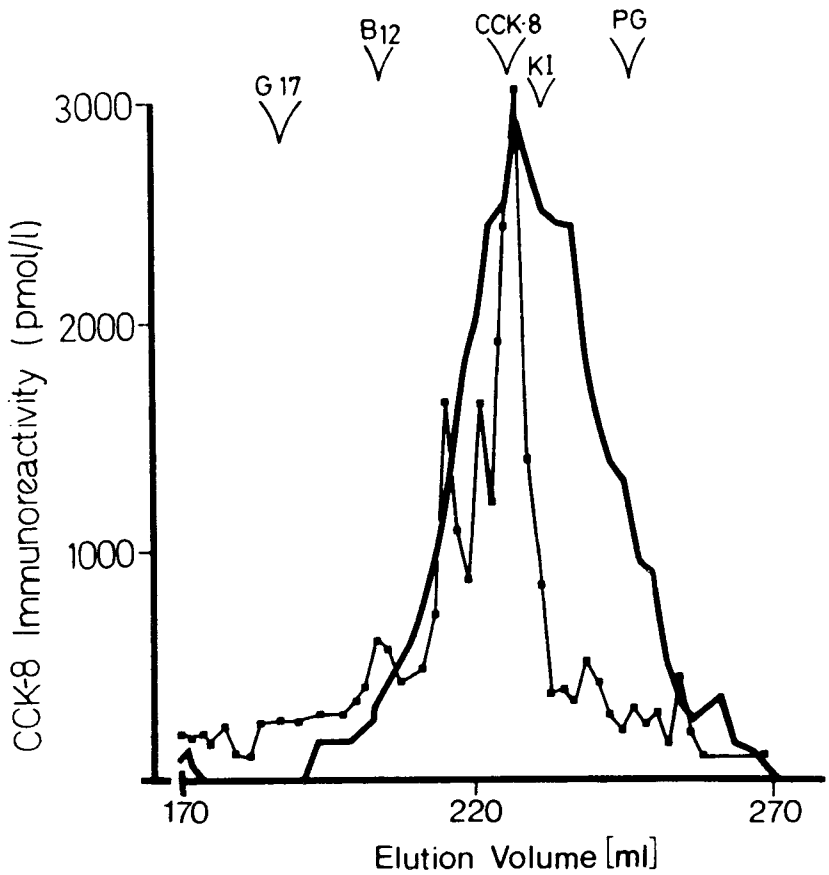
Perifusion with 100 mM KCl in one column and acetyl choline (10^{-6}M) in three parallel columns caused significant release of CCK_8 from the synaptosomes. In addition, eserine, 100 mM and $[^{14}C]$ choline added to all columns after stimulation caused a second peak of CCK_8 release.

Fig 5.10



Boiling water extracts of cortex coelute as a single peak with CCK_8 standards on HPLC.

Fig 5.11



Gel chromatography of boiling water extracts of cortex (——) and the cortical synaptosome release material (■——■) on Sephadex G-50 (sf). The column was equilibrated with synthetic human heptadecapeptide gastrin (SHG-17-1), cyanocobalamin (B12), cholecystokinin octapeptide (CCK_8), pentagastrin (PG) and potassium iodide (KI).

DISCUSSION

The use of a perifusion system to study release of peptides from isolated synaptosomes has several advantages over the conventional incubation method. These are the low basal release of peptide, the low rate of peptide degradation and the ability to elicit multiple stimuli from the same preparation.

In previously described continuous synaptosome superfusion techniques, the synaptosome fraction was layered on a G-15 Sephadex bed and samples collected for 60 (Bosse and Kuchinsky, 1978) and 90 (Mulder, Van Den Berg and Stoof, 1975) min after commencement of the perifusion.

The system described here differs from these in that the synaptosomes are distributed throughout the Bio-gel mesh which acts as a bed matrix and appears to provide a stable protective support for the synaptosomes, which remain functionally viable for at least $2\frac{1}{2}$ hours.

Using this technique we have been able to demonstrate that there is a high release of CCK_8 during the equilibration period which probably reflects the removal of peptides released from synaptosomes during their preparation and from non intact synaptosomes. During the first 45 min of perifusion, $\left[^{14}C\right]$ choline and $\left[^{3}H\right]$ noradrenaline uptake was lower than in subsequent periods. The 45 min equilibration period thus allows the synaptosomes to return to an optimal functional level and permits the removal of non-occluded peptide. Using a static incubation technique early release during the equilibration period is included in the basal release and thus the relative increase of the stimulation may be poor. Another advantage of the present system is that we have been able to demonstrate that the synaptosomes respond fully for at least 160 min after the beginning of the perifusion and moreover the same preparation may be stimulated a second and possibly even a third time. Lastly, the perifusion technique potentially has yet another advantage in that exposure to released degradative enzymes is obviated by the

continuous flow and rapid boiling of the perifusate. We have demonstrated 90% recovery of CCK_8 in the absence of protease inhibitors which compares to 20% reported by Dockray, Dodd, Edwardson et. al. (1980) using the static system. A disadvantage of this system however, is the large number of samples generated for each experiment.

The exposure of synaptosomes to 100 mM KCl or 75 μM veratrine stimulated the release of CCK_8. As discussed in Chapter Four, increasing the extracellular concentration of K^+ reduces the potential difference across the membrane whereas veratrine is thought to open Na^+-channels, both generating an action potential (Li and White, 1977). This veratrine-stimulated release can be blocked by its specific antagonist, tetrodotoxin. The Ca^{++} dependence of this release is demonstrated by the inhibition of K^+ stimulated release by the Ca^{++} chelating agent EGTA.

To date there have been no reports regarding neurotransmitter control of release of CCK_8 from brain. We have shown dopamine and noradrenaline stimulated CCK_8 release from isolated incubated hypothalami (Chapter Six). The finding that CCK_8 release occurred in response to dopamine and acetylcholine at 10^{-6}M but not in response to the higher concentrations implies that high (10^{-4}M) dopamine and acetylcholine are able to reverse the effect at 10^{-6}M. Whether this is due to actions on different types of receptors (Kebabian and Calne, 1979) with different effects or due to opposite effects of neurotransmitters on different populations of CCK synaptosomes from different areas, cannot be determined in this study. A similar finding was made by Bennett, Edwardson, Marcano De Cotte et. al. (1979) using hypothalamic synaptosomes where they reported that 10^{-8}M dopamine stimulated somatostatin release, whereas 10^{-4}M dopamine inhibited release. Denef, Manet and Dewals (1980) have reported both stimulatory and inhibitory effects of high and low dose dopamine on prolactin release from cultured pituitary cells. Hökfelt, Rehfeld, Skirboll et. al. (1980) have shown the co-existence of CCK-like peptides in a subpopulation of dopaminergic neurones in

rat and man. These neurones project to mesolimbic areas in forebrain. CCK fragments have been shown to inhibit dopamine release in the nucleus accumbens and tuberculum olfactorium region. Hökfelt, Rehfeld, Skirboll et. al. (1980) suggested that the peptide released together with the main neurotransmitter dopamine, may be part of an inhibitory system modulating dopamine release via an action on autoreceptors. The data presented here which demonstrates that CCK_8 is released by dopamine is consistent with such an autoreceptor hypothesis.

In conclusion we have devised a new technique for perifusing brain synaptosomes dispersed within a Bio-gel P2 column which obviates many of the difficulties experienced with static incubation systems. Rat cortical synaptosomes release CCK_8 in response to depolarising stimuli of 100 mM KCl and 75 μM veratrine and the release is calcium-dependent. Moreover the demonstration that neurotransmitters affect CCK_8 release in physiological concentrations provides further evidence for CCK_8 being a modulator of neuronal function.

CHAPTER SIX

CHOLECYSTOKININ OCTAPEPTIDE RELEASE FROM INCUBATED RAT HYPOTHALAMI

INTRODUCTION

CCK was found to be widely distributed throughout rat brain (Chapter Three). The highest concentrations of CCK have been found in the cerebral cortex but in addition high concentrations are present in the hypothalamus. Immunocytochemical studies have shown the presence of CCK-like immunoreactivity in the dorsomedial nucleus of the hypothalamus (Innes, Corrêa, Uhl et. al., 1979; Larsson and Rehfeld, 1979) whilst the presence of CCK_8 in rat hypothalamic synaptosomes and the release of CCK_8 from the synaptosomes by depolarising stimuli has been demonstrated in Chapter Four. Morley, Melmed, Briggs et. al. (1979) have demonstrated that CCK_8 releases growth hormone from isolated rat pituitaries whilst Domschke, Lux and Domschke (1980) demonstrated that the structurally related peptide, pentagastrin, stimulates growth hormone secretion in man. These studies suggest a possible hypophysiotrophic role for this peptide. In view of this possible role of CCK as regulator of GH we have examined the regulation of CCK release from hypothalami. The hypothalamic block was dissected to include parts of the paraventricular, dorsomedial, ventromedial, suprachiasmatic and arcuate nuclei as well as the median eminence (Hirooka, Hollander, Suzuki et. al., 1978) all of which have been shown by immunohistochemistry to contain CCK (Table 1.3).

MATERIALS AND METHODS

Hypothalamic blocks, 1mm deep and bounded by the optic chiasm, lateral grooves and mamillary bodies, were dissected from male Long-Evans rats within 1 - 2 min and placed in a glass incubating dish

(100ml) containing incubation medium.

The incubation medium consisted of :

NaCl	126	mM
$NaHCO_3$	22	mM
KCl	6	mM
$CaCl_2$	1.45	mM
NaH_2PO_4	1	mM
$MgSO_4 \cdot 7H_2O$	0.88	mM
D-glucose	11	mM
Gelatine	0.1	% w/v (Hopkins and Williams)

The hypothalami, at all times, were incubated in a shaking water bath at 37°C, in an atmosphere of 95% O_2, 5% CO_2. Following a 60 min preincubation period the hypothalami were individually placed in stoppered glass vials (10ml) containing 0.5ml of incubation medium or 0.5ml of test medium. After 30 min of incubation the medium was decanted, boiled, snap frozen and stored at -20°C until assayed for CCK_8 immunoreactivity.

A. Viability of the incubating hypothalami was assessed by the following studies :

A 1) O_2 Consumption

Their ability to consume oxygen using the Warburg constant volume manometer (Chapter Two). The incubation medium was gassed with 100% oxygen, bicarbonate being replaced by phosphate (Na_2HPO_4, 22 mM).

A 2) Protein Synthesis

Their capacity to synthesise proteins as assessed by the incorporation of a labelled $\left[{}^3H\right]$ amino acid mixture into the perchloric acid precipitable protein fraction and the inhibition of this incorporation by specific inhibitors of protein synthesis. Individual hypothalami were incubated in a shaking water bath at 37°C in 0.5ml of medium (as for incubation experiments) containing 20 μCi $\left[{}^3H\right]$ amino acid mixture (code TRK 440 ; Batch 26 ; radioactive concentration

of mixture 1mCi/ml) (Radiochemical Centre, Amersham, England).

In some experiments, puromycin (Sigma) 1 000 µg/ml and cycloheximide (BDH) 500 µg/ml were added to the incubation medium. At time 2, 30, 60 and 90 min hypothalami were removed and placed in $[^3H]$ amino acid free medium to wash off any excess label from the surface of the block and then homogenised in 2ml ice-cold distilled water. 200 µl of cold 1M perchloric acid was added to 200 µl of the homogenate to precipitate protein and centrifuged for 10 min at 1.7×10^3 g at 4°C. The precipitate was washed three times by resuspending in 200 µl of cold 0.5 M perchloric acid and recentrifuging. The final pellet was resuspended in 200 µl of distilled water, the tube washed out with a further 200 µl of water and transferred to a 25 ml glass scintillating vial (Packard). The sample was solubilised by the addition of 800 µl of soluene 350 (Packard). 5ml of scintillation fluid, Dimilume (Packard) was added to all samples and the radioactivity determined by liquid scintillation counting with a Packard Tricarb Z650 appropriately set for $[^3H]$.

B. In preliminary experiments the preincubation time of 60 min was determined by incubation in medium for 30, 45, 60 and 90 min while the post stimulation time of 30 min was determined by incubation with and without 60 mM KCl for 15, 30 and 45 min.

C. 10ng synthetic CCK_8 was added to the medium of 8 incubating hypothalami. After 30 min of incubation the recovery of the added CCK_8 was compared to the endogenous CCK_8 released from a parallel set of hypothalami without the addition of synthetic CCK_8.

D. To show identity of the released material to CCK_8, serial dilutions were prepared in assay buffer and measured for CCK_8 immunoreactivity. In addition the elution profile of the material was compared after gel chromatography, Sephadex G-50 (sf) with that

of synthetic CCK_8 (Chapter Two).

E. To study the release of immunoreactive CCK_8 in response to depolarising stimuli and the calcium dependence of this release. The following substances were individually made up in the previously described incubation medium to give final concentrations of :

KCl 60 mM

Veratrine 75 μM (Sigma)

KCl 60 mM + Ethyleneglycol - bis - (β - aminoethyl ether) NN^1 - tetra-acetic acid (EGTA, Sigma) 1 mM

Veratrine 75 μM + tetrodotoxin 1 μM (Calbiochem)

Calcium ionophore A23187 0.1 μM (courtesy of Lilly Laboratories).

Medium containing no Ca^{++} was prepared by substituting choline chloride for $CaCl_2$.

F. Neurotransmitters used to study the modulation of CCK_8 release in vitro were :

1. Dopamine $10^{-4}M$, $10^{-6}M$, $10^{-8}M$ and $10^{-10}M$
2. Noradrenaline $10^{-4}M$, $10^{-6}M$, $10^{-8}M$ and $10^{-10}M$
3. Serotonin $10^{-6}M$ and $10^{-8}M$
4. Acetyl Choline $10^{-6}M$ and $10^{-8}M$

0.01% ascorbic acid was added to each neurotransmitter to prevent oxidation.

G. The release of CCK_8 in response to dopamine $10^{-6}M$, which was found to release CCK_8 was studied following preincubation of the hypothalamus with the following preparation for 10 min.

1. rat growth hormone (rGH) NIAMDD - rat EH-RP-I
2. somatostatin (SRIF) Beckman Laboratories
3. phentolamine (CIBA) $10^{-6}M$ and 10^{-8} concentrations

Dopamine was then added to give a final concentration of $10^{-6}M$ and the incubation continued as previously described.

CCK_8 was measured using the CP6 antiserum (Chapter Two).

H. Significant differences between the release of $iCCK_8$ in response to the test substance and the release observed in normal medium (control) were evaluated by the students t test. Due to variation in basal release of $iCCK_8$ between experiments, control results in each experiment were corrected to a mean absolute value while test results within the same experiment were similarly corrected using the correction factor derived for that experiment.

RESULTS

A 1) Oxygen consumption from the incubated hypothalami was linear over the 120 min period studied ($r = 0.89$, $p < 0.001$ (Fig 6.1). Mean oxygen consumption was 114.1 ± 6.5 µmol O_2 / g,hr (mean $\pm$ SEM, $n = 6$).

A 2) The hypothalami incorporated $[^3H]$ amino acids into the protein precipitable fraction in a linear fashion, over the 90 min of incubation (Fig 6.2) ($r = 0.92$, $p < 0.001$). The addition of puromycin 1 000 µg/ml and cycloheximide 500 µg/ml resulted in $78.3 \pm 0.8\%$ inhibition (mean $\pm$ SEM, $n = 6$) of incorporation of $[^3H]$ amino acid mixture at 90 min.

B. CCK_8 release was uniform from 60 min - 90 min preincubation. A preincubation time period of 60 min was used for all subsequent experiments. The time-course of release showed that CCK_8 release increased up to 30 min. 60 mM KCl caused significant increase in release relative to control at this time period. Total release of CCK_8 up to 45 min was diminished (Fig 6.3). Degradation or reuptake of released CCK_8 probably occurring after 30 min of incubation.

C. The recovery of synthetic CCK_8 added to the medium of the incubating hypothalami for 30 min was $87.5\% \pm 8.1\%$ (mean $\pm$ SEM, $n = 8$).

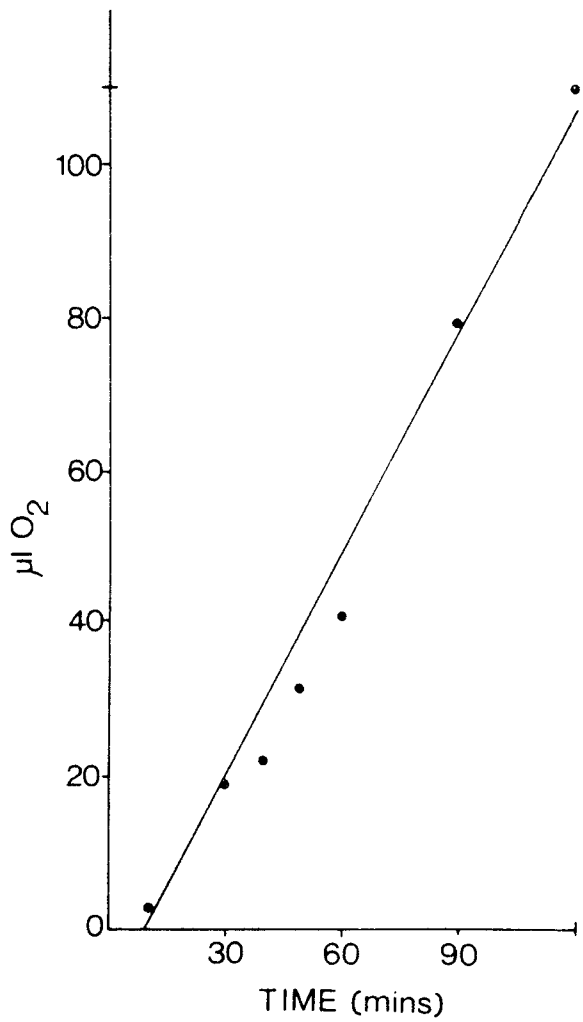
D. The released material caused displacement of ^{125}I-G-17-1 parallel to that of CCK_8 standards (Fig 6.4) and coeluted with synthetic CCK_8 on gel chromatography (Fig 6.5)

E. Release of CCK_8 was significantly increased by the depolarising stimuli, 60 mM KCl and 75 μM veratrine. The release in response to high K^+ was inhibited in the presence of the calcium chelating agent EGTA and the release in response to veratrine inhibited by its specific antagonist, tetrodotoxin. The calcium ionophore A23187 significantly stimulated release. No release occurred in the absence of Ca^{++} (Fig 6.6).

F. Dopamine at 10^{-4}M, 10^{-6}M and 10^{-8}M concentrations caused a significant release of CCK_8 with the maximal effect caused by 10^{-6}M ($p < 0.001$) (Fig 6.7). Results obtained with noradrenaline were inconsistant at all the concentrations used and no conclusions could be drawn. There was no significant release of CCK_8 in response to any of the other neurotransmitters at the concentrations used (Table 6.1).

G. SRIF at all concentrations did not significantly alter the dopamine effect. Similarly rGH which has been shown to cause SRIF release from the isolated hypothalamus (Sheppard, Kronheim and Pimstone, 1978) did not alter 10^{-6}M dopamine-stimulated CCK_8 release. Phentolamine 10^{-6}M and 10^{-8}M which is a specific α-adrenergic blocker significantly inhibited dopamine-stimulated CCK_8 release ($p < 0.001$) (Table 6.2).

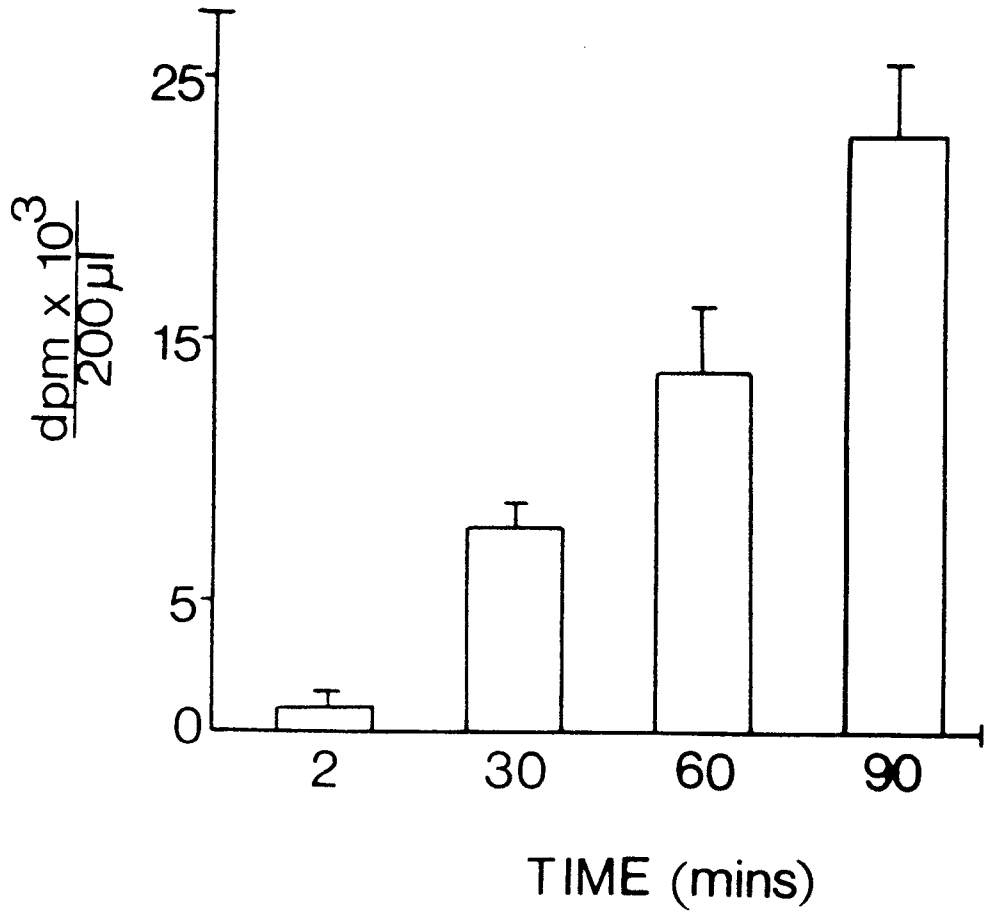
Fig 6.1



Oxygen consumption (µl O_2) by hypothalami

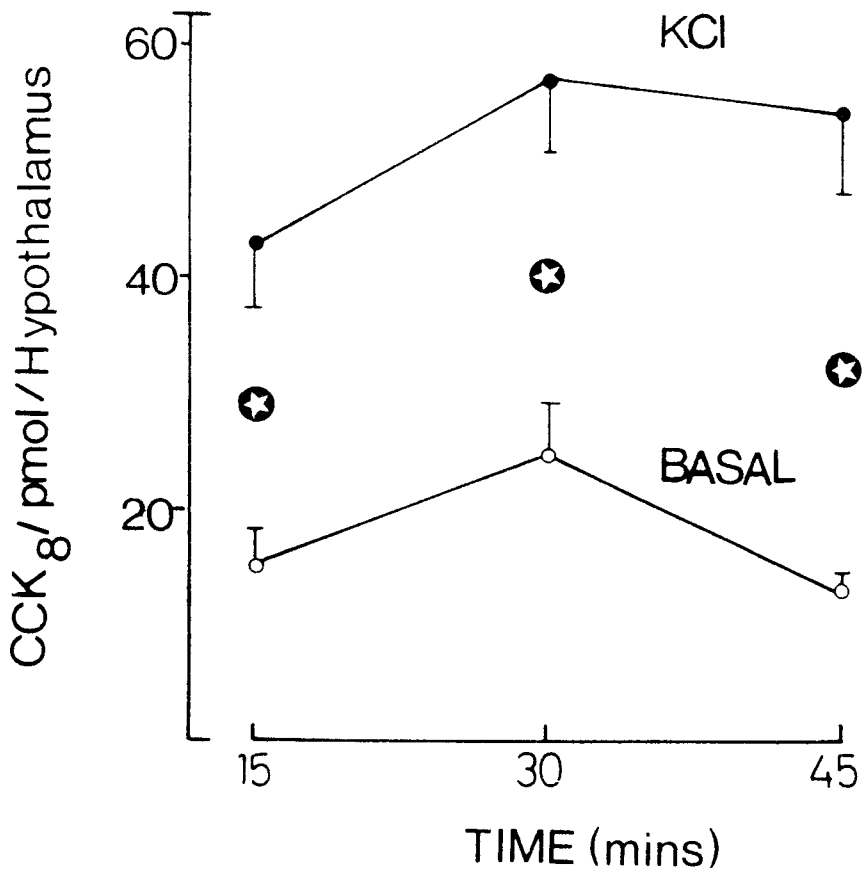
(r = 0.8865, $p < 0.001$, results from 5 experiments)

Fig 6.2



Incorporation of $[^3H]$ amino acids (dpm x 10^3/ 200 μl) by incubated hypothalami into the perchloric acid-precipitate. Histograms represent mean $\pm$ SEM of 6 experiments at each time point ($r = 0.9169$, $p < 0.001$)

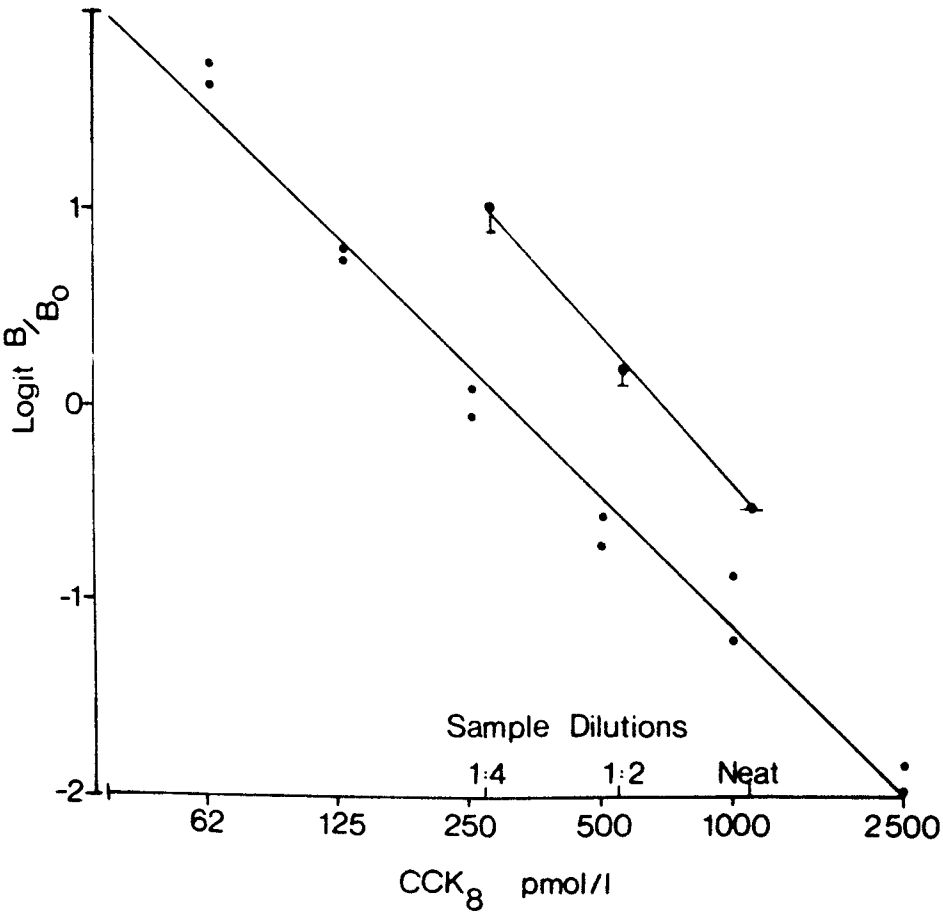
Fig 6.3



Time course of release of CCK_8 immunoreactivity from incubated hypothalami

* $p < 0.01$

Fig 6.4

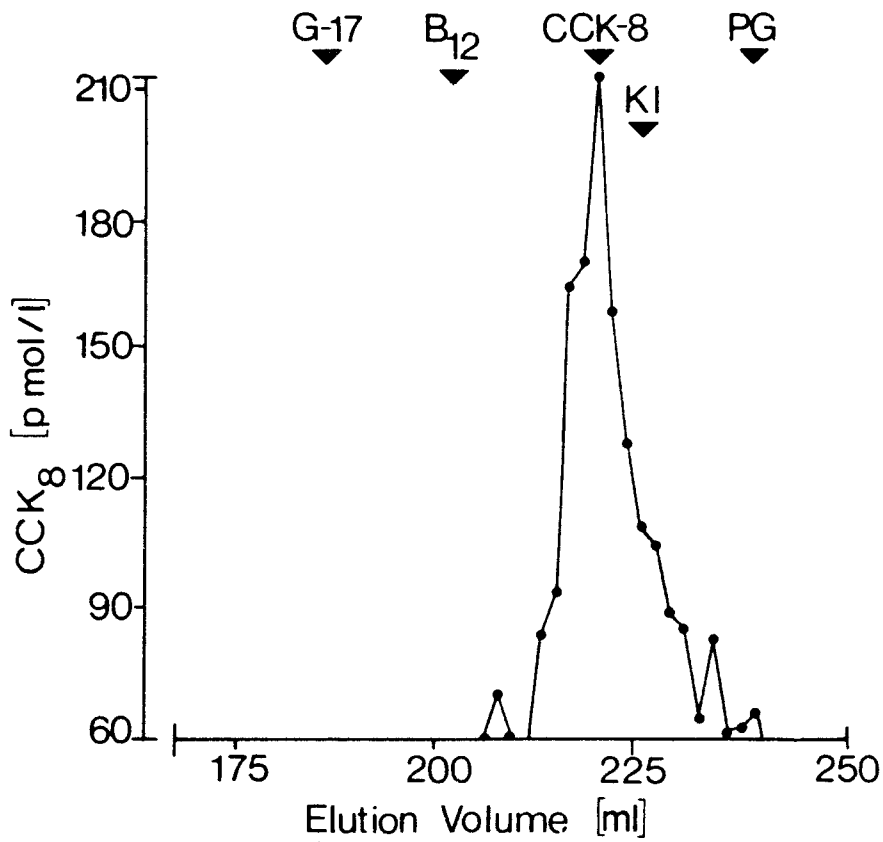


$^{B}/_{B_o}$ (Logit) vs $\log_e$ of CCK_8 standards (r = - 0.1) with displacement of ^{125}I-G-17-1 by serial dilutions of released material of incubated rat hypothalami (r = - 1.2)

F = 4.257 (df 1.24) by analysis of covariance.

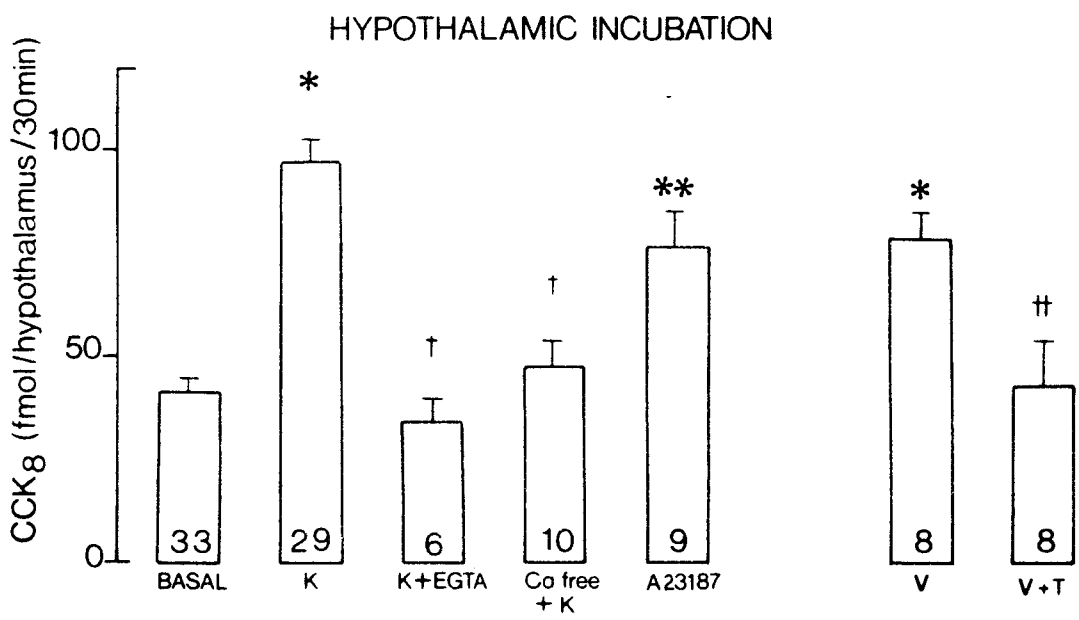
p > 0.05

Fig 6.5



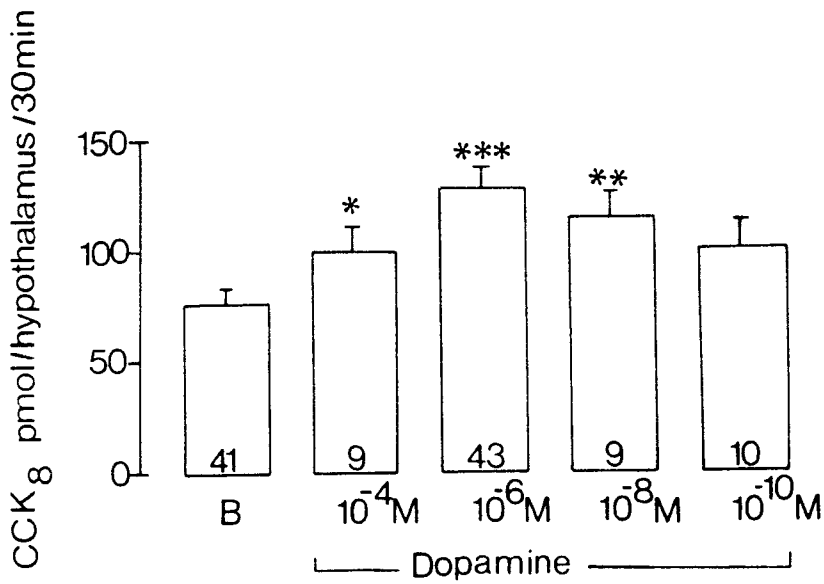
Gel chromatography of released material of incubated rat hypothalami on Sephadex G-50 (sf). The column was equilibrated with synthetic human heptadecapeptide gastrin (SHG-17-1), cyanocobalamin (B_{12}), cholecystokinin octapeptide (CCK_8), pentagastrin (PG) and potassium iodide (KI).

Fig 6.6



Effect of two depolarising stimuli (60 mM KCl and 75 μM veratrine) on the release of CCK_8 and the inhibition of release by 1 μM tetrodotoxin and 1 mM EGTA. In the absence of Ca^{++} ions and in the presence of the Ca^{++} ionophore A23187 release of CCK_8 was significantly decreased. Histograms represent mean $\pm$ SEM of number of experiments indicated. Significant differences between test and control release results are shown by * ($p < 0.01$)

Fig 6.7



Effect of Dopamine (10^{-4}M, 10^{-6}M, 10^{-8}M and 10^{-10}M) on immunoreactive CCK_8 release from rat hypothalami. Histograms represent mean $\pm$ SEM of number of experiments indicated. Significant release over control release is shown by * ($p < 0.05$) ** ($p < 0.01$) *** ($p < 0.001$)

Table 6.1

Concentration		Basal	10^{-6}M	10^{-8}M
ACETYL CHOLINE	Mean	68.2	86.9	95.9
	SEM	9.6	12.1	14.4
	n	16	13	16
SEROTONIN	Mean	74.2	119.4	93.55
	SEM	11.7	21.6	17.8
	n	18	14	14

Effect of Acetyl Choline and Serotonin at 10^{-6}M and 10^{-8}M concentrations on CCK_8 release from isolated hypothalami.

Values given are p mol CCK_8 released/ Hypothalamus/ 30 min.

Table 6.2

	WITHOUT DOPAMINE			WITH DOPAMINE 10^{-6}M			
Somatostatin concentrations	control	$4X10^{-7}$M	$8X10^{-8}$M	control	$4X10^{-7}$M	$8X10^{-8}$M	$8X10^{-4}$M
Mean		76.13	71.1		130.2	138.2	129.1
SEM		13.5	16.6		17.5	12.2	19.9
n		15	12		17	6	7
rGH concentrations		$2.5X10^{-8}$M			$2.5X10^{-8}$M	$5X10^{-9}$M	$5X10^{-10}$M
Mean	7 5	94.08		128.4	122.6	126.3	95.9
SEM	7.4	7.8		8.95	11.9	19.5	19.9
n	41	16		43	9	9	9
Regitine concentrations		10^{-6}M			10^{-6}M	10^{-8}M	
Mean		60.5			43.2***	51.2***	
SEM		7.7			7.2	4.2	
n		7			6	6	

Effect of Somatostatin, rGH and phentolamine with and without Dopamine (10^{-6}M) on CCK_8 release from isolated hypothalami. Values given are p.mol CCK_8 released/ hypothalamus/ 30 minutes.

*** p 0.001 Compared to Dopamine 10^{-6}M alone

DISCUSSION

A prerequisite of physiological studies on CCK release by hypothalami is the establishment of systems for the viable maintenence of the hypothalami over the period of incubation. It has been shown by other workers that incubated hypothalami are capable of consuming oxygen over a three hour period (Bridges, Hillhouse and Jones, 1976 ; Bigdeli and Snyder, 1978 ; Hirooka, Hollander, Suzuki et. al. , 1978 ; Berelowitz, Kronheim, Pimstone et. al., 1978). We have verified this with the use of the Warburg constant volume manometer and found oxygen consumption to be linear over a 120 min period at a rate of 114.1 μmol oxygen/g.hr similar to the 68.9 - 120 μmol oxygen/g,hr reported by Bradbury, Burden, Hillhouse et. al. (1974).

We have selected protein synthesis as an additional and probably more reliable index of tissue viability since it is dependent on a wide spectrum of biochemical pathways as well as the maintenance of subcellular structure. Incorporation of a $[^3H]$ amino acid mixture occurred over a 90 min time period and could be inhibited by puromycin (Which acts by inhibiting assembly of new proteins by ribosomes), and cycloheximide (which acts by inhibiting DNA and RNA synthesis) (Vasquez, 1974). Hypothalamic tissue incubated under these conditions thus shows considerable metabolic activity.

The thickness of the hypothalamic block was kept to $\pm$ 1mm so that the maximum diffusion distance of oxygen to reach all cells was 0.5mm which is considered adequate for metabolic requirements (Bradbury, Burden, Hillhouse et. al. , 1974 ; McIlwain, 1975).

A preincubation time of 60 min was chosen because by this time release of CCK_8 had decreased to a low value. This time period was well within that established for viability. A time-course of release showed both basal and K^+ induced release of CCK_8 occurred throughout a 30 min period of incubation, release being diminished between 30 - 45 min. Thus 30 min was chosen as the maximum acceptable incubation time, degradation or reuptake of released

CCK_8 probably occurring after that time period. The good recovery of added CCK_8 after 30 min of incubation suggests that degradative enzymes were not active in the medium during the first 30 min, either because of the period of preincubation in a large volume of medium, or because of the use of a tissue block rather than a broken cell suspension.

Using this system we have shown that CCK_8 can be released by the depolarising stimuli, 60 mM KCl and 75 μM veratrine (the mechanisms of action have been discussed in Chapter Four). The calcium-dependence of this release is demonstrated by the inhibition of 60 mM KCl stimulated release in a low Ca^{++} medium or in the presence of the Ca^{++} chelating agent EGTA. A classical role for Ca^{++} in the neurosecretory process is also suggested by the stimulation of release elicited by the Ca^{++} ionophore A23187. However, since this ionophore is also known to increase fluxes of other cations through membranes it is also possible that the effect is due to a depolarisation.

The material released, from the hypothalami, coeluted with CCK_8 on Sephadex G-50 (sf) chromatography and diluted in parallel to CCK_8 standards in the radioimmunoassay. No gastrin-like material was found even though low concentrations have been found in the hypothalamus (Rehfeld, 1978c). In addition, other CCK moieties such as CCK_{33} and CCK_4 crossreact poorly with the antiserum used, thus rendering the assay relatively specific for CCK_8.

The function of CCK_8 in the hypothalamus is as yet unknown. CCK_8 has been reported to cause satiety when injected into various animals and this may occur via the hypothalamic satiety centre. Vijayan, Samson and McCann (1979) demonstrated that CCK_8 when injected into the third ventricle of rats caused release of GH and suppression of LH and TSH but could not demonstrate a direct effect of CCK_8 on the isolated pituitary. This was confirmed by Berelowitz et. al., (personal communication). In contrast Morley, Melmed, Briggs et. al. (1979) have demonstrated GH release from incubated rat pituitaries in response to CCK_8. Our data shows

that CCK_8 is released in response to dopamine. We were unable to demonstrate any negative feedback on dopamine-stimulated release by either GH or SRIF. Dopamine causes SRIF release in these preparations and inhibits GH secretion in the rat in vitro. It was of interest, however, that the dopamine-stimulated CCK_8 release appeared to be dependent upon noradrenergic modulation as demonstrated by our findings that phentolamine at physiological concentrations inhibited dopamine-stimulated release. This noradrenergic modulation could be via a number of pathways.

1. That the effect is not dopamine mediated but is transformed within the tissue by the enzyme dopamine-β-hydroxylase to noradrenaline and thus is a noradrenaline effect. Without specific experiments using dopamine agonists and/or a dopamine-β-hydroxylase blocker this possiblility cannot be excluded (Fig 6.8 a).

2. That the dopamine effect is mediated through an intervening noradrenergic neurone. The blocking of this neurone by phentolamine blocks both the dopamine and noradrenaline stimuli (Fig 6.8 b).

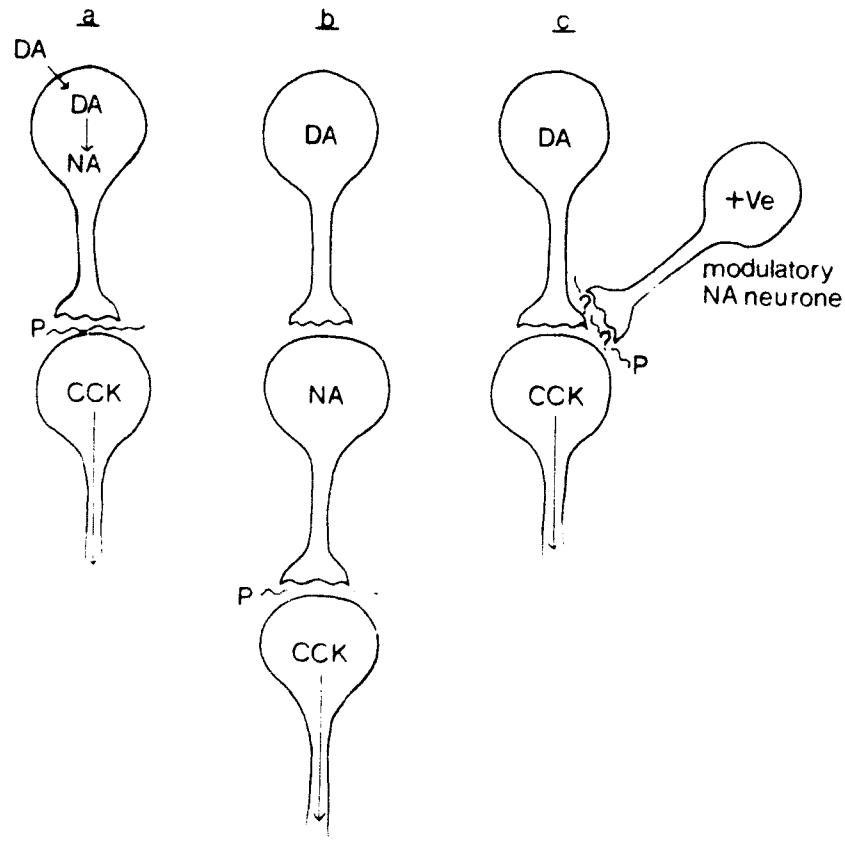
3. That the dopamine has a direct effect on CCK_8 release but is dependent on :
   i) a pre-synaptic positive modulatory effect of a noradrenergic neurone or
   ii) on a post-synaptic modulation of the effect of dopamine on the CCK neurone (Fig 6.8 c).

This data is not sufficient to preclude the possibility of CCK_8 from having an effect on GH secretion either at a hypothalamic or pituitary level. Further investigations need to be done to investigate the interrelationship of the dopamine and noradrenaline pathways in the control of GH secretion.

Thus in conclusion the rat hypothalamus appears viable in vitro under the conditions of incubation, as assessed by linear oxygen consumption and linear incorporation of labelled amino acids over time. Depolar-

rising stimuli of 60 mM KCl and 75 μM veratrine caused release of CCK_8 which was calcium-dependent. Dopamine at physiological concentrations, stimulated CCK_8 release via a noradrenergic pathway as demonstrated by the inhibition of release with phentolamine.

Fig 6.8



Noradrenergic modulation of dopamine-stimulated CCK_8 release could be via :

a) transformation of dopamine (DA) to noradrenaline (NA) within the neurone.

b) an intervening noradrenergic neurone.

c) a pre- (?) or post-synaptic (?) positive modulatory noradrenergic neurone.

P represents inhibition of release of CCK_8 by phentolamine (10^{-6}M and 10^{-8}M).

SUMMARY OF CONCLUSIONS

The aim of the study was to show that CCK_8 was both a neuromodulator and a hypothalamic hormone in the central nervous system.

In order to define a possible neuromodulatory or neurotransmitter role for CCK_8, the presence and release of the peptide throughout the central nervous system was examined. The uneven and widespread distribution of CCK_8 in rat brain was confirmed along with its localisation in the synaptosome fraction of rat brain homogenates prepared from hypothalamus, striatum, cortex and thalamus. A calcium-dependent release of CCK_8 from these areas has been demonstrated.

CCK_8 was released in a calcium-dependent manner from perifused cortical synaptosomes. Dopamine and acetyl choline caused a significant release of CCK_8, thus showing neurotransmitter control of CCK_8 release. No release occurred in response to the higher concentrations implying that high dopamine and acetyl choline concentrations are able to reverse the effect of the lower concentrations. It has been shown that CCK-like peptides and dopamine coexist in the same neurone and thus it has been suggested that the peptide is released together with dopamine and may be part of an inhibitory system modulating dopamine release via an action on autoreceptors.

Evidence for CCK_8 being a hypothalamic hormone relates to experiments involving the <u>in vitro</u> incubated hypothalamus. CCK_8 was shown to be released from the <u>in vitro</u> incubated hypothalamus by depolarising stimuli in a calcium-dependent manner. The demonstration in this laboratory that CCK_8 causes GH release from isolated rat pituitaries suggests that CCK is a possible regulator of GH. The dopamine-stimulated release of CCK_8 which appeared to be dependent upon a noradrenergic pathway could not be inhibited by rGH or SRIF. Thus, if CCK_8 is a GH releasing factor, the regulation of GH does not appear to operate via inhibition of CCK

release by rGH or SRIF. This data does not however preclude the possibility of CCK_8 from having an effect on GH secretion either at a hypothalamic or pituitary level.

Identity of the released immunoreactive material was demonstrated by coelution on Sephadex G-50 (sf) chromatography with synthetic CCK_8 and parallelism with dilutions of synthetic CCK_8. The low crossreactivity of other CCK Moieties with the antisera used in this study does not enable conclusions to be drawn regarding the importance of other molecular weight forms of CCK which may be present.

In conclusion the results of the in vitro experiments presented in this study suggest a probable important role for CCK_8 in the rat CNS. The demonstration that neurotransmitters affect CCK_8 release provides further evidence for CCK_8 being a modulator of neuronal function.

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