THE UNIVERSITY OF LEICESTER

STUDIES OF CIRCADIAN PATTERNS IN CRF SECRETION

FROM THE RAT HYPOTHALAMUS ISOLATED AND INCUBATED *IN VITRO*

by

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INTRODUCTION

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I.   Hormones of the Hypothalamo-Pituitary-Adrenal Axis

a)   Corticosteroids

Although a search for the function of the adrenal gland was
initiated as early as 1563 by Eustacchio, no meaningful conclusions
were reached until Addison described, in 1855, a disease associated
with a disturbed condition of the suprarenal capsules.   Early
animal experimentation raised questions as to whether the adrenal
glands are essential for life (Brown-Sequard, 1856), and as to the
nature of the functions of the adrenal cortex.   The first effective
extracts of adrenocortical tissue were prepared in 1930 by Swingle
and Pfiffner (1930), and by Hartman and Brownell (1930) - these lipid
extracts were capable of maintaining adrenalectomized animals
indefinitely.   Elucidation of the chemistry of the adrenocortical
extracts was initiated by the demonstration that numerous steroids
could be crystallized from extracts of the adrenal cortex (Pfiffner,
1942), and this in turn led to the identification of the actions of
the different steroids, and to the synthesis of the steroids (Kendall,
1949).

        Preliminary evidence of the involvement of cholesterol in the
biosynthesis of the adrenal steroids came from the demonstration that
injection of pituitary extracts containing corticotrophin (adreno-
corticotrophic hormone - ACTH) into rats leads to an immediate fall in
adrenal ascorbic acid and cholesterol (Sayers et al, 1944).   The
existence of a pituitary hormone involved in regulation of adreno-
cortical activity had previously been shown by experiments in which
administration of anterior pituitary extracts prevented the atrophy

of the adrenal cortices which follows hypophysectomy (Smith, 1927)
and produced hypertrophy of the adrenal cortex (Houssay et al, 1933;
Anselmino et al, 1934). The role of acetate and cholesterol as
precursors in the biosynthesis of corticosteroids was shown in
experiments in which radioactive steroids were isolated from adrenal
tissue and in the perfusates of adrenal glands perfused with ^{14}C-
cholesterol and ^{14}C-acetate (Hechter and Pincus, 1954). Perfusion
experiments demonstrating the increased formation of progesterone,
corticosterone and cortisol in adrenals perfused with pregnenolone
led to inclusion of pregnenolone and 17-hydroxypregnenolone as inter-
mediates in the biosynthesis of corticosterone and cortisol from
cholesterol (Hechter and Pincus, 1954). Conversion of cholesterol
to pregnenolone, a rate-limiting step in steroid biosynthesis, is
regulated by ACTH (Koritz and Hall, 1964; Davis and Garren, 1968;
Farese et al, 1969) and involves oxidation followed by enzymatic side
chain cleavage. Conversion of pregnenolone to the glucocorticoids
corticosterone and cortisol involves 17α-hydroxylation as an initial
step in the formation of cortisol, dehydrogenation and isomerization
by microsomal enzymes to progesterone and 17α-hydroxyprogesterone,
21-hydroxylation by a microsomal enzyme to 11-deoxycorticosterone
(DOC) and 11-deoxycortisol (substance S of Reichstein), transport
across the mitochondrial membrane and 11-hydroxylation by a mitochon-
drial enzyme to corticosterone and cortisol. In the rat and mouse
the initial 17α-hydroxylation is low, and corticosterone is the
principal glucocorticoid. The latter biosynthetic pathway was
deduced for the most part by studies with radioactive steroids and
steroid precursors (reviewed by Samuels and Nelson, 1975), and
quantitatively is the most important in the formation of cortico-
steroids, there being numerous other hydroxylation and dehydrogenation
pathways and sequences which vary from species to species. Of the

three groups of enzymes involved in the biosynthetic pathways the 3β-hydroxysteroid dehydrogenase utilizes NAD^+, or at a lower rate $NADP^+$, as a hydrogen acceptor, and the hydroxylases and lysases utilize NADPH and molecular oxygen, but the isomerases have no known coenzyme requirement. The hydroxylase systems (or mixed function oxidases, since two substrates are oxidised, NADPH and the steroid) involve a scheme of electron transport, the components of which include a flavoprotein dehydrogenase specific for NADPH, an iron-containing nonheme protein, and cytochrome P-450 (Omura et al, 1965).

A number of procedures have been devised for the separation of steroids involving either extraction or chromatographic procedures. The assay of corticosteroids is accomplished by a variety of methods which involve spectrophotometry, double isotope derivative methods, saturation analysis techniques utilizing antibodies or binding proteins, or bioassay (reviewed by Moore and Heftmann, 1962; Dorfman, 1962; and Sönksen, 1974).

b)   Corticotrophin (ACTH)

Li and co-workers, and Sayers and co-workers, in the early 1940s, succeeded in making active preparations of an "ACTH protein" with a molecular weight of approximately 20,000 (Li et al, 1942; Li et al, 1943; Sayers et al, 1943). Although these preparations satisfied several of the criteria for homogeneity it was subsequently found that they represented the ACTH molecules associated with a specific inert protein, as shown by the behaviour of the biological activity of these ACTH preparations when subjected to conditions such as partial peptic and acid hydrolysis, ultrafiltration, dialysis, and electrodialysis. This material was not "big" ACTH (molecular weight $> 20,000$) identified more recently by Yalow and Berson (1971). The

first purified corticotrophin preparation from sheep pituitary glands
was obtained in 1954, and was shown to be homogeneous by subjecting
the material to countercurrent distribution, partition chromatography
and zone electrophoresis on starch; ultracentrifugation studies gave
a molecular weight of 5,360 for the trichloroacetate salt (Li et al,
1954; Li, 1956). Amino acid sequence determination of the prepara-
tion revealed a peptide consisting of 39 amino acids (Fig. 1).
Purification and identification of the amino acid sequence of porcine
and bovine ACTH was accomplished by White and Landmann (1955) and Li
et al (1958) respectively. The sequence proposed by these workers
has subsequently been revised and Fig. 1 shows the amino acid sequence
of human, porcine, bovine, and ovine ACTH (Schwyzer, 1977). Pepsin,
under conditions of "mild" digestion, attacks mainly the acidic COOH-
terminal portion of the ACTH molecule, producing several fragments
that are biologically active (Bell et al, 1956: Li, 1956). Integrity
of the entire ACTH molecule is not required for biological activity,
the active peptide fragments residing in the NH_2-terminal amino acid
residues of the molecule. In the in vitro incubation technique
$ACTH^{1-24}$ has been shown to have the same molar potency as $ACTH^{1-39}$;
a fragment as small as $ACTH^{5-10}$ hexapeptide is capable of triggering
the steroidogenic receptors to their full extent; however, part of
the molar potency in vivo is lost by omitting the COOH-terminal
sequence 25-39, and the COOH-terminal is necessary to prevent losses
due to degradation, since protection of the COOH-terminus by amide
formation increases activity above that of the full 1-39 ACTH molecule
(Ramachandran, 1973).

It has become clear that the concept of heterogeneity
exemplified by earlier work on human parathyroid hormone, insulin and
gastrin, is also applicable to ACTH, and that ACTH exists in a variety

	HUMAN	PORCINE	OVINE/BOVINE
	Ser		
	Tyr		
	Ser		
	Met		
5 –	Glu		
	His		
	Phe		
	Arg		
	Trp		
10 –	Gly		
	Lys		
	Pro		
	Val		
	Gly		
15 –	Lys		
	Lys		
	Arg		
	Arg		
	Pro		
20 –	Val		
	Lys		
	Val		
	Tyr		
	Pro		
25 –	Asn		
	Gly		
	Ala		
	Glu		
	Asp		
30 –	Glu		
	Ser	Leu	
	Ala		
	Glu		Gln
	Ala		
35 –	Phe		
	Pro		
	Leu		
	Glu		
39 –	Phe		
	OH		

FIGURE 1

The amino acid sequence of human ACTH, and comparison with the molecules from other species. Only the amino acid replacements are shown; the molecules are otherwise identical with the human sequence.

(Schwyzer, 1977)

of molecular forms in the pituitary gland of a number of species

(Yalow, 1976). Gel filtration and measurement of bioactivity and

immunoreactivity have revealed the presence of, in addition to

"small" $ACTH^{1-39}$ (molecular weight 4541 - Human ACTH), "intermediate"

ACTH (molecular weight between 10,000 and 15,000) in rabbit, rat and

mouse pituitary extracts, "big" ACTH (molecular weight about 23,000)

in mouse, human, rat and porcine pituitaries, and "very big" ACTH

(molecular weight about 34,000) in mouse pituitary tumour cells

(Eipper and Mains, 1975). The biological activity of these larger

ACTH molecules is not, however, necessarily the same as their immuno-

reactivity, even when the antiserum is directed against amino acid

sequences at the NH_2 terminal of the molecule. Thus, in the study of

Yalow (1976), although the biological and immunological activities of

"little" ACTH obtained by gel filtration of a bronchiogenic tumour

extract agreed, the biological activity of "big" ACTH from the same

extract was less than 4% of the activity measured by radioimmunoassay.

It is also significant that trypsin digestion of "big" ACTH produces

a peptide resembling "little" ACTH physicochemically, and which shows

full corticotrophic bioactivity (Yalow, 1976). Similar data have

been obtained with "big" ACTH extracted from mouse pituitary tumour

cells, "big" ACTH possessing only 20-25% of the steroidogenic activity

of "little" ACTH (Eipper and Mains, 1975). It has also been shown

that mouse and human "big" ACTH are glycopeptides; in the mouse,

"intermediate" ACTH is also a glycopeptide (Eipper et al, 1976; Orth

and Nicholson, 1977).

This diversity of ACTH-like peptides is further complicated by

the presence of a COOH-terminal peptide, discovered by the simultaneous

measurement of NH_2-terminal (13-18) and COOH-terminal (33-39) immuno-

reactivity in rat, pig and guinea-pig pars intermedia and pars nervosa

extracts. Purification and amino acid analysis of the peptide, corticotrophin-like intermediate lobe peptide (CLIP), revealed that it is indistinguishable from the amino acid sequence 18-39 of the ACTH molecule of the corresponding species (Scott et al, 1973). CLIP has not been shown to have ACTH bioactivity and is confined to the intermediate and posterior lobes of the pituitary. It has been proposed, in the light of the similarities between the ACTH molecules, α-melanocyte stimulating hormone (α-MSH), and β-lipotrophin (β-LPH) and because of the localization of these peptides in the pituitary gland, that "big" ACTH may represent a precursor molecule for a family of peptides including "little" and "intermediate" ACTH, CLIP, α-MSH, enkephalin and β-endorphin, the biosynthetic pathway for each hormone being regulated by cleavage of the precursor molecule by peptidases (Bradbury et al, 1976; Lowry et al, 1977). This hypothesis has been recently reinforced by the determination of the nucleotide sequence of a cloned c-DNA insert encoding the bovine ACTH-β- lipotrophin precursor m-RNA. The amino acid sequence corresponding to the c-DNA nucleotide sequence includes 4 repetitive sequences each containing an MSH peptide sequence, and the amino acid sequences corresponding to bovine ACTH (1-39) and β-LPH (42-134) and their respective fragments α-MSH (1-13), CLIP (18-39), and γ-LPH (42-101). β-MSH (84-101), methionine-enkephalin (104-108) and β- endorphin (104-134) were also identified in the nucleotide code (Nakanishi et al, 1979).

c) Action of Corticotrophin (ACTH) on the Adrenal Cortex and the Role of Secondary Messengers

The immediate effects of ACTH upon adrenal corticosteroido-genesis have been shown to involve a "secondary messenger", the adenylate cyclase - cyclic 3', 5'-adenosine monophosphate (cyclic-AMP) system. Using in vitro incubation of rat adrenal glands, it has been

shown that cyclic-AMP induces steroidogenesis in the absence of ACTH (Haynes et al, 1959), and that incubation with ACTH increases tissue levels of the nucleotide (Haynes, 1958). Further, an increase in cyclic-AMP levels is seen before an increase in rate of cortico-steroidogenesis following administration of ACTH, and doses of ACTH that produce a graded response in steroid secretion also cause a graded increment in cyclic-AMP levels; it has also been shown that stimulation of steroidogenesis, both by ACTH and cyclic-AMP, require protein synthesis, but that ACTH-stimulated increases in cyclic-AMP levels are not blocked by protein synthesis inhibitors (Grahame-Smith et al, 1967; Schulster et al, 1970). Cycloheximide and chloramphenicol cause a decay of a previously established steroido-genic effect, produced either by ACTH or cyclic-AMP, with a half-life of 7 to 10 minutes in in vivo experiments (Garren et al, 1965), but with a half-life of 25 to 45 minutes in sections of adrenal tissue in vitro (Farese et al, 1969; Schulster et al, 1970) and of 2 to 4 minutes in isolated adrenal cells in vitro (Schulster and Jenner, 1975) - this effect is virtually identical in ACTH or cyclic-AMP stimulated adrenals, implying cyclic-AMP mediation in activating a protein. From these investigations it has been proposed that ACTH binds with a receptor, activating membrane adenylate cyclase to generate cyclic-AMP, which in turn affects a number of processes involved in steroidogenesis, including a "regulator protein" which facilitates the conversion of cholesterol to corticosteroids (Schulster et al, 1976).

A variety of ACTH analogues may occupy the ACTH receptor and yet have little or no steroidogenic potency (Seelig and Sayers, 1973), and it is clear that the relation between binding and activation of adenyl cyclase is not simple. In a study of displacement of ^{125}I-labelled ACTH binding to adrenal cell membrane preparations, it was

found that the ability to displace binding of a number of ACTH

analogues (e.g. $ACTH^{6-39}$, $ACTH^{9-24}$) was the same as their ability to

produce inhibition of adenylate cyclase stimulation produced by

$ACTH^{1-39}$ (Ways et al, 1976). However, nitrophenylsulphenyl (NPS)

ACTH increases steroidogenesis in rat adrenal cell suspensions

without the concomitant increase in cyclic-AMP observed with ACTH;

further, this analogue competes with ACTH for its receptor, producing

a decreased cyclic-AMP response to ACTH (Seelig et al, 1972). These

experiments, therefore, place some doubt on the obligatory mediatory role

of cyclic-AMP; recent experiments also suggest that whilst cyclic-AMP

may mediate the steroidogenic effect of ACTH at low affinity receptors

for ACTH, calcium ions mediate the steroidogenic effect of ACTH at

high affinity receptors for ACTH (Yanagibashi et al, 1978; Yanagi-

bashi, 1979).

Binding studies with ^{125}I-labelled ACTH indicate the presence

of a heterogeneous population of ACTH binding sites with different

binding affinities; this has been observed in extracts of mouse

adrenal tumour cells (Lefkowitz et al, 1970), and in rat dispersed

adrenal cells (McIlhenny and Schulster, 1975). The concentration of

ACTH required for half maximal stimulation of cyclic-AMP in rat

isolated adrenal cells is 35 times greater than that required for half

maximal stimulation of steroidogenesis (Seelig and Sayers, 1973),

indicating that the adrenal membrane also contains an excess of

receptor-adenyl cyclase complexes above the requirement for maximal

stimulation of steroidogenesis. Similarly, the concentration of

^{125}I-ACTH required for half-maximal binding in rat dispersed adrenal

cells is several times greater than that required for half-maximal

steroidogenesis (McIlhenny and Schulster, 1975). The effect of ACTH

and cyclic-AMP on steroidogenesis in rat adrenals is unaffected by

over 70% inhibition of protein synthesis with actinomycin D

(Halkerston et al, 1965); greater inhibition of protein synthesis

than of steroidogenesis during stimulation with cyclic-AMP was

similarly demonstrated using a variety of concentrations of puromycin

and cycloheximide (Koritz and Wiesner, 1975).

Availability of NADPH for the enzyme system involved in

cholesterol side chain cleavage has been shown to be of importance

in regulation of adrenal steroidogenesis (Halkerston et al, 1959).

Reducing the glucose concentration of the incubation medium surrounding

adrenal quarters from 10mM to 1mM reduces the amount of cyclic-AMP

formed in response to ACTH, and the amount of corticosterone formed in

response to ACTH and cyclic-AMP, but not that formed in response to

NADPH - this suggests glucose facilitates the formation of cyclic-AMP

in response to ACTH and in addition might provide a source of glucose-6-

phosphate which is utilized by the pentose pathway, increasing

reduction of $NADP^+$ to NADPH (Jones et al, 1970) - activation of

glucose-6-phosphate dehydrogenase has been implicated in ACTH-

produced stimulation of steroidogenesis (McKerns, 1964).

Calcium ions are also required as a co-factor in ACTH-induced

steroidogenesis in vitro (Birmingham et al, 1953). One hypothesis

on the role of calcium is that ACTH activates adenylate cyclase by

dissociation of Ca^{2+}, and causes the redistribution of Ca^{2+} to an

active site in the cell which couples the biosynthesis and release of

corticosteroids (Rubin et al, 1972). This hypothesis that calcium

ions act, like cyclic-AMP, as a secondary messenger is also supported

by experiments which show that calcium ions may mediate the steroido-

genic effect of high affinity receptors for ACTH (Yanagibashi et al,

1978; Yanagibashi, 1979).

Prostaglandins have been implicated in adrenal steroido-

genesis, but their exact role is as yet unclear (Shaw and Tillson, 1974).

Perfusion of rat adrenal glands with PGE_1, $PGF_{2\alpha}$, or PGE_2 increases the

amount of corticosterone released into the medium, PGE_2 being the most

potent in this effect (Flack et al, 1969). Similar results have been

obtained with cat dispersed adrenal cells (Warner and Rubin, 1975).

The effect of PGE_2 on steroidogenesis is transient by comparison with

that of ACTH or cyclic-AMP, stimulation decaying by 4 hours of

incubation (Flack et al, 1969; Flack and Ramwell, 1972). Cyclo-

heximide blocks the effect of PGE_2 in rat adrenal slices (Flack et al,

1969), and PGE_2 and PGE_1 stimulate an increase in intracellular cyclic-

AMP levels in beef adrenal slices (Saruta and Kaplan, 1972), human and

ovine membrane preparations (Dazord et al, 1974), and rat dispersed

adrenal cells (Rubin and Warner, 1972). Specific binding of ^3H-

labelled PGE_1 and PGE_2 in subcellular preparations of human and ovine

adrenals has been demonstrated; this, and the facts that ACTH does

not inhibit binding of prostaglandins (Dazord et al, 1974), and that

ACTH and prostaglandins have an additive effect on adenyl cyclase

activity (Flack and Ramwell, 1972; Dazord et al, 1974) suggest adrenal

prostaglandin receptors are different from those of ACTH. The

evidence relating to the block of PGE_2 stimulation by cycloheximide both

in vitro (Flack et al, 1969) and in vivo (Shaw and Tillson, 1974), the

timing of maximal activation of steroidogenesis by prostaglandins

(Flack and Ramwell, 1972), the stimulation of cyclic-AMP levels by

prostaglandins (Saruta and Kaplan, 1972; Dazord et al, 1974), and

the similarity of the effect of PGE_2 to that of ACTH in radioactive

acetate incorporation studies (Warner and Rubin, 1975), together

suggest that although prostaglandins bind to a receptor distinct from

that of ACTH, PGE_2-induced activation of steroidogenesis involves a

similar process to that involved in ACTH-induced steroidogenesis.

However, the exact manner in which prostaglandins, in particular PGE_2,

stimulate steroidogenesis may be different from ACTH; firstly, because

of the transient nature of the effect of PGE_2 on steroidogenesis (Flack

and Ramwell, 1972); and secondly, because of the difference in cyclic-

AMP levels stimulated by equipotent doses of PGE_2 and ACTH (Warner and

Rubin, 1975).

d)   Assay of Corticotrophin (ACTH)

Corticotrophin may be assayed by a variety of experimental

principles, the earliest of which relied upon changes in adrenal

weight or histology produced by administration of ACTH to the hypophy-

sectomized rat (Sayers et al, 1943). The widely used adrenal ascorbic

acid depletion method depends on the depletion of adrenal ascorbic acid

caused by the injection of ACTH into hypophysectomized animals (Sayers

et al, 1948). A linear relationship exists between the adrenal

ascorbic acid depletion and logarithm of the dose of ACTH administered

(Sayers et al, 1948), and a physico-chemical method is used to deter-

mine the ascorbic acid concentration of the gland (Roe and Kuether,

1943). The method is sensitive to 0.1mU ACTH, but lacks specificity

in that vasopressin and α-MSH also cause ascorbic acid depletion (Royce

and Sayers, 1958). Further, ascorbic acid changes in the adrenal

gland are merely one aspect of the action of ACTH on the adrenal gland,

depletion being only indirectly related to steroid biosynthesis, such

that changes in corticosteroidogenesis can occur independently of changes

in adrenal ascorbic acid (Rerup and Hedner, 1961; Montanari and
                        Hodges and Sadow, 1969;
Stockham, 1962; /Hodges and Mitchley, 1970a). The procedure has been

used in the assessment of the potency of various ACTH preparations and

synthetic ACTH peptides. The cytochemical bioassay technique (Daly

et al, 1977) measures the optical density of adrenal tissue sections

when stained with an agent which precipitates with cellular reducing

agents. Tissue sections may be incubated with ACTH and plasma

preparations, and a linear relationship exists between the log concentration of ACTH and the extinction caused by the reaction between the stain and reducing agents. The assay is sensitive to 50fg/ml of ACTH, and is specific to the extent that α- and β-MSH, luteinizing hormone (LH), and $ACTH^{18-39}$ do not react in the system. Cyclic-AMP is inactive, but dibutyryl cyclic-AMP (db-cyclic-AMP) does produce a response, presumably because of its increased ability to cross membranes.

A large number of assays which depend on adrenal corticosteroidogenesis, either in vitro or in vivo, exist. A number of in vivo assays utilize the increment in blood corticosterone, either in the general circulation or adrenal effluent blood supply, caused by injection of ACTH to the hypophysectomized rat. In its original form, ACTH is injected into 24-hour hypophysectomized rats and blood collected from the circulation 15 minutes later, and corticosterone estimated by a sulphuric acid fluorescence technique (Guillemin et al, 1958; Guillemin et al, 1959a). Variants of the method include those of Lipscomb and Nelson (1962), using 2-rather than 24-hour hypophysectomized rats, and collection of blood from the adrenal vein, thereby increasing sensitivity; of Vernikos-Danellis et al (1966), using injection of ACTH into the jugular vein rather than subcutaneous injection, and measurement of adrenal corticosterone concentration; and of Nicholson and Van Loon (1973), using 24-or 48-hour hypophysectomized rats primed with ACTH, thereby increasing sensitivity to 70pg/ml of ACTH.

In vitro corticosteroidogenesis and radioimmunoassay methods for ACTH, however, are now most commonly used, both because of the large number of samples that can be assayed, and because of the specificity, sensitivity and precision that can be established. An

early in vitro corticosteroidogenesis technique utilizes the production

of corticosteroids from rat adrenal quarters incubated in vitro with

ACTH; at the end of the incubation period the steroid concentration

of the medium is measured either by optical densitometry (Saffran

and Bayliss, 1953), or better, by more sensitive sulphuric acid

fluorescence (Guillemin et al, 1958; Guillemin et al, 1959a). A

linear dose-response relation is established between corticosteroid

concentration and ACTH concentration (Saffran and Schally, 1955), and

by preincubating the adrenal quarters the assay sensitivity is

increased to 2ng ACTH per 100mg adrenal tissue (Saffran and Schally,

1955). The method is relatively specific in that growth hormone

(GH), thyotrophin (TSH) and MSH show little activity (Saffran and

Schally, 1955). The main attributes of the assay are the use of

tissue of common origin for both test and standard doses of ACTH, and

the reproducibility of estimates of potency.

The technique of incubating adrenal quarters with ACTH has

been modified into a continuous flow system, coupled with an auto-

mated fluorimetric method for the determination of corticosterone in

the superfusate (Saffran and Rowell, 1969; Saffran et al, 1971).

The system suffers from lack of sensitivity, as does static incubation

of adrenal quarters (which is also subject to end-product inhibition - see

e.g. Schulster et al, 1970), and requires a recovery period for each test
dose of ACTH.

A number of dispersed adrenal cell methods have been developed,

and have the advantages that inter-animal variation is eliminated ,

diffusion barriers between adrenal cells are removed, and sensitivity

is increased to picogram quantities of ACTH (Sayers et al, 1971;

Richardson and Schulster, 1972). The assay involves dispersion of

adrenal quarters with trypsin or collagenase, incubation of aliquots

of dispersed cells with standard and test doses of ACTH or synthetic

ACTH peptides, extraction of the steroids produced, and estimation of corticosterone by a sulphuric acid fluorescence or binding assay method. The effects of trypsin dispersion on adrenal cells are such that sensitivity to ACTH is not reduced when compared to in vivo assays, and production of steroids on a weight basis is similar to that in vivo. A linear relationship between corticosterone production and log dose of ACTH may be established and the responses are specific in that vasopressin, oxytocin, and angiotensin II are inactive at mg concentrations (Sayers, 1977).

The development of radioimmunoassay methods for ACTH has resulted in a large number of investigations, an example of which are the studies outlined above, which utilize the techniques of radio-immunoassay in the detection and characterization of a variety of peptides related to "big" ACTH. In the radioimmunoassay, antibodies to ACTH are raised in rabbits and guinea-pigs, and test and standard doses of unlabelled ACTH are incubated with the antibodies, and with a standard quantity of ^{131}I-labelled ACTH. The assay depends on the decrease of ^{131}I-labelled ACTH bound to antibody when increasing concentrations of unlabelled ACTH are added. Free and antibody-bound radioactivity (labelled ACTH) are separated by paper strip electrophoresis, by precipitation as salt complexes, or by adsorption onto charcoal, talc, or polyethylene glycol, and bound activity counted. A standard curve of bound-to-free labelled ACTH against known, standard amounts of ACTH added is plotted and the amount of test ACTH is calculated from the curve (Yalow et al, 1964; Demura et al, 1966; Rees et al, 1971; Liotta and Krieger, 1975; Marton et al, 1978). Although radioimmunoassay procedures are sensitive to 10pg ACTH, and show good accuracy, they may be criticized for a number of reasons. Firstly, they require preliminary extraction of ACTH in order to concentrate the hormone, and to remove proteolytic enzymes and other

possible plasma constituents which interfere in the assay. Secondly,
the antibodies recognise a certain sequence of the whole ACTH molecule,
and the sequence recognised by the antibody is not necessarily that
required for activation of the adrenal membrane receptor. Immuno-
logical activity and bioactivity are not therefore always related when
the two systems are compared, especially if antibodies are raised to
the COOH-terminal portion of the ACTH molecule. It is therefore
necessary to characterise the antibody recognition site with ACTH
analogues and make a comparison of the immunoassay with a bioassay.
Most radioimmunoassays now use NH_2-terminal antisera.   These problems
are compounded by the fact that a variety of peptides exist such as
α-MSH, β-lipotrophin, and "intermediate" and "big" ACTH which have
peptide sequences in common with ACTH and may bind to the antibody,
yet have a low biological potency.

e)   The Release of   Corticotrophin   (ACTH) from the Pituitary
        Gland

        Corticotrophs have been localized to the anterior and inter-
mediate lobes of the pituitary gland of the rat using histochemical,
immunohistochemical and immunofluorescence techniques (Moriarty, 1977).
Using conventional microscopy, histochemical and quantitative changes
following adrenalectomy demonstrate that the corticotroph is stellate
in shape with secretion granules of 200nm average diameter situated
near the plasma membrane (Siperstein and Miller, 1973). The
distribution of corticotrophs is similar to that of growth hormone (GH)
containing cells (Nakane, 1975), but their storage granules are
distinct, GH storage granules being 300 to 450nm in diameter (Ishikawa
et al, 1972). β-lipotrophin has been localized to the same secretory
granules as those containing ACTH, using immunohistochemical staining,
both in the anterior and intermediate lobes of the pituitary gland

(Pelletier et al, 1977). Further, a parallel association between release of β-endorphin and ACTH may be shown under a variety of conditions such as adrenalectomy, dexamethasone, or acute stress in vivo, and noradrenaline, vasopressin or purified corticotrophin releasing factor (CRF) in vitro, suggesting ACTH and β-endorphin are released concomitantly by the pituitary gland (Guillemin et al, 1977; Vale et al, 1978).

The physiological function of intermediate lobe ACTH is unclear, especially as a large proportion of ACTH COOH-terminal immunoactivity is due to corticotrophin-like intermediate peptide (CLIP) (Scott et al, 1973). Adenohypophysectomy, but not incomplete adenohypophysectomy or neurohypophysectomy, causes a reduction in plasma ACTH immunoactivity (ACTH$^{11-24}$ antiserum) and corticosterone responses to tourniquet stress and ether stress, showing that the adenohypophysis, but not the pars intermedia or neurohypophysis, is the chief source of plasma ACTH (Greer et al, 1975). Intermediate lobe bioactive ACTH is not affected by adrenalectomy, either 24 hours or 21 days following the operation, ether stress, or by ether stress 24 hours after adrenalectomy - only the neurogenic stress of loud noise and strobo-scopic light results in a decrease in intermediate lobe content of bio-active ACTH (Moriarty and Moriarty, 1975). This suggests intermediate lobe bioactive ACTH is primarily regulated by neurogenic stress, but not by circulating glucocorticoids, and is not released in functionally significant quantities. Hypothalamic extracts cause a non-specific release of bioactive ACTH from the intermediate lobe in vitro since liver and cerebral cortex extracts are just as effective - in a super-fusion system, however, hypothalamic extracts cause a specific response which is not abolished by antisera to vasopressin (Briaud et al, 1978).

The mechanism involved in release of ACTH contained in

secretory granules is uncertain but may conform to the stimulus-secretion coupling hypothesis, in which releasing hormones, or other agents, interact with the plasma membrane to cause a change in membrane chemistry, resulting in increased membrane permeability, depolarization, and entry of Ca^{2+} ions. Increased intracellular Ca^{2+}, or redistribution of intracellular Ca^{2+}, would then lead to hormone release, possibly by the contraction of the intracellular microtubule-microfilament system, and fusion of granular and cellular phospholipid membranes (Lacy et al, 1968; Douglas, 1974). Although K^{+} or vasopressin elicited release of ACTH is inhibited by short periods of incubation in Ca^{2+}-free media, periods of incubation exceeding one hour in Ca^{2+}-free media are necessary to inhibit hypothalamic extract, theophylline, or dibutyryl cyclic-AMP induced secretion of ACTH (Kraicer et al, 1969; Zimmerman and Fleischer, 1970; Milligan and Kraicer, 1974). No extracellular or loosely bound Ca^{2+} is therefore required for ACTH release stimulated by hypothalamic extracts or theophylline - this does not deny the possibility that redistribution of intracellular Ca^{2+} may be involved in the release process (Milligan and Kraicer, 1974).

Cyclic-AMP has been shown by a variety of criteria to be involved in the secretion of ACTH. Firstly, incubation of pituitary halves with vasopressin or theophylline causes an increase in cyclic-AMP levels, but insulin does not. Secondly, cyclic-AMP and dibutyryl cyclic-AMP stimulate the release of ACTH. Thirdly, theophylline increases ACTH release, and potentiates the stimulatory effect of vasopressin and hypothalamic extracts (Fleischer et al, 1969; Sadow, 1973; Vale and Rivier, 1977). The release of ACTH induced by cyclic-AMP and theophylline is dependent upon tightly-bound intracellular Ca^{2+} (Zimmerman and Fleischer, 1970; Milligan and Kraicer, 1974). The manner in which cyclic-AMP is involved in releasing factor or vasopressin induced

release of ACTH is not certain, but it may mediate a redistribution of

intracellular Ca^{2+} following activation of a membrane receptor,

resulting in activation of a protein kinase involved in the release

process (Kraicer, 1975).

A third factor implicated in the release mechanism of ACTH,

in addition to Ca^{2+} and cyclic-AMP, are the prostaglandins (Sandow

and Babej, 1974; Hedge, 1977). Intravenous injection of PGE_1 or

PGE_2 to pentobarbital (Peng et al, 1970) or pentobarbital-chlorpromazine

(De Wied et al, 1969) treated rats results in activation of adrenal

corticosteroidogenesis. The effect of intravenous injection of PGE_1

is, however, inhibited in animals pretreated with morphine, hypophy-

sectomized animals, neurohypophysectomized animals, and animals bearing

lesions of the median eminence (De Wied et al, 1969; Peng et al, 1970).

This suggests the stimulatory effect of PGE_1 and PGE_2 on the pituitary-

adrenal axis in vivo is primarily via the central nervous system. PGE_1

and PGE_2 have no direct effect on ACTH release in vitro (De Wied et al,

1969; Sadow and Penn, 1972), and microinjection of PGE_1, $PGF_{1\alpha}$, $PGF_{2\alpha}$,

PGA_1, and PGB_1 to pentobarbital anaesthetized animals, in doses which

are effective in the median eminence, are ineffective when injected

directly into the anterior pituitary (Hedge and Hanson, 1972; Hedge,

1976). Although prostaglandins do not have any corticotrophin

releasing activity they appear to modulate the stimulatory effect of

hypothalamic extracts on ACTH release. In vivo, prior microinjection

of PGB_1, PGE_1 and $PGF_{1\alpha}$ into the anterior pituitary has an inhibitory

effect on the corticosteroidogenesis produced by subsequent micro-

injection of a hypothalamic extract (Hedge, 1976), and in vitro PGE_1

potentiates the effect of hypothalamic extracts on ACTH release from

preincubated pituitaries, which are not responsive in the absence of

PGE_1 (Sadow and Penn, 1972). In the study of Vale et al (1971),

however, PGE_1 was found to have a direct ACTH releasing affect in vitro,

and the presence of prostaglandin receptors was suggested by the inhibition of K^+ induced release of ACTH produced by a prostaglandin antagonist, 7-oxa-13-prostynoic acid. PGE_1 stimulates adenyl cyclase activity and raises cyclic-AMP levels in pituitaries incubated in vitro (Zor et al, 1972), suggesting the effects of prostaglandins in modulating pituitary release of ACTH may be on intracellular cyclic-AMP levels.

f)   Neurohumoral Regulation of ACTH Release from the Pituitary
        Gland

The concept that specialised neurons of the hypothalamus might have a neurosecretory endocrine function, secreting proteins that are conveyed to the pituitary was proposed by Scharrer in 1928 from work on a teleost fish (Scharrer, 1975). Scharrer noted that the supra-optic and paraventricular nuclear cells in a variety of species are distinct in that they are multinucleate, show protein-containing colloid-like vacuoles and granules, and have a unique relationship between each cell and its surrounding capillary network.

In 1930, Popa and Fielding noted the existence of a portal capillary network between the hypophysis and hypothalamus which they suggested conveyed blood from the adenohypophysis to the hypothalamus. The direction of blood flow in the portal system was subsequently shown to occur from the hypothalamus to the adenohypophysis in blood vessels on the ventral aspect of the pituitary (Green and Harris, 1947), but a blood flow from the adenohypophysis to the brain, along the dorsal aspect of the pituitary, also exists (Török, 1964). Recent evidence suggests that a microcirculatory system may exist within the pituitary; a number of long portal vessels conveying blood from the infundibulum to the adenohypophysis; short portal vessels then conveying blood from the adenohypophysis to the neurohypophysis; and

a large number of vessels carrying blood from the neurohypophysis to the brain (Bergland and Page, 1978).

In 1950, Harris and Jacobsohn transplanted the hypophysis of 1 to 10 day old rats into the temporal subarachnoid space, or the sella turcica, of their hypophysectomized mothers - animals with transplants in the sella turcica, that is, in apposition with the median eminence, showed normal adrenal activity, in contrast to those with transplants under the temporal lobe. Both kinds of implants were vascularized, but only grafts supplied by the portal blood supply and not by the systemic circulation were capable of secreting ACTH.

The findings that hypothalamic neurones may secrete substances involved in pituitary trophic function, that the hypophyseal portal system is necessary for normal hypophyseal function, and that the pars distalis receives few nerve fibres from the hypothalamus, led Harris (Green and Harris, 1947; Harris, 1955) to propose that the hypothalamus may influence anterior pituitary hormone secretion by a two-link chain, nerve fibres passing from the hypothalamus to the median eminence portal capillary network; the portal capillaries then passing from the infundibular stem to the pars distalis. This system would convey stimuli from the hypothalamus by means of a humoral agent released from the hypothalamic neuron terminal.

Further evidence of a neural, hypothalamic involvement in control of secretion of pituitary adrenocorticotrophin was provided by electrical stimulation and lesion experiments in various structures of the basal region of the brain (De Groot and Harris, 1950; Harris, 1955). The first direct evidence that a corticotrophin releasing factor exists in the portal blood supply came from Porter and Jones (1956), who showed that portal blood, but not blood from the carotid artery, collected from hypophysectomized dogs, causes adrenal ascorbic

acid depletion when injected into cortisol pretreated rats.

In 1955 Guillemin and Rosenberg, and Saffran et al demonstrated the presence of an ACTH releasing peptide, distinct from vasopressin, in extracts of posterior hypothalamic and neurohypophyseal tissue, by stimulating the release of ACTH from pituitary tissue cultures (Guillemin and Rosenberg, 1955), and from pituitaries incubated in vitro (Saffran et al, 1955). Subsequently the peptide became known as Corticotrophin Releasing Factor (CRF). The activity of a CRF preparation was also demonstrated in morphine-nembutal blocked, and median eminence lesioned rats, CRF showing a linear dose-response curve distinct from that of lysine vasopressin, and was effective in doses having less pressor activity than the minimum effective dose of purified lysine vasopressin. Further, injection of the preparation into 24-hour hypophysectomized rats confirmed that the activity was not due to the presence of any ACTH (Guillemin et al, 1959b).

Using ion-exchange chromatography, column electrophoresis, countercurrent distribution and gel filtration, Schally et al (1960) succeeded in demonstrating the presence of two CRF's, α-CRF and β-CRF, in posterior pituitary extracts. α -CRF was further separated by Schally et al (1960) into two peptides, α_1- and α_2-CRF, both having an amino acid composition similar to that of α-MSH, which were separable on carboxymethyl cellulose. β -CRF was later further analyzed, and a structure similar to (Fig. 2), but not identical with, vasopressin was proposed by Schally and Bowers (1964). These authors noted that although the peptides had a potent ACTH-releasing effect, α_2-CRF had a very high MSH activity, and β-CRF had some intrinsic pressor activity. Purification of ovine hypothalamic extracts by gel filtration and further fractionation by countercurrent distribution, also yielded two zones of CRF activity with the same

β-CRF

Acetyl-Ser-Tyr-CyS-Phe-His-(Asp-Glu)-CyS-(Pro-Val)-Lys-Gly

NH₂ NH₂ NH₂

$$\text{Acetyl-Ser-Tyr-CyS-Phe-His-(Asp-Glu)-CyS-(Pro-Val)-Lys-Gly}$$

Lysine Vasopressin

CyS-Tyr-Phe-Glu-Asp-CyS-Pro-Lys-Gly

NH₂ NH₂ NH₂

Arginine Vasopressin

CyS-Tyr-Phe-Glu-Asp-CyS-Pro-Arg-Gly

NH₂ NH₂ NH₂

Arginine Vasotocin

CyS-Tyr-Ileu-Glu-Asp-CyS-Pro-Arg-Gly

NH₂ NH₂ NH₂
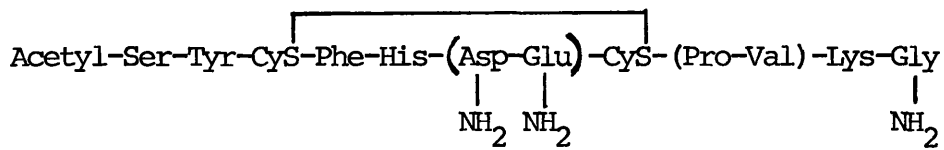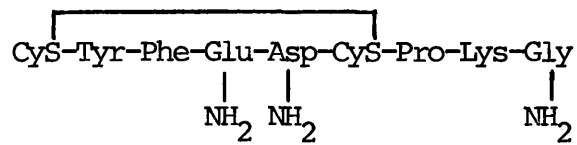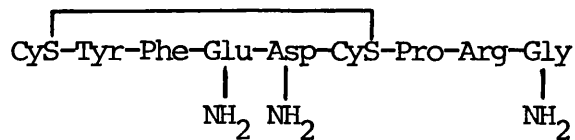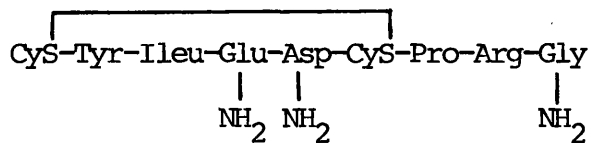
FIGURE 2

Postulated structure of β-CRF (Schally and Bowers, 1964), and structures of lysine and arginine vasopressin, and of arginine vasotocin (Heller and Pickering, 1970).

partition coefficients as those found for α-CRF and β-CRF of neuro-
hypophyseal origin (Guillemin and Schally, 1963). Determination of
the amino acid composition of CRF of hypothalamic origin has not, to
date, been accomplished; however, something is known of its
characteristics.

In the study of Chan et al (1969b), extracts of rat median
eminence yielded an active zone of CRF activity on thin-layer chroma-
tography with a distribution including the zone of lysine vasopressin -
however, the pressor activity of the zone of CRF activity was too low
to account for the presence of any vasopressin. On gel filtration,
some CRF activity was found in the unretarded peak, suggesting the
presence of a large molecule with CRF activity, but most of the
activity was found in a peak not associated with peptide material and
of low molecular weight. In another study, also of extracts of rat
median eminence, gel chromatography also revealed the presence of two
peaks of CRF activity. However, neither peak had CRF activity of
its own, unless combined with a fraction of the other peak (Pearl-
mutter et al, 1975). Yet other workers, using extracts of rat hypo-
thalamus (Seelig and Sayers, 1977) have found only a single peak of
CRF activity, but which was not contaminated with ACTH. Using
porcine hypothalamic extracts, CRF activity has been clearly resolved
into one peak, of molecular weight less than 1500, and distinct from
that of ACTH, using a solution of dimethyl formamide as the eluent,
which is strongly hydrophobic in contrast to acetic acid used in other
studies (Cooper et al, 1976). This suggested that the higher molecular
weight CRF might represent an aggregated form of lower molecular weight
CRF. Using CRF obtained by incubation of rat hypothalami Jones et al
(1977) found two peaks of CRF activity, neither contaminated with ACTH,
which were resolved by gel chromatography and which had molecular weights

of 2500 and 1300 - neither peak of activity released thyrotropin (TSH),
luteinizing hormone (LH), follicle stimulating hormone (FSH) or pro-
lactin from rat pituitaries in vitro.

Although the existence of a CRF molecule distinct from other
known neurohumors has been demonstrated for over 20 years, it is also
true that a number of substances have CRF activity in a variety of
test systems. Most prominent of these is vasopressin (Fig. 2) which
shows characteristics like CRF, in particular β-CRF of neurohypophyseal
origin, on chromatography. Vasopressin has been localized to granules
in hypophyseal stalk homogenates which show a similar distribution on
sucrose gradient centrifugation to granules containing CRF activity
(Ishii et al, 1969). Vasopressin and CRF activity in synaptosomal
preparations are, however, separable, suggesting CRF and vasopressin
are synthesized and released from separate neurones in the hypothalamus
(Mulder et al, 1970). Using immunohistochemical techniques, the cell
bodies of vasopressin and neurophysin containing neurones have been
localized to the supraoptic, paraventricular, and suprachiasmatic
nuclei of the rat (Defendi and Zimmerman, 1978); CRF bioactivity,
however, has been localized (in addition to the median eminence) to the
arcuate, dorsomedial, ventromedial, and periventricular nuclei of the
hypothalamus, whereas CRF bioactivity in the supraoptic and para-
ventricular nuclei is low (Krieger et al, 1977a), lending support to
the theory that CRF and vasopressin are synthesized in distinct neural
elements.

The contribution of vasopressin to normal physiological
stimulation of ACTH release is unclear. It has been suggested on
the basis of experiments demonstrating quenching of CRF activity of
rat stalk-median eminence extracts by antisera to arginine vasopressin,
and the presence of a peak of CRF activity on gel filtration with

immunological characteristics identical with arginine vasopressin, that CRF may represent vasopressin modulated by a hypothalamic factor(s) which is present in a fraction obtained on gel filtration, and which potentiates the CRF activity of synthetic arginine vaso- pressin to the level of that of crude stalk-median eminence extract (Gillies et al, 1978a; Gillies and Lowry, 1979). This hypothesis, however, remains to be validated, since it has repeatedly been shown that CRF and vasopressin show similar behaviour on gel chromatography (Guillemin and Schally, 1963; Schally and Bowers, 1964; Schally et al, 1968; Chan et al, 1969b), and that the vasopressin present in whole medial basal hypothalamic extracts may be suppressed with specific arginine vasopressin antisera, without significant inactiv- ation of specific CRF activity (Lutz-Bucher et al, 1977; Briaud et al, 1978). Further, the vasopressin content can only account for 30% of the biological CRF activity of crude stalk-median eminence extracts (Gillies and Lowry, 1979), and of purified hypothalamic extracts (Synetos et al, 1978).

Vasopressin is, however, involved in the physiological release of ACTH in vivo, to the extent that in Brattleboro rats, which lack vasopressin, the corticosterone response to ether, or bleeding and restraint stress is attenuated (McCann et al, 1966), the extent of the deficiency depending on the intensity of the stress administered (Yates et al, 1971). Brattleboro rats also show a diminished increment in plasma ACTH following both ether stress and the stress of a sound stimulus (Mialhe et al, 1979), and their hypothalamic CRF release in vitro (Buckingham and Leach, 1979) and content (Krieger et al, 1977a; Gillies et al, 1978b) is diminished, indicating that, in part, their deficiency may be attributable to a lack of CRF in addition to vasopressin although this is likely to be small (Pearlmutter et al,

1980). Further, pituitaries obtained from Brattleboro rats are less responsive to hypothalamic extracts than those of normal rats, but are more responsive to vasopressin (Krieger and Liotta, 1977; Lutz-Bucher et al, 1977). Prior injection of vasopressin into the anterior pituitary potentiates the effect of a subsequent injection of CRF (Yates et al, 1971; Hiroshige et al, 1977), and the presence of glucocorticoid sensitive arginine vasopressin, which is absent in Brattleboro rats, has been demonstrated in the rat anterior pituitary (Chateau et al, 1979), suggesting vasopressin may have a function at the anterior pituitary in modulating the release of ACTH. The adrenocortical and blood ACTH response to neurogenic stress is diminished by neurohypophysectomy (De Wied, 1961a; Greer et al, 1975), indicating posterior lobe vasopressin is involved in the response to neurogenic stress, as the studies with Brattleboro rats imply. An effect of vasopressin in several in vivo assay systems in which the endogenous release of CRF is blocked by steroids, hypothalamic lesions, or tranquilizers, is also evident (Guillemin et al, 1959b; Arimura et al, 1967; Hiroshige et al, 1968; Chan et al, 1969a; Cheifetz et al, 1969; De Wied et al, 1969; Dhariwal et al, 1969). In each instance however, vasopressin displays characteristics different from those of CRF.

That vasopressin has a direct effect on the adenohypophysis in vivo is also shown by the finding that animals with pituitary grafts under the renal capsule respond to injections of lysine vasopressin with an elevation of plasma corticosterone; no response is elicited in hypophysectomized animals, and CRF content is unchanged in the hypothalamus, hypophyseal stalk, and neurohypophysis after injection of vasopressin, implying the effect of vasopressin is neither directly on the adrenal glands nor on CRF content in the hypothalamus (Yasuda et al, 1978). The amount of vasopressin present in the anterior

pituitary, 2 to 4mU (Chateau et al, 1979) casts some doubt on the physiological significance of some of the in vivo results with vasopressin, since doses between 10mU intravenously (Hiroshige et al, 1968), 20mU injected into the carotid artery (Chan et al, 1969a), or 100mU intra-peritoneally (Yasuda et al, 1978) have been used. On the other hand, doses as small as 2mU injected into the anterior pituitary or median eminence (Dhariwal et al, 1969) and 0.1mU injected intravenously (Cheifetz et al, 1969) have a significant effect on plasma corticosterone levels.

The manner in which vasopressin exerts its effect on the anterior pituitary has to some extent been investigated in vitro. Pituitaries from Brattleboro rats are less responsive to hypothalamic extracts, indicating that vasopressin is necessary in the anterior pituitary for the response to CRF (Krieger and Liotta, 1977). That the diminution in response is due to the absence of vasopressin, and not to decreased ability to release ACTH in response to a stimulus, is shown by the fact that the concentration of ACTH is greater in the dispersed cells of Brattleboro rats than in those of normal rats, and by the fact that cells from Brattleboro rats are more responsive to vasopressin (Krieger and Liotta, 1977) and respond to vasopressin without a preincubation period (Lutz-Bucher et al, 1977). Acute removal of pituitary vasopressin by preincubating pituitaries for 3 hours, however, has no effect on the response to hypothalamic extracts (Lutz-Bucher et al, 1977), suggesting the effect of vasopressin is not one on immediate CRF receptor activation, but in some way, on "priming" of the corticotroph, as suggested by in vivo studies (De Wied, 1961a; Yates et al, 1971; Jones and Hillhouse, 1977), and by studies in vitro (Portanova and Sayers, 1973a; Yasuda and Greer, 1976a; Buckingham and Hodges, 1977a; Mialhe et al, 1979) which

demonstrate the additive or potentiating effects of vasopressin, or

pitressin, and hypothalamic extracts, and dose related effects of

vasopressin on the response to CRF preparations. That vasopressin

acts on a receptor distinct to that to CRF is suggested by the

difference in dose-response characteristics of vasopressin and CRF

extracts both in vivo (Guillemin et al, 1959b) and in vitro (Chan et

al, 1969a; Portanova and Sayers, 1973a; Krieger et al, 1977a;

Lutz-Bucher et al, 1977; Seelig and Sayers, 1977; Vale and Rivier,

1977; Gillies et al, 1978a) by the finding that in a number of in

vitro systems preincubation of pituitaries is necessary for vaso-

pressin to produce a response but is not necessary for the effect of

hypothalamic extracts (Fleischer and Vale, 1968; Sadow and Penn,

1972; Lutz-Bucher et al, 1977), and by the demonstrations that the

effect of vasopressin reaches a plateau after 20 minutes of incubation,

whereas the effect of hypothalamic extracts persists for at least one

hour in vitro (Lutz-Bucher et al, 1977), and that the peak of ACTH

release in vivo caused by CRF extracts occurs within 5 to 10 minutes

and that for vasopressin occurs between 15 and 20 minutes (Chan et al,

1969a). This evidence, however, is not unequivocal and it is equally

possible that vasopressin may act as a partial agonist at the CRF

receptor as a number of in vitro studies suggest (Chan et al, 1969a;

Portanova and Sayers, 1973a; Gillies et al, 1978a), and in view of

the similarity in antigenic properties of vasopressin and CRF (Gillies

et al, 1978b; Mialhe et al, 1979), whilst noting that the antigenic

and bioactive determinants of a molecule may be distinct. Thus the

exact role of vasopressin at the anterior pituitary is still unclear,

but it may be concluded that CRF and vasopressin are distinct mole-

cules with different physico-chemical characteristics and different

activities as ACTH releasing agents. It may be added that the total

quantities of arginine vasopressin present in the anterior pituitary

(2mU - Chateau et al, 1979), hypophyseal blood (5mU - Zimmerman et al, and
1973), neurohypophysis (340mU - Rosenbloom and Fisher, 1975) make it

necessary to differentiate between the effect of vasopressin and CRF

in those systems which are sensitive to vasopressin. The amount of

vasopressin released by the whole hypothalamus incubated in vitro for

10 minutes (0.126mU - Bridges et al, 1975), however, is insufficient

to account for any CRF activity due to vasopressin.

A hypothalamic site of action of vasopressin on CRF secretion

has also been proposed from studies demonstrating an effect of intra-

hypothalamic injections of vasopressin on peripheral corticosterone

production (Dhariwal et al, 1969; Yates et al, 1971). These studies

may, however, be criticized in that diffusion of vasopressin to the

anterior pituitary is likely. Further, physiological doses of vaso-

pressin have no effect on the production of CRF from hypothalami in

vitro (Jones and Hillhouse, 1977).

Although the highest concentrations of CRF activity in the

central nervous system (CNS) are confined to the pituitary stalk,

neurohypophysis, median eminence, arcuate nucleus, periventricular

nuclei, dorsomedial nucleus, and ventromedial nucleus, CRF bio-

activity is also detectable in other regions of the CNS, in particular

the thalamus, cortex and supraoptic nucleus (Krieger et al, 1977a;

Yasuda et al, 1977). The nature of this CRF activity is unknown,

but CRF extracts obtained from cortical tissue and the whole posterior

pituitary show dose-response characteristics different from those of

extracts of median eminence, pituitary stalk, and neurohypophysis

(Yasuda et al, 1977), indicating there may be qualitative differences

in the CRF activity obtained from different tissues. Non-specific

CRF activity has been demonstrated in liver extracts (Witorsch and

Brodish, 1972; Briaud et al, 1978) which is not due to an ACTH-preserving effect (Uemura et al, 1976; Briaud et al, 1978). CRF activity is also found in the blood of hypothalamic lesioned rats which are hypophysectomized and laparotomized (Lymangrover and Brodish, 1973). The term "tissue-CRF" has been applied to the latter, in order to distinguish it from hypothalamic CRF in view of its non-hypothalamic origin and instability, and because of its potency and prolonged effect by comparison to median eminence CRF when injected into lesioned assay animals (Brodish, 1977b). The nature of this substance is unknown, but it appears to be under physiological control in that administration of ACTH suppresses the amount of tissue CRF in the blood of donor animals (Brodish, 1977a).

Oxytocin (Saffran et al, 1955; Buckingham and Hodges, 1977a) and angiotensin II (De Wied et al, 1969; Jones and Hillhouse, 1977) have little or no effect on ACTH release in vitro. Another peptide which has been considered for CRF activity is arginine vasotocin (Fig. 2), which causes the release of ACTH in vitro in physiological doses (Portanova and Sayers, 1973a; Buckingham and Hodges, 1977a; Gillies et al, 1978a). Substance P has been found either to have a stimulatory effect (Guillemin et al, 1957) or an inhibitory effect (Guillemin et al, 1957; Jones et al, 1978) on basal ACTH release, in vitro, and an inhibitory effect on ACTH release stimulated by CRF or lysine vasopressin in vivo and in vitro (Jones et al, 1978).

Serotonergic, dopaminergic, noradrenergic, and cholinergic neurones have been identified in the external layer of the median eminence and in the pars intermedia of the pituitary gland (Bridges et al, 1973; Björklund et al, 1973; Hökfelt et al, 1978). The manner in which monoamines and acetylcholine may contribute directly to the release of ACTH from the pituitary is unclear - they may

innervate cells of the pars intermedia or be carried in the hypophyseal

portal circulation. In vivo, intra-pituitary injections of acetyl-

choline, noradrenaline, dopamine, and serotonin into nembutal-blocked

rats are without effect, whereas histamine is a potent releasor of

ACTH (Hiroshige and Abe, 1973); similar results have been obtained

with intravenous injections into the median eminence lesioned rat,

with the difference that carbachol was effective but not when tested

on pituitaries in vitro (De Wied et al, 1969). Adrenaline, noradren-

aline, acetylcholine, dopamine, serotonin, and histamine have no effect

on the release of ACTH from pituitary fragments in vitro (Saffran et al,

1955; Guillemin et al, 1957; Buckingham and Hodges, 1977a). If,

however, the intermediate lobe of the pituitary is incubated in

isolation, serotonin has a stimulatory effect on ACTH release (Kraicer

and Morris, 1976b), but lack of an effect has also been reported

(Smelik and Tilders, 1978). Dopamine, noradrenaline, and adrenaline

appear to have an "inactivating" effect on pars intermedia ACTH

(Kraicer and Morris, 1976b) and a similar effect has been demonstrated

with dopamine on ACTH released from the adenohypophysis (Van Loon and

Kragt, 1970). The role of neurotransmitters in the pars intermedia

is therefore unclear, if neurotransmitters have any direct effect on

ACTH release at all (Wurtman, 1970).

g)   Assay of Corticotrophin Releasing Factor (CRF)

As the chemical nature of corticotrophin releasing factor is

unknown and pure preparations are not available, the measurement of

CRF is dependent on bioassay methods utilizing its ability to stimulate

pituitary corticotrophic activity either in vivo or in vitro. The

general applicability of studies of CRF activity is therefore hampered

both because of the widely differing nature of the CRF preparation

used in different laboratories, and because of differences in the

characteristics of bioassays. In the first instance, CRF bioassay requires a method of measuring pituitary release or pituitary content of ACTH - this has been discussed in a previous section. Of the methods outlined, in vivo corticosteroidogenesis, in vivo ascorbic acid changes, changes in adrenal content of corticosteroids induced in vivo, in vitro corticosteroidogenesis, and radioimmunoassay have been incorporated into CRF bioassays. The objectives of a bioassay for CRF, in addition to establishing a suitable method of measuring changes in corticotrophic function, are therefore to ensure that endogenous CRF activity in the animal in vivo does not interfere with the response of the pituitary gland to test doses of CRF, that the response is due to true CRF bioactivity and not a non-specific effect either on the test animal, degradation of ACTH, responsiveness of the adrenals to ACTH, or some other secondary effect, and that the method in which the pituitary gland is prepared does not compromise either the specificity or sensitivity of its response to CRF.

In order to suppress endogenous CRF release, in vivo CRF assays have used either median eminence lesioned animals (Guillemin et al, 1959b; De Wied, 1961b; Chan et al, 1969a; De Wied et al, 1969; Witorsch and Brodish, 1972; Lymangrover and Brodish, 1973; Bradbury et al, 1974; Jones et al, 1976), animals treated with central nervous system tranquilizers (Guillemin et al, 1959b; Arimura et al, 1967; Dhariwal et al, 1969; De Wied et al, 1969), or animals treated with suppressive doses of glucocorticoids (Vernikos-Danellis, 1964; Hiroshige et al, 1968). Each of these procedures may be criticized for fundamental reasons.

The distribution of CRF in the hypothalamus is so extensive (Krieger et al, 1977a; Yasuda et al, 1977) that in order to block endogenous CRF release under stress, lesions must be correspondingly

large (Brodish, 1963; Martini et al, 1968), resulting in an animal with disturbed hypothalamic regulatory function and pituitary blood supply. In practice, the hypothalamic lesioned animal shows non-specificity to large doses to vasopressin (Guillemin et al, 1959b; De Wied, 1961b; Chan et al, 1969a; De Wied et al, 1969), and is still subject to "sensitization" or loss of specificity by prior manipulation (Witorsch and Brodish, 1972).

In the "pharmacologically blocked"rat, specificity is poorer than in the lesioned animal (De Wied et al, 1969). The mode of action of CRF on the pituitary corticotroph has to some extent been described in previous sections - that is, the involvement of calcium, cyclic-AMP, prostaglandins, vasopressin, and neurotransmitters. That the levels of these substances be standardized is as much a prerequisite in the CRF bioassay as the removal of the effects of endogenous CRF, and for this reason pharmacological pretreatment will inevitably result in a loss of specificity. Although pretreatment with glucocorticoids either on their own (Vernikos-Danellis, 1964) or in conjunction with nembutal (Hiroshige et al, 1968; Dhariwal et al, 1969; De Wied et al, 1969) is an effective means of suppressing endogenous release of CRF, glucocorticoids also inhibit the response to administered CRF (Vernikos-Danellis, 1964; De Wied, 1964; Russell et al, 1969).

In the in vitro assay the site of action of test doses of CRF is better defined, and specificity is thereby improved - this is also true of pituitary microinjection methods in vivo (Hiroshige et al, 1977). Since the pituitary tissue is isolated in vitro the effect of endogenous CRF is removed (Saffran et al, 1955; Guillemin et al, 1957), and this is further corrected by preincubation of the pituitary frag-ments (Chan et al, 1969a; Buckingham and Hodges, 1977a; Lutz-Bucher et al, 1977), dispersion of the pituitary cells (Portanova et al, 1970;

Mulder and Smelik, 1977; Gillies and Lowry, 1978), tissue culture
(Vale et al, 1972; Takebe et al, 1975), and by handling of the
animal prior to use in the assay (Thomas and Sadow, 1975a). Pituitary
cell dispersion systems are responsive to vasopressin (Portanova and
Sayers, 1973a; Vale and Rivier, 1977; Vale et al, 1978; Gillies
and Lowry, 1978), as are methods using pituitary fragments (Saffran
et al, 1955; Chan et al, 1969a; Buckingham and Hodges, 1977a; Lutz-
Bucher et al, 1977), but only under certain conditions. Yasuda and
Greer (1976a), using a dispersed cell technique found vasopressin to
be ineffective, whereas Fleischer and Vale (1968), Sadow and Penn
(1972), and Lutz-Bucher et al (1977) found the effect of vasopressin
to be a function of duration of preincubation of the pituitary tissue.
Although the specificity, precision, and sensitivity of in vitro
methods are satisfactory (De Wied et al, 1969; Saffran et al, 1973;
Hiroshige et al, 1977), they are regarded as "less physiological" -
in practice, however, they have proved to be more convenient, sensitive
and specific than in vivo methods (De Wied et al, 1969; Takebe et al,
1975; Hiroshige et al, 1977), the fundamental difference being in the
choice of method of attaining specificity, in vivo by the removal or
suppression of the tissue containing the material being measured, and
in vitro by testing the isolated tissue (Schally and Bowers, 1964).
That the pituitary in vitro is able to respond with de novo synthesis
of ACTH is shown by work demonstrating an increase in content of ACTH
in pituitary fragments (Buckingham and Hodges, 1977a) and in pituitary
tissue cultures (Takebe et al, 1975) in response to CRF, in agreement
with the demonstration in vivo of the acute effect of CRF and stress
on pituitary ACTH content (Vernikos-Danellis, 1964; Vernikos-Danellis,
1965).

h)   In Vitro Collection of Hypothalamic Corticotrophin Releasing
        Factor (CRF)

Hypothalamic CRF is prepared for assay either by extraction
techniques (Chan et al, 1969b; Hiroshige et al, 1969; Portanova and
Sayers, 1973b; Yasuda and Greer, 1976a; Buckingham and Hodges, 1977a;
Lutz-Bucher et al, 1977; Mulder and Smelik, 1977; Gillies and Lowry,
1978), extraction and purification (Saffran et al, 1955; Guillemin et
al, 1957; Chan et al, 1969b; Dhariwal et al, 1969; De Wied et al,
1969; Vale and Rivier, 1977), by incubation of whole hypothalami in
vitro (Bradbury et al, 1974; Thomas and Sadow, 1975a; Jones et al,
1976; Buckingham and Hodges, 1977b; Vermes et al, 1977) or by
incubation of hypothalamic synaptosomes (Edwardson and Bennett, 1974).
Extraction procedures permit evaluation of the balance between CRF
synthesis and release, and measure the total hypothalamic CRF content
at the time of excision of the tissue. The manner in which CRF is
biosynthesized and stored in neurosecretory granules (Mulder et al,
1970; Fink et al, 1972; Mialhe et al, 1979) is unknown, but crude
extracts may contain pro-hormones or precursors to CRF, and CRF in
association with a neurophysin (Vandesande et al, 1974; Bock, 1977).
Whether the latter is true of CRF secreted from the hypothalamus in
vitro is also unknown, but CRF obtained from incubated hypothalami is
separable into two peaks of different molecular weight, 1500 and 2500,
neither of which would be consistent with binding to a neurophysin
(Jones et al, 1977).

In vitro incubation techniques have the advantage that, in
conjunction with a sensitive bioassay for CRF, they permit a direct
measure of the ability of the hypothalamus to secrete CRF following
treatment of the donor animal in vivo (Bradbury et al, 1974; Jones
et al, 1976; Jones and Hillhouse, 1976; Thomas, 1977; Buckingham,

1979), or following the direct addition of agents in vitro (Bradbury
et al, 1974; Jones et al, 1976; Jones and Hillhouse, 1976;
Buckingham and Hodges, 1977b; Vermes et al, 1977). The viability
of hypothalami incubated in vitro has been assessed by their oxygen
consumption, and is linear over a 3-hour incubation, irrespective of
whether whole or quartered hypothalami are used (Bradbury et al, 1974).
However, intracellular potassium is lost, sodium and chloride
accumulated, presumably resulting in depolarization, and intra- and
extra-cellular swelling develops (Bradbury et al, 1974). Nevertheless,
CRF producing neurones appear capable of responding to depolarizing currents,
and to neurotransmitters, with altered secretion of CRF (Bradbury et al,
1974; Jones et al, 1976; Buckingham and Hodges, 1977b; Vermes et al,
1977). Both basal and stimulated CRF release are sensitive to the
calcium concentration of the incubating or superfusion medium (Jones
and Hillhouse, 1976; Vermes et al, 1977), and the effect is specifically
on calcium channels to the extent that the stimulus of high calcium
concentrations on basal release is blocked by manganese and mimicked
by strontium (Jones et al, 1976), implying influx of calcium is part
of the CRF release process (Lederis and Jaysena, 1970; Douglas, 1975).

Neurotransmitters (Hubbard et al, 1969) and neurohypophyseal
hormones (Lederis and Jaysena, 1970; Burford et al, 1973) are
distributed between at least two pools in their respective nerve
terminals, a readily released pool, and a less readily released pool.
That this may be so in CRF containing neurones is suggested by two
lines of evidence. Firstly, differential gradient and sucrose
gradient centrifugation reveal the presence of CRF in at least three
subcellular particle fractions - further, hypotonic disruption of
synaptosomes is found to release "free" cytoplasmic CRF, which may
represent a maximum of 50 to 60% of the total CRF activity stored in

nerve endings (Mulder et al, 1970). Secondly, experiments with cycloheximide both in vivo (Hiroshige et al, 1977; Fujieda and Hiroshige, 1978) and in vitro (Jones and Hillhouse, 1977) demonstrating a block of delayed CRF release following stress, and a block of serotonin stimulated CRF release, suggest at least part of the CRF that is releasable is recently synthesized. CRF is rapidly synthesized in vitro since hypothalami may be repeatedly stimulated over two or three hours without alteration of CRF stores (Jones and Hillhouse, 1977), and CRF content is increased within ten minutes when incubated with stimulatory agents (Buckingham and Hodges, 1977b). Neurotransmitters (Hubbard et al, 1969), neurohypophyseal hormones (Burford et al, 1973) and insulin (Howell and Taylor, 1967) are also released preferentially from a newly synthesized pool, and this may, therefore, also be the case with CRF. The release and synthesis of CRF are also separable under experimental conditions in which corticosterone causes an inhibition of acetylcholine induced release of CRF and an increase in CRF content (Jones and Hillhouse, 1976).

In the studies described here, CRF release was investigated during various periods of incubation, and during two consecutive 15 minute incubations, at a number of times in the 24-hour cycle. It was hoped that this would provide information about the dynamics of CRF release in vitro under non-stimulated conditions and about circadian rhythmicity in CRF release. Further, hypothalamic CRF content was measured at three times in the day in order to determine if this in any way paralleled CRF release. In a further experiment CRF release was measured in calcium-free, magnesium-elevated, incubation media in order to determine whether CRF release is dependent on a calcium ion-linked process, since it may represent the "leakage" of CRF from damaged neurones or some other non-specific release process.

II.   Circadian Periodicity in Brain-Pituitary-Adrenal Activity

a)   Circadian Rhythms in Adrenocortical Function

A circadian periodicity in urinary excretion of 17-keto-
steroids in man was originally described in 1943 (Pincus, 1943), and
later in 17-hydroxycorticosteroids (Laidlaw et al, 1954). Diurnal
rhythmicity in plasma 17-hydroxycorticosteroids is also well documen-
ted (Perkoff et al, 1959; Krieger, 1975a). In man, this rhythmicity
cannot be ascribed to rhythmicity in plasma protein binding since this
is reported either to remain constant over the day (De Moor et al,
1962) or to be entrained by rhythmicity in plasma cortisol levels
(Angeli et al, 1978); nor to variations in adrenal sensitivity to
ACTH, since administration of ACTH produces a greater effect during
the day and not overnight or the early morning, the time of the peak
of plasma 17-hydroxycorticosteroids (Forsham et al, 1955; Perkoff et
al, 1959), and when ACTH is infused constantly over the 24-hour period,
eliminating any "priming" by the circadian rhythm in ACTH, no
periodicity in adrenal sensitivity is apparent (Perkoff et al, 1959;
Nugent et al, 1960); nor can it be ascribed to rhythmicity in meta-
bolic clearance rate of corticosteroids since this is reported either
to be invariant (Perkoff et al, 1959), or to vary in a direction
opposite to that which would be expected if it were responsible for
the circadian rhythm in plasma corticosteroids (Hellman et al, 1970;
De Lacerda et al, 1973); nor are variations in glucocorticoid feed-
back sensitivity responsible, since this is greatest at midnight or
the early morning (Nichols et al, 1965; Angeli, 1974). This suggests
the origin of circadian rhythmicity in plasma glucocorticoids is in the
stimulus provided by brain-pituitary function.

Diurnal rhythmicity in plasma corticoids is disturbed in
patients with neurological and psychiatric disease (Perkoff et al,

1959; Krieger, 1975a). In blind, or partially sighted, subjects a normal peak of 11-hydroxycorticosteroids is associated with transition from sleep to wake, but peaks are found at other times of the day, and circadian patterns are not reproducible in these subjects (Krieger and Rizzo, 1971). Reversal of light and sleep-wake cycles results in reversal of the peak of 17-hydroxycorticosteroids in plasma (Perkoff et al, 1959). Separation of the effects of activity and light cycles, by arranging the hours of darkness in the middle of the waking day, results in two peaks of 17-hydroxycorticosteroids, one at the usual time of awakening, and another at the time of illumination in the evening (Orth and Island, 1969). By restricting light to only one hour in the evening, the peak of plasma cortisol on awakening is delayed, and a second major peak is found during the one hour of illumination (Orth and Island, 1969), and in blind subjects the morning peak may be in advance of the normal 8 a.m. peak (Krieger and Rizzo, 1971), suggesting that although transition from sleep to wake is important in the genesis of circadian rhythms, they are synchronized by light stimuli. Restriction of feeding in man to breakfast only, dinner only, or several meals has no effect on the normal rhythm of plasma cortisol (Haus, 1976).

Pharmacological evidence of a central involvement in plasma corticoid rhythmicity in man is provided by results demonstrating a greater susceptibility to the combined stimulus of insulin hypoglycaemia, thyrotropin releasing hormone (TRH), and luteinizing hormone-releasing hormone (LHRH) when given at midnight or 6 a.m. than when given at other times of the day (Rastogi et al, 1976), and to the stimuli of a synthetic vasopressin analogue (CRA-41) (Clayton et al, 1963), or of insulin (Takebe et al, 1969) when given at midnight.

Circadian periodicity in plasma corticosteroids has been

described in the rat (Guillemin et al, 1959c), mouse, (Halberg et al,

1959), rhesus monkey (Leshner et al, 1978), dog (Galicich et al,

1964), and the pig (Schulke et al, 1970). In contrast to man, where

the evidence suggests brain-pituitary activity is the dominant

entrainer of circadian rhythms, in the rat there is evidence to

suggest circadian rhythmicity may be inherent in adrenocortical

function. In the rat and mouse, nocturnal animals, adrenocortical

responses to administered ACTH are greatest at night, both when ACTH

is administered in vivo (Haus, 1964; Ottenweller et al, 1978;

Dallman et al, 1978), and to adrenals in vitro (Ungar and Halberg, 1963).

In the mouse, however, the timing of peak adrenal sensitivity to ACTH

is several hours after the peak in corticosterone production (Ungar

and Halberg, 1963; Haus, 1964) and may be due, therefore, to a priming

effect of the peak in plasma ACTH (Perkoff et al, 1959; Ungar and Halberg,

1963). In the rat, on the other hand, adrenal sensitivity to ACTH is

increased at the time of the circadian peak of corticosterone levels,

and is not dependent on rhythmicity in ACTH secretion to the extent

that increased sensitivity to administration of ACTH in the evening

persists both in hypophysectomized animals, in animals treated 15

hours prior to sampling with suppressive doses of dexamethasone, and

in adrenals in vitro obtained from hypophysectomized animals (Ungar,

1967; Meier, 1976; Dallman et al, 1978; Ottenweller et al, 1978).

The timing of peak adrenal sensitivity in the rat is, however, still

unclear, as in vitro, like the mouse, it is several hours after the

peak in plasma corticosterone levels (Ungar, 1967), but in vivo it

appears to be at the onset of darkness (Engeland et al, 1977;

Wilkinson et al, 1979). Although a circadian peak in ACTH secretion

is not necessary for the appearance of a rhythm in adrenal sensitivity,

it may be necessary for its timing, since peak sensitivity is shifted

in phase to an earlier time when adrenals incubated in vitro are

obtained from hypophysectomized rats (Ungar, 1967), and in the hypo-

thalamic lesioned rat, peak adrenal sensitivity is greater at 8 a.m.

than at 8 p.m. both in vivo and in vitro (Jones et al, 1979b). The

circadian variation in responsiveness to ACTH may involve a process

associated with the activation of adrenal adenylate cyclase, since

injection of ACTH in the evening produces a greater increment in

adrenal cyclic-AMP levels than does a similar injection in the

morning (Dallman et al, 1978); further, the rate of association of

cholesterol with the cholesterol side chain cleavage form of adrenal

cytochrome P450 is greater in the evening than in the morning

(Brownie et al, 1979).

Rhythmicity in corticosteroid production in the absence of

ACTH has been shown in hamster adrenals in vitro (Shiotsuka et al,

1974), but not in monolayer cultures of rat adrenals (O'Hare and

Hornsby, 1975), and is absent in the hypophysectomized rat not given

ACTH (Meier, 1976) and in rat autotransplanted adrenals (Ottenweller

et al, 1978). It has been suggested from the latter that direct

neural connections (Engeland and Dallman, 1975; Dallman et al, 1977a)

may also be necessary for the appearance of a rhythm in adrenal

corticosteroidogenesis (Ottenweller et al, 1978).

Thus, ACTH is necessary for the appearance of rhythmicity in

adrenal corticosterone secretion (Fig. 3). Although rhythmicity in

plasma ACTH levels may not be necessary for the maintenance of a

rhythm in adrenal sensitivity to ACTH, it may be necessary for its

timing (Ungar, 1967), and is necessary for the full expression of a

circadian corticosterone peak since the latter is greatly attenuated

in the hypophysectomized rat given ACTH and thyroxine (Meier, 1976),

and in adrenals obtained from hypophysectomized rats and incubated

FIGURE 3

Block diagram of the Hypothalamo-Pituitary-Adrenal (H-P-A) axis.   O, +, - indicate values which each signal may take.

If = immediate stimulation of corticosteroidogenesis by ACTH.

pf = trophic effect of ACTH.

ISF = Interstitial Fluid.

In the rat, circadian rhythms are evident in the neural input to the hypothalamus, hypothalamic CRF content, pituitary ACTH content, plasma ACTH levels, adrenal sensitivity to ACTH, adrenal corticosterone content, plasma corticosterone levels, metabolism of corticosterone, and plasma glucose levels.   The circadian rhythmicity of the system is also susceptible to the negative feedback effects of ACTH and of corticosterone.   See text.

(Diagram modified from Yates and Maran, 1974.)
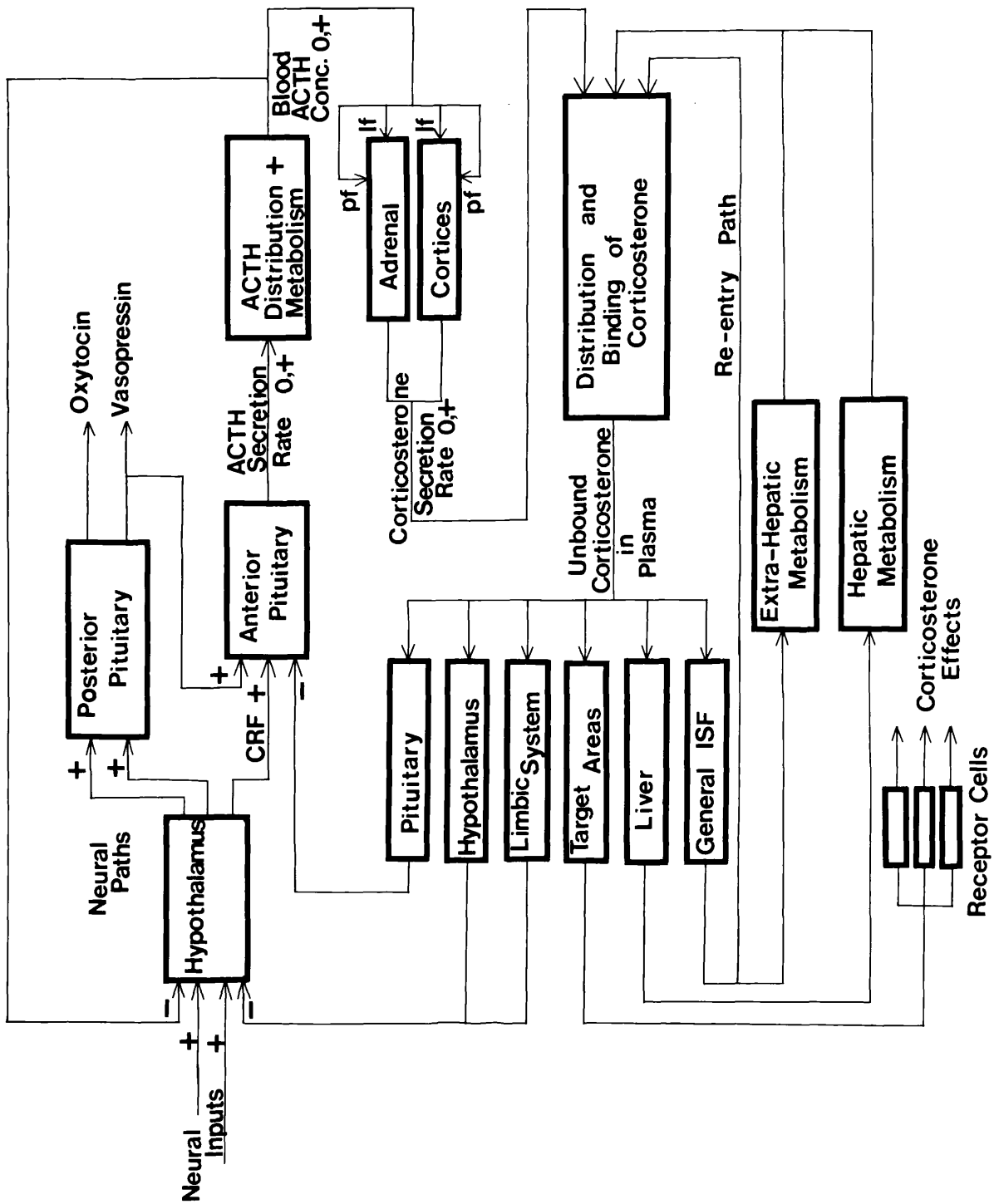
with ACTH (Ungar, 1967).

Short, episodic, secretion of cortisol, however, may be functionally dissociated from secretion of ACTH in that in man, peaks of plasma ACTH appear without a concomitant rise of cortisol levels (Krieger, 1975a), and conversely, in the rhesus monkey ultradian rhythmicity of cortisol secretion persists in the presence of supra-maximal infusions of ACTH (Holaday et al, 1977).

Further evidence of a central involvement in adrenocortical rhythmicity is provided by experiments demonstrating that diurnal rhythmicity appears later in development than responsiveness to stress, both in man (Franks, 1967) and in the rat (Hiroshige and Sato, 1971). In the rat, rhythmicity appears with the development of periodicity in hypothalamic CRF content (Hiroshige and Sato, 1970) and maturation of neurosecretory activity (Fiske and Leeman, 1964). During this period, exposure to high levels of corticosteroids (Turner and Taylor, 1976) or ACTH (Lorenz et al, 1974) may delay the onset of circadian rhythmicity. Lesion studies also demonstrate a central involvement in adrenal circadian rhythmicity, since a number of lesions in the hypothalamus may abolish corticosteroid periodicity without a reduction in "basal" corticosteroid levels (Slusher, 1964; Allen et al, 1972) or in a loss of responsiveness to stress (Slusher, 1964; Palka et al, 1969), and vice versa lesions of the medial forebrain bundle temporarily abolish corticosteroid periodicity without inhibiting the response to ether stress (Heybach et al, 1979).

b)   Circadian Rhythmicity in Pituitary Corticotrophic Function

A large, tenfold, variation in ACTH, measured both by bioassay and radioimmunoassay, is present over the 24-hour light cycle in man (Demura et al, 1966; Krieger, 1975a, 1975b), consisting of small

episodic peaks of ACTH and a circadian peak before awakening. In the rat, rhythmicity in plasma ACTH is also evident but to a much smaller extent, a twofold variation (Dallman et al, 1978), or less (Yasuda et al, 1976; Engeland et al, 1977; Wilkinson et al, 1979) being reported in immuno-active ACTH, and a four to five-fold variation in ACTH measured by bioassay (Retiene et al, 1968; Jones et al, 1979b), the peak plasma ACTH levels in each case being at the time of peak corticosterone levels, that is, the early evening. There is also a circadian variation in pituitary ACTH content but reports of the time of peak values vary widely. In mice, peak values occurred between 8 a.m. and 12 noon in one study (Ungar and Halberg, 1963), but at 4 p.m. in another (Ungar, 1967). In female rats, peak content was found between 5 and 6 p.m. (Retiene et al, 1968; Retiene and Schulz, 1970), but in male rats peak values have been found at midnight (Retiene et al, 1968), in the morning (Halasz et al, 1967b), and between 4 p.m. and 7 p.m. (Chiappa and Fink, 1977; Ixart et al, 1977).

Circadian periodicity in plasma ACTH in humans with central nervous system disease is disturbed (Krieger, 1975a) but there is little other data relating central nervous system lesions and effects on plasma ACTH rhythmicity directly, inference being made from cortico-steroid levels in most studies. Circadian rhythmicity in pituitary sensitivity to a vasopressin analogue (CRA-41) has been shown in man (Clayton et al, 1963), and in the intact, and to a lesser extent in the 48-hour hypothalamic lesioned rat, the pituitary-adrenal response to CRF is greatest in the evening (Jones et al, 1979b).

Although there is only a two to five-fold increase in plasma ACTH concentrations between morning and evening in the rat, cortico-sterone levels increase nine to ten-fold (Retiene et al, 1968; Yasuda et al, 1976; Engeland et al, 1977; Dallman et al, 1978; Jones et al,

1979b). Rhythmicity in adrenal sensitivity per se is not sufficient to account for the diurnal rhythm in plasma corticosterone, as is evident from data in hypophysectomized animals (Ungar, 1967; Meier, 1976). If there is a threefold variation in adrenal sensitivity as shown by Dallman et al (1978) and Ottenweller et al (1978), and plasma ACTH concentrations vary two to five-fold, then the ten-fold increase in plasma corticosterone levels may be partially explained, assuming the system behaves in a linear manner. Rhythmicity in corticosterone metabolism (Fig. 3) may also contribute to the overall plasma corticosteroid rhythmicity in the rat, since disappearance of exogenous and endogenous corticosteroids is greater during the day than at night (Saba et al, 1963a; Marc and Morselli, 1969; Dallman et al, 1978; Wilkinson et al, 1979). As stated by Engeland et al (1977) rather than the log dose ACTH - linear corticosterone response usually seen in the bioassay of ACTH, between morning and evening the relationship appears more nearly linear dose ACTH - log corticosterone response, emphasizing the importance of circadian rhythmicity in adrenal sensitivity to ACTH and in metabolism of corticosterone in the genesis of rhythmicity in plasma corticosterone levels in the rat (Fig. 3).

Circadian rhythmicity in hypothalamo-pituitary-adrenal (H-P-A) activity is sensitive to the influence of blood corticosteroid levels (negative feedback elements, Fig. 3). This feedback effect of adrenal steroid production is often referred to as feedback of "basal" hypo-thalamo-pituitary-adrenal function, in contrast to feedback of stress induced activity (Hodges et al, 1968; Critchlow, 1972). Specific binding of corticosteroids has been demonstrated in cytoplasmic and nuclear fractions of rat anterior pituitary cells (De Kloet et al, 1974; Koch et al, 1975), and in plasma membrane preparations (Koch et al, 1978), and a correlation between occupancy of cell nuclear

receptors and inhibition of basal ACTH release (De Kloet et al, 1974; Rotsztejn et al, 1975) and of CRF stimulated ACTH release (Koch et al, 1975) may be shown. This delayed negative feedback may involve an effect at the genomic level (McEwen et al, 1978), and dexamethasone inhibition of CRF induced ACTH release is prevented by actinomycin D (Arimura et al, 1969) and cycloheximide (Vale and Rivier, 1977). Cytoplasmic and nuclear binding of corticosteroids has similarly been demonstrated in the hypothalamus, hippocampus, amygdala, and septum (Stevens et al, 1973; De Kloet et al, 1974; Rotsztejn et al, 1975; McEwen et al, 1978), and a circadian rhythm in binding of radio-labelled corticosterone to such receptors has been shown (Stevens et al, 1973). The presence of glucocorticoid receptors in the hypo-thalamus and hippocampus is also inferred from studies in which corticosteroids applied iontophoretically affect a change in firing of neurons (Steiner et al, 1969; Segal, 1976; Ben Barak et al, 1977).

The sensitivity of the circadian, "basal", secretion of CRF and ACTH to a feedback of glucocorticoids is evident in a number of experimental situations. Betamethasone, a synthetic glucocorticoid, administered in drinking water for 24 hours (Hodges and Mitchley, 1970b) or 2 weeks (Thomas and Sadow, 1976) abolishes the circadian peak in plasma corticosterone in rats; recovery of the afternoon peak in plasma corticosterone levels occurs before recovery of the response to stress (Hodges and Mitchley, 1970b). In man, dexamethasone given either orally (Nichols et al, 1965; Asfeldt and Buhl, 1969), or infused intravenously (Ceresa et al, 1969) produces an acute (within 8 hours) inhibition of the "basal" circadian peak in corticosteroid secretion, administration of the steroid at night being the most effective time in producing this inhibition. In the rat, injection of dexamethasone subcutaneously also produces an inhibition of the

circadian rise in corticosterone levels occurring 4 hours later, whilst
not inhibiting the adrenocortical response to stress, implying a dis-
sociation in the feedback sensitivity of circadian — and ether
stress-induced activation of the hypothalamo-pituitary-adrenal system
(Retiene et al, 1967; Zimmerman and Critchlow, 1969a; Dunn and
Carrillo, 1978). Implants of dexamethasone in the anterior and
posterior hypothalamus, midbrain tegmentum, and thalamus abolish the
afternoon peak in plasma corticosterone 8 hours after implantation,
but not at 32 hours, without suppressing the response to ether stress
(Zimmerman and Critchlow, 1969b). Implants of cortisol in the hypo-
thalamus, ventral hippocampus, and midbrain also suppress the circadian
rhythm of plasma corticosterone but for periods of 5 days or more
(Slusher, 1966; Davidson et al, 1968). It should be noted, however,
that the site of action of intracerebral implants of corticosteroids
may extend to the pituitary gland because of diffusion via the portal
system (Bogdanove, 1963; Russell et al, 1969) or via the general
circulation (De Kloet et al, 1974). Pituitary implants of dexametha-
sone and cortisol, however, are ineffective in producing inhibition
of both basal (Corbin et al, 1965; Stark et al, 1968; Zimmerman and
Critchlow, 1969b) and stress induced (Smelik and Sawyer, 1962) cortico-
sterone secretion and inhibition of compensatory adrenal hypertrophy
(Davidson and Feldman, 1963), whilst similar implants in the brain are
effective. This does not necessarily exclude the pituitary gland as a
site of negative feedback inhibition of basal hypothalamo-pituitary-
adrenal activity, since when dexamethasone is injected bilaterally
into the pituitary gland, an inhibitory effect is observed on CRF
induced ACTH secretion (Russell et al, 1969).

Endogenously released steroids may also have an effect on
circadian rhythmicity in the H-P-A axis. Sound, ether, and dien-
cephalic stimulation stress procedures applied at 9.45 a.m. abolish

the afternoon rise in plasma corticosteroids (Slusher, 1964). The

stress of placement in a stereotaxic apparatus at noon also has an

inhibitory effect on the circadian peak in plasma corticosterone in

rats (Zimmerman and Critchlow, 1969b). On the other hand, electric

shock given to the sciatic nerve at 9 a.m. has no effect on the

afternoon rise in plasma corticosterone (Dallman and Jones, 1973).

The stress of repeated immobilization in the rabbit results in a

phase advance of the peak of pituitary-adrenal activity (Kawakami

et al, 1972) and chronic mild stress in the rat is also reported to

result in a phase shift of the circadian rhythm in CRF content

towards an earlier time (Sakakura, 1974; Brodish, 1974). Whether

these effects are due to corticosteroid feedback per se, or to a re-

setting of the circadian rhythm in the hypothalamus is unclear.

However, in the reverse situation, that is, the effect of circadian

rhythmicity on the stress response, it is clear that although there

is a small effect of "basal" plasma corticosteroids at the time of

stress, stress responses are largely determined by the "neural

sensitivity" of the animal at the time in the 24 hour cycle at which

stress is applied, a finding which also applies to studies of feedback

inhibition of the stress response (Smelik, 1963; Hodges and Jones,

1963; Hodges and Sadow, 1967; Dallman et al, 1972; Dallman and

Jones, 1973; Sato et al, 1975). Thus the plasma corticosterone

response of the intact animal to injection of saline (Haus, 1964),

handling (Ader and Friedman, 1968), ether anaesthesia (Dunn and

Carrillo, 1978) or laparotomy (Engeland et al, 1977) is greater in

the morning than the evening, a time when adrenal sensitivity to ACTH

is low (Haus, 1964; Ottenweller et al, 1978; Dallman et al, 1978).

Similarly, the plasma ACTH response to ether (Yasuda et al, 1976),

histamine or laparotomy (Engeland et al, 1977) is greater in the

morning, when pituitary ACTH content is low (Retiene et al, 1968;

Petiene and Schulz, 1970), and responsiveness to CRF is low (Jones
et al, 1979b). Increases in CRF content to ether with laparotomy
stress is also greater in the morning than in the evening (Hiroshige
et al, 1969; Takebe and Sakakura, 1972), when CRF content is lowest
but release in vitro in response to serontonin is greatest (Dallman
et al, 1977a). Adrenalectomy, thus removing the fast feedback
component of the stress response (Dallman and Jones, 1973; Yates
and Maran, 1974; Jones et al, 1974) has no effect on the greater
response in plasma ACTH to histamine or laparotomy in the morning
than in the evening, but the response to the milder stress of saline
injection which is greater in the evening in the intact animal,
becomes greater in the morning in the absence of corticosteroid feed-
back (Engeland et al, 1977). The response in hypothalamic CRF
content to ether with laparotomy also remains greater in the morning
than in the evening in the absence of corticosteroid feedback (Takebe
and Sakakura, 1972). Conversely, ether (Gibbs, 1970; Dunn and
Carrillo, 1972) or histamine (Engeland et al, 1977) stress which are
most effective in the morning in the intact animal, elicit a greater
response in the afternoon in animals treated with dexamethasone.
Thus, in the absence of corticosteroid feedback the response to stress
is greatest in the morning, and in its presence this is "phase-delayed"
to the afternoon. Circadian rhythmicity in the brain-pituitary-
adrenal axis therefore appears to result in an increased responsiveness
to stress in the morning whilst hypothalamic CRF content is low, but
ability to respond to neurotransmitters is greatest.

Apart from the above-mentioned interrelation between stress
and circadian activity in the H-P-A axis, negative feedback and
circadian periodicity under basal conditions appear to be interrelated.
The sensitivity of circadian rhythmicity to corticosteroids both

administered exogenously or raised by stress has already been outlined and suggests that corticosteroids may have a role in determining the timing of the circadian peak in hypothalamo-pituitary function and its amplitude. Negative feedback, in principle, would be expected to decrease the sensitivity of the H-P-A axis to the influence of an oscillator (e.g. the hypothalamus), but it also permits oscillations to be transmitted upstream from the oscillator (e.g. the limbic system), because the feedback signal is negative (Yates, 1974), and the gain, or sensitivity, of the axis to be varied (Yates, 1967) (Fig. 3). How corticosteroid feedback would affect the phase relations of circadian rhythms in the H-P-A axis has not been considered from the systems analysis approach (Yates, 1974; Yates and Maran, 1974), but adrenalectomy results in a shift of the circadian peak of plasma ACTH (Cheifetz et al, 1968) and of hypo-thalamic CRF content (Hiroshige and Sakakure, 1971; Takebe and Sakakura, 1972) to an earlier time. In man, like the rat, circadian rhythmicity in plasma ACTH, and presumably CRF, persists in the absence of steroid feedback in patients with Addison's disease, but there does not appear to be a phase shift in the plasma ACTH peak (Krieger, 1975b).

"Short-loop" feedback of ACTH (Fig. 3) (Martini et al, 1968; Hodges et al, 1968; Motta et al, 1969; Seiden and Brodish, 1971; Penn and Sadow, 1972; Takebe et al, 1974; Jones et al, 1976) like corticosteroid negative feedback, appears to have no role in CRF periodicity per se, since a diurnal rhythm of hypothalamic CRF content persists after hypophysectomy; but also like corticosteroid feedback, removal of ACTH feedback from the pituitary gland, as distinct from ACTH in the brain (Krieger et al, 1977b; Watson et al, 1978), moves the circadian peak of CRF content to an earlier time (Seiden and Brodish, 1972; Takebe et al, 1972).

In addition to the corticosteroid "long" feedback loop and

ACTH "short" feedback loop, there is also an interaction between gonadal steroids and hypothalamo-pituitary-adrenal circadian rhythmicity (Yates, 1974; Hiroshige and Wada, 1974). Orchectomy in the rat results in increased release of ACTH from the pituitary in vitro when CRF is added, whereas ovariectomy has the opposite effect - that is, oestradiol appears to increase release of ACTH in response to CRF, and testosterone to decrease release of ACTH. Gonadectomy in both males and females has no effect on hypothalamic CRF content in the rat, but in the hamster results in reduced CRF content (Coyne and Kitay, 1969; Gaskin and Kitay, 1971). In the female rat, the afternoon rise in plasma corticosteroids is less gradual and greater than that in the male, but in both sexes peak corticosteroid levels are attained at the same time (4 p.m. in a 6 a.m. to 6 p.m. light schedule). The rhythmicity in hypothalamic CRF content, however, is markedly different in the female from that in the male rat. CRF content in the female is greatest in the morning and least in the evening, CRF content falling sharply as plasma corticosteroid levels rise (Hiroshige et al, 1973). In addition, as the rat goes through its 4 day oestrus cycle starting from the day of pro-oestrus, the elevated CRF content in the morning decreases and the low CRF content in the evening increases, so that by the day of late-dioestrus morning and evening hypothalamic CRF content is no longer different (Hiroshige and Wada-Okada, 1973). Ovariectomy results in a movement of the peak of hypothalamic CRF content from 8 a.m. to noon, that is, towards the time of the peak CRF content in males (Hiroshige et al, 1973). Further, the rhythm in CRF content in neonates, when it appears in days 21 to 28 of life, is similar in both males and females, that is, CRF content is greater in the evening and neonates appear to begin their CRF rhythm with a male pattern of CRF content rhythmicity (Hiroshige and Wada, 1974). How these findings relate to the release

of CRF and ACTH and the overall regulation of the hypothalamo-pituitary-
adrenal axis in the female is unclear, and can only be guessed at from
measurements of CRF content rather than of CRF release.

c)   Circadian Rhythmicity in the Central Nervous System in relation
        to Corticotrophin Releasing Factor

        Circadian rhythms in hypothalamic CRF content have been
extensively investigated in the rat under a variety of experimental
conditions - in the intact animal (Ungar, 1967; Hiroshige et al, 1969;
David-Nelson and Brodish, 1969; Yasuda and Greer, 1976b; Chiappa and
Fink, 1977; Ixart et al, 1977), adrenalectomized animal (Cheifetz et
al, 1969; Hiroshige and Sakakura, 1971; Takebe et al, 1972), hypophy-
sectomized animal (Takebe et al, 1972; Seiden and Brodish, 1972),
neonatal animal (Hiroshige and Sato, 1970), and female animals (Retiene
and Schulz, 1970; Hiroshige et al, 1973), the amplitude of the rhythm
and its phase relation of the lighting schedule varying according to
the experimental animal (viz. previous section), and also according
to the laboratory.   A circadian rhythm in CRF content is also detect-
able in the pigeon hypothalamus, the peak CRF content occurring at
6 a.m. as the pigeon is active diurnally (Sato and George, 1973).   A
fluctuation in CRF secretion from the rat hypothalamus in vitro in
response to serotonin is also evident, the peak secretory response
being reported to be phase reversed from the peak CRF content
(Dallman et al, 1977b; Jones et al, 1979b).   To date, no reports of
CRF secretion at serial intervals in the 24 hour, circadian cycle have
been made.   Further, since Dallman et al (1977b) and Jones et al
(1979b) studied CRF secretion at only two time points, it is possible
that their data may represent "phase-shifted" hypothalamo-pituitary-
adrenal rhythmicity, especially since CRF secretion was found to be
greatest in the morning in both intact and adrenalectomized animals.

Evidence from lesion studies suggests the circadian rhythm in hypothalamo-pituitary-adrenal activity is not inherent to the hypothalamus, but that the hypothalamus may behave as a circadian oscillator entrained by a "Zeitgeber", or "time-giver" in the form of photic neural input (Fig. 3), possibly from the retinohypothalamic tract (Menaker et al, 1978). Isolation of the mediobasal hypothalamus results in abolition of circadian rhythmicity in plasma corticosteroid levels in rats (Halasz et al, 1967a; Palka et al, 1969; Allen et al, 1972), and in rhesus monkeys (Krey et al, 1975), in the rat frontal deafferentation (Halasz et al, 1967a; Lengvari and Liposits, 1977) or lesions (Slusher, 1964; Moore and Eichler, 1972) being effective in this respect. Two afferent pathways entering the mediobasal hypothalamus anteriorly have been implicated in hypothalamo-pituitary-adrenal circadian rhythmicity, the medial corticohypothalamic tract, originating from the hippocampus and passing adjacent to the suprachiasmatic nuclei to the arcuate nucleus and preoptic nucleus, and a projection from the suprachiasmatic nucleus dorsally and caudally through the periventricular area and retrochiasmatic region to the arcuate nucleus and median eminence (Szentagothai et al, 1968; Moore, 1979).

Of the neurotransmitters, serotonin, acetylcholine, dopamine, and noradrenaline have been the most extensively investigated in relation to control of CRF secretion (Wurtman, 1970; Hiroshige and Abe, 1973; Krieger, 1973a; Jones et al, 1976). Acetylcholine concentrations in whole brain undergo a circadian fluctuation with a peak 2 to 6 hours after the onset of light and a trough 2 to 6 hours after the onset of darkness (Hanin et al, 1970; Moroji et al, 1973). Similarly, serotonin concentrations demonstrate a circadian rhythm in the amygdala, hippocampus, midbrain, and hypothalamus (Scapagnini et al, 1971; Vermes et al, 1974; Simon and George, 1975). Peak

serotonin concentrations have been found to occur at midday (Moroji et al, 1973; Vermes et al, 1974; Simon and George, 1975), or in the evening (Scapagnini et al, 1971), in the hypothalamus, hippocampus, and amygdala. In contrast, peak levels of noradrenaline in the caudate nuclei, midbrain, and hypothalamus are found in the dark period (Friedman and Walker, 1968; Moroji et al, 1973), but also have been found during the daylight period in the striatum and pons (Simon and George, 1975). Circadian rhythmicity in dopamine concentrations is found in the hypothalamus, amygdala, septum, striatum, midbrain and pons - in each of these regions a peak occurs at midday and a secondary peak is also seen at midnight, except in the hypothalamus where only a midday peak is found (Simon and George, 1975).

That circadian rhythms in brain neurotransmitter agents are causally related to rhythmicity in hypothalamo-pituitary-adrenal rhythmicity has not been proven. However, systemic injection of atropine at 6 p.m. in the cat abolishes the circadian rise in plasma 17-hydroxycorticosteroids between midnight and 4 a.m., injections 16 hours before, at 8 a.m., being ineffective (Krieger et al, 1968) - these doses of atropine were without effect on the response to stress, and imply a cholinergic link in the afferent pathway controlling circadian release of CRF. Although, in vivo, cholinergic agonists appear to have little effect on plasma corticosterone levels (Makara and Stark, 1976), in vitro acetylcholine readily stimulates CRF release (Bradbury et al, 1974; Jones et al, 1976). Further, intra-venous or intraperitoneal injection of 3-(2-aminobutyl)-indole acetate (a monoamine oxidase inhibitor, which in the cat selectively increases serotonin, but not noradrenaline levels), parachloroamphetamine (which depletes central nervous system serotonin levels), 2'-(3-dimethylamino-propylthio)-cinnamanilide (a competitive inhibitor of serotonin at the

receptor), cyproheptadine (a serotonin antagonist) and para-chloro-
phenylalanine (which depletes central nervous system serotonin levels)
all abolish the circadian rise in plasma corticosterone levels
(Krieger and Rizzo, 1969; Scapagnini et al, 1971), increases in
serotonin levels apparently being as effective as decreases.
Serotonin injected intraventricularly causes an increase in plasma
corticosterone levels (Abe and Hiroshige, 1974); serotonin also
stimulates the release of CRF from the hypothalamus in vitro (Jones
et al, 1976), an effect which is blocked by cyproheptadine (Buckingham
and Hodges, 1978; Jones et al, 1979a). Thus, a serotonergic
afferent pathway is implicated in hypothalamo-pituitary-
adrenal circadian rhythmicity. Reserpine (which depletes central
monoamines), 6-hydroxydopamine (which lesions catecholaminergic
neurons and depletes catecholamines), α-methyl-para-tyrosine (which
inhibits dopamine and noradrenaline biosynthesis), and methamphetamine
(which depletes central noradrenaline) do not abolish the circadian
rhythm in plasma corticosteroids either in the cat (Krieger and Rizzo,
1969) or in the rat (Abe and Hiroshige, 1974; Asano and Moroji, 1974;
Kaplanski et al, 1974; Thomas and Sadow, 1975b). Reserpine does not
affect the circadian fluctuation of hypothalamic CRF content (Abe and
Hiroshige, 1974) nor the basal release of CRF from the hypothalamus
in vitro at 13.30 hr (Thomas and Sadow, 1975b), whilst in both
instances the response to stress is affected. Similarly, intravent-
ricular 6-hydroxydopamine does not abolish the circadian rhythm of
hypothalamic CRF content (Abe and Hiroshige, 1974). Dopamine and
noradrenaline therefore seem less likely to be involved in the
regulation of H-P-A circadian rhythmicity.

Circadian rhythms in pineal indole metabolism have been
implicated in the regulation of H-P-A circadian rhythmicity (Martini,

1974; Jones et al, 1976). There is also a circadian rhythm in pineal arginine vasotocin in the rat (Calb et al, 1977). However, the evidence suggests that neither pineal serotonin, melatonin, or arginine vasotocin are involved in H-P-A circadian rhythms in the rat. Firstly, melatonin in the rat (Jones et al, 1976) and arginine vasotocin in the cat (Pavel et al, 1977) inhibit the secretion of CRF, yet peak levels of melatonin (Klein, 1979) and vasotocin (Calb et al, 1977) are found at night. Similarly, serotonin stimulates the secretion of CRF (Jones et al, 1976; Buckingham and Hodges, 1977b) yet is at its lowest level in the pineal at night (Snyder et al, 1967). Secondly, pinealectomy does not abolish the circadian rhythm either in plasma corticosterone levels (Vernes et al, 1974; Takahashi et al, 1976), or in food intake (Takahashi et al, 1976) in the rat.

In the experiments described here, the release of CRF from the hypothalamus in vitro, devoid of its neural and humoral connections with the brain and pituitary, was studied during incubation for varying lengths of time, and at different times of the day, immediately after removal from the animal. The release of CRF when studied in this manner is sensitive to alterations in corticosteroid feedback and neurotransmitter levels and to stress applied to the animal prior to sacrifice (Jones et al, 1976; Thomas, 1977; Buckingham, 1979). Circadian rhythmicity in the afferent connections to the mediobasal hypothalamus is suggested from the effects of isolation of the mediobasal hypothalamus on rhythmicity in peripheral plasma corticosteroid levels (Halasz et al, 1967a; Palka et al, 1969; Allen et al, 1972; Krey et al, 1975). That such rhythmicity is mediated by rhythmicity in CRF release has not yet been shown; still less that CRF release is sensitive to a circadian rhythm in its afferent

input, the only study on this matter being that of Jones et al (1979b)

at 8 a.m. and 8 p.m. which does not give any information about the

phase relations of CRF release with respect to pituitary and adrenal

circadian rhythms. In addition, CRF release was studied under

conditions of reversed lighting in order to establish whether any

rhythmicity observed is dependent on light-related cues in the environ-

ment. The circadian rhythm in plasma corticosterone levels in the

mouse, and in the rat, is reversed when the light cycle is phase-

reversed (Haus, 1964; Morimoto et al, 1977; Krieger and Hauser, 1978),

but this has not been shown for CRF release.

METHODS

1)   Animals

Male Wistar rats, weighing 100 to 125 g were obtained from
Bantin and Kingman Ltd, Grimston, North Humberside.   Prior to delivery
to the laboratory in Leicester, the animals were kept in natural day-
light, and were handled during weighing and sexing procedures before
weaning at 20 to 21 days of age;   the latter procedures were kept as
uniform as possible since handling at an early age influences the
development of circadian rhythms in adrenocortical activity and
adrenal stress responsiveness in mature rats (Ader, 1969;   Sieck and
Ramaley, 1975).

On delivery to Leicester, the animals were housed two per cage.
This number of animals was chosen in order to prevent the activation
of the hypothalamo-pituitary-adrenal (H-P-A) axis found in animals
caged more than 2 per cage (Grant and Chance, 1958;   Barrett and
Stockham, 1963) which is caused by the establishment of a rank order
(Grant and Chance, 1958).   Animals caged in pairs do not establish a
rank order (Grant and Chance, 1958) and have lower plasma corticosterone
levels than animals housed singly (Clark and Nowell, 1978).   All the
cages were solid-sided, preventing animals from seeing each other, but
translucent, allowing the passage of light.   Following caging in
pairs, the animals were handled daily, at approximately 16.30 hr each
day, for two weeks until 24 hours before sacrifice (at which time the
animals weighed 180 to 220 g).   Food (Diet 41B;   Lane-Petter and Dyer,
1952) and water was available ad libitum, and was checked daily.   The
animals were kept on stainless steel grids, and the soil trays were
cleaned out 3 times per week in order to minimise activation of the
H-P-A axis caused by ammonia from stale urine (Clough and Gamble, 1976).

During the course of this study, the rats were housed in two
different animal laboratories, the second laboratory being used after

the completion of the Medical Sciences Building, University of Leicester, in December 1977. The caging and the regime established for the animals in the second laboratory was the same as that in the first laboratory. A control experiment was performed to check that the reactivity of the tissues from animals kept in the second location was similar to that in the first. The standard output of pituitary-adrenal hormones monitored from animals kept in the two locations was found to be the same (see Appendix A). In both Animal Houses the animals were kept in sound-insulated rooms with a background "white noise" of 40 dB supplied by the ventilation system, sound levels occasionally fluctuating between 40 dB and 60 dB (Barrett and Stockham, 1963; Guha et al, 1976). Room temperatures were maintained at $21 \pm 1^{\circ}C$: when the temperatures fell below $20^{\circ}C$ or rose above $26.7^{\circ}C$ due to failures in the heating or ventilation systems, the experiments were postponed until normal temperatures were established for at least 24 hours. The relative humidity of the rooms was set at 45%. The rooms were illuminated by artificial-fluorescent lighting and the light/dark cycle was set at 07.20 hr "lights on" and 19.20 hr "lights off", that is, the light period extended between 7.20 a.m. and 7.20 p.m. (see also Reversed Light Cycle Experiments, below). The overall experimental conditions in both laboratories were therefore such as to minimise, in every way possible, activation of the H-P-A axis by extraneous environmental factors.

The animals were not disturbed for at least 17 hours prior to any experiment (Barrett and Stockham, 1963). On the day of the experiment, at the appropriate time (see below), one animal from a pair was removed from each cage.

This procedure was adopted for obtaining, in separate circumstances, both hypothalamic and pituitary tissue. The rats were de-

capitated with tailors' scissors within 7 seconds of removal from

their cage, since animals removed from their cages for a period as

short as 5 seconds show increased adrenocortical activity 15 minutes

later (Ader and Friedman, 1968). The required tissue, either

pituitary or hypothalamus, was then removed rapidly and placed in

Krebs-Ringer bicarbonate glucose solution (KRBG) (see below). Both

of the adrenal donor animals from each cage were removed consecutively

from their cage and swiftly decapitated; this was done since the

time lapse between the removal and decapitation of the first and

second animals from a cage is not sufficient to result in increased

adrenocortical activity in the second animal (Ader and Friedman, 1968;
Phillips and Poolsanguan, 1978).
Both of the adrenal glands from each animal were removed by the dorsal

route, and placed in order on filter paper moistened with KRBG. The

glands were then rapidly cleaned of fat and connective tissue on a

dry white tile, quartered, the quarters randomized, weighed, and

placed in KRBG (see Adrenal Tissues in Assay of CRF, below). Pre-

cautions were taken to minimise noise throughout the experimental

procedure, to prevent arousal and activation of the H-P-A axis of the

remaining animals in the experimental group since animals decapitated

later in a sequence show greater adrenocortical activity than animals

decapitated earlier (Ader and Friedman, 1968).

When animals were decapitated for hypothalamic tissue or blood

samples in the dark period of the light/dark cycle (see Circadian

Rhythm Experiments and Reversed Light Cycle Experiments, below) either

(i) a covered lamp was arranged in a corner of the animal room so that

it permitted illumination only of the area at which the animals were

decapitated; this lamp was arranged at least 24 hours before any

experiment was performed; or (ii) the animals were decapitated in

darkness. In either case, the lights in the room adjacent to the

animal room were switched off before entry into the animal room.

Following decapitation, the heads of the animals were taken to the adjacent room in less than 10 seconds, the lights switched on again, and the hypothalamic tissues removed and placed in KRBG (see next section). The duration of the decapitation procedure in the dark period was no longer than in the light period, and the time between decapitation and removal of hypothalamic tissues was still less than two minutes (see Removal of Hypothalamic Tissue, below). In all other respects the procedures adopted for obtaining hypothalamic tissues and blood samples in the dark period, and the incubation procedures (see Incubation of Hypothalami, below) performed in the adjacent preparation room, were identical with the procedures used in the light period.

2)   Removal of Hypothalamic Tissue

A minimum of two and a maximum of three animals were decapitated and their hypothalami incubated or extracted for CRF in any single experiment. This number of animals was chosen for the following reasons:-

(i)   In order to ensure the validity of the data obtained such that by assaying the pooled CRF release or content of hypothalami obtained from two or more animals, interanimal variation, especially the possibility of asynchronous episodic fluctuations in CRF release or content in individual animals, is eliminated.

(ii)   In order to avoid "alerting" and activating the H-P-A axis of animals used for hypothalamic tissues, despite the animals being decapitated with the minimum of noise within 7 seconds and precautions being taken to contain the smell of blood by the use of absorbent towels. Animals decapitated later in a sequence have higher plasma corticosterone levels than animals

sacrificed earlier in a sequence (Ader and Friedman, 1968).

The number of animals used in any single collection of hypo-

thalamic tissues was therefore kept to a ~~minimum~~ max of three, or

where necessary, to two groups of two or three animals (see

below).

(iii) In order to minimise the time between/death the animal's and commencement

of incubation or homogenization of the tissues to under two

minutes, and thereby optimize the viability of the tissues.

Following decapitation the skull was cut open, the brain

reflected, and the whole of the mediobasal hypothalamus, extending

from the anterior border of the optic chiasm to the mamillary bodies,

was carefully removed with an angled knife.   Each hypothalamus was

then placed individually in a pyrex tube containing KRBG for incubation,

or appropriately, 0.1 N HCl for extraction of CRF, and the time

recorded (see Incubation of Hypothalami, below).

In early experiments carried out in the laboratory in order to

investigate the dose-response characteristics of CRF released from

the median eminence the block of tissue removed included only the

median eminence and parts of the adjacent arcuate nucleus (see Assay

of CRF in Results; Thomas and Sadow, 1975a; Thomas, 1977) and weighed

approximately 15 mg.   Data obtained using this block of tissue are

presented only in Fig. 6 and Fig. 7 of the results.   Otherwise, all

the experiments used a larger block of hypothalamic tissue which

included most of the mediobasal hypothalamus.

The extent of the block of tissue was established by the

following methods:-

(i)   By the examination of parasagittal, haematoxylin-eosin stained

sections of tissue (prepared by Dr P Thomas; Thomas, 1977).

The extent of the block of tissue was determined by the

FIGURE 4

Parasagittal section of the basal hypothalamus of the rat (De Groot, 1959). The line X————X indicates the extent of the hypothalamic area excised for incubation.

DBB: Diagonal Band of Broca

POA: Preoptic Area

CO: Optic Chiasm

SC: Suprachiasmatic Nucleus

PVH: Paraventricular Nucleus

AHA: Anterior Hypothalamic Area

VMH: Ventromedial Nucleus

DMH: Dorsomedial Nucleus

PH: Posterior Hypothalamic Nucleus

MM: Medial Mamillary Nucleus

MP: Posterior Mamillary Nucleus

ARH: Arcuate Nucleus

ME: Median Eminence

ADH: Anterior Hypophysis
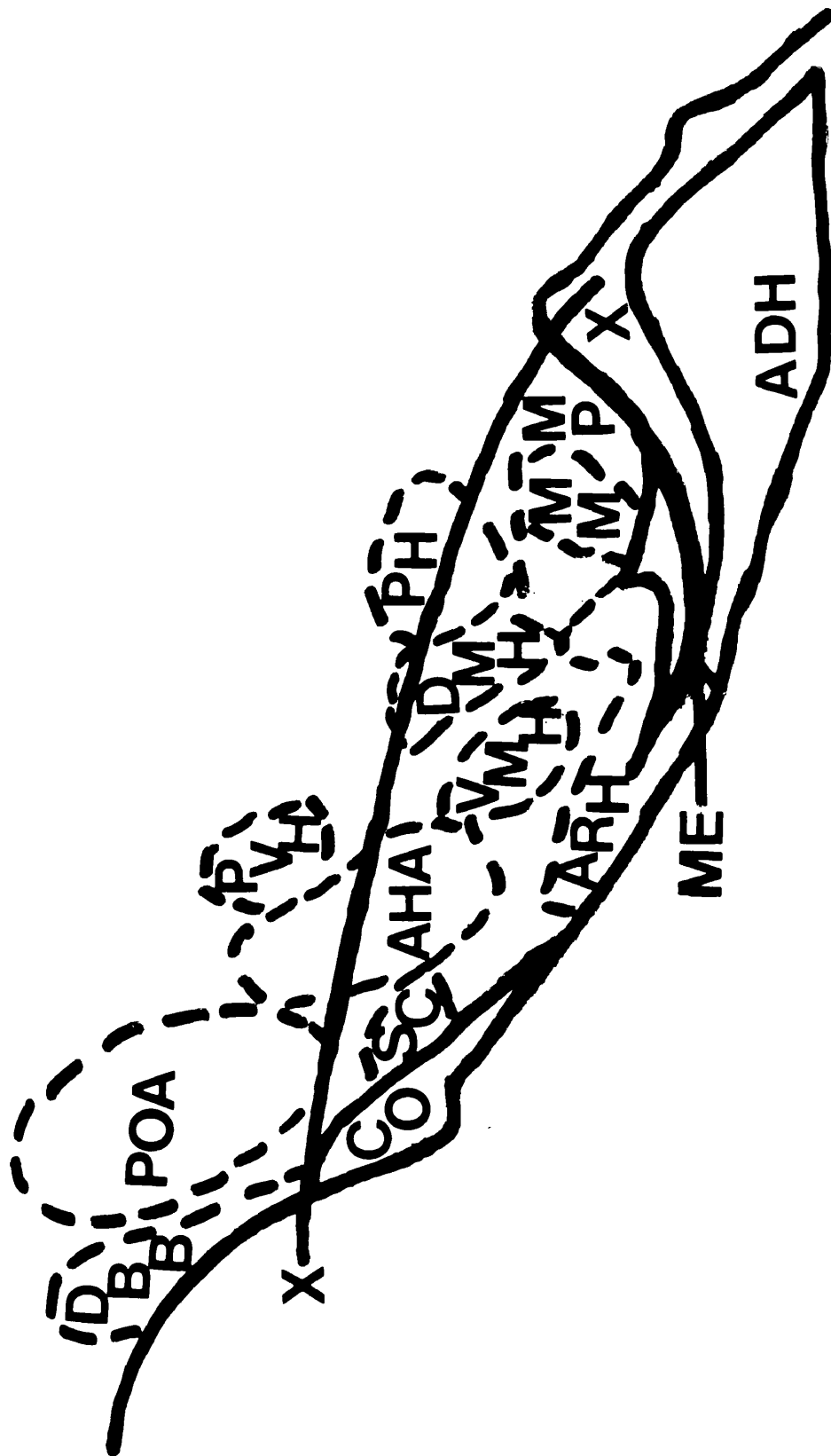
identification of hypothalamic nuclei under light miscroscopy.

(ii) By the measurement in situ of the block of tissue removed,

using a stainless steel probe attached to the electrode

carrier of a stereotaxic frame. The measurements were plotted

on a stereotaxic atlas (De Groot, 1959).

The extent of the tissue block determined by the two methods

agreed, and is shown in Fig. 4. The tissue included the optic

chiasm, suprachiasmatic nucleus, ventromedial nucleus, arcuate nucleus,

median eminence, dorsomedial nucleus, ventral posterior hypothalamic

nucleus, dorsal premamillary nucleus, and medial and posterior mamillary

nuclei. The block of tissue weighed 32.05 $\pm$ 2.92 mg (Mean $\pm$ standard

error of mean of 6 hypothalami). The results are expressed as the

amount of CRF released or extracted per hypothalamus, since the

amount of tissue removed included the whole of the CRF producing

elements of the mediobasal hypothalamus of each animal (see Introduction).

3) Circadian CRF Content Experiments - Extraction of CRF

Immediately following removal from the brain, at 07.00 hr, 13.30

hr, or 16.00 hr; groups of 3 hypothalami were ground in 4.5 ml 0.1 N

HCl at 4°C (AnalaR, Fisons) in a pyrex homogenizer. The homo-

genate was neutralized with 0.1 N NaOH (AnalaR, BDH) and then

centrifuged at low speed for 10 minutes. The supernatant was then

collected, the pH checked and adjusted to 7.0 with 1 N HCl or NaOH if

necessary, and stored at -20°C for assay within 7 days, usually 5 days

(see Storage of CRF in Methods and Results). CRF extracts appear

to be stable if stored at 4°C (Guillemin et al, 1957; Chan et al,

1969b) or at -20°C (Yasuda and Greer, 1976c; Gillies et al, 1978a)

for periods exceeding 7 days.

4)   Incubation of Hypothalami

In all hypothalamic incubations the following general procedure
was used:-

Immediately following removal from the brain, groups of 2 to 3
hypothalami (see Removal of Hypothalamic Tissue, above) were incubated
individually in pyrex tubes each containing 3 ml of Krebs-Ringer
bicarbonate glucose (KRBG) medium (see Appendix B) maintained at $37^{O}C$
in an incubation bath, and through which a saturated 95% O_2/5% CO_2 gas
mixture was bubbled continuously with polyethylene tubing.   At the
end of the specified incubation period the media from 2 to 6 hypo-
thalami (in some instances hypothalami were incubated in two groups
of two or three hypothalami, each group starting incubation within 5
minutes of the other, see below) were decanted and pooled.   The pooled
media were assayed for CRF immediately, or, if the incubation was
performed other than at the time of the CRF assays (13.30 hr - see
Assay of CRF, below) the pooled media were stored at $-20^{O}C$ for assay
within 7 days (see Storage of CRF in Methods and Results).   The times
at which the incubations were performed, their duration, and other
details are specified in the following sections.

5)   Time-Course-of-CRF-Release Experiments

13.30 hr

Immediately following removal from the animals at 13.30 hr,
groups of 2 to 3 hypothalami were incubated for one of the following
lengths of time: 0 minutes (instantaneous wash in 3 ml KRBG), 1
minute, 3 minutes, 5 minutes, 10 minutes, 15 minutes, 20 minutes, 25
minutes, 30 minutes, 45 minutes, 60 minutes, 90 minutes, or 120 minutes.
At the end of each respective incubation period, the media from 2 to 4
hypothalami (groups of 2 or 3 hypothalami incubated simultaneously, or

2 groups of 2 hypothalami incubated consecutively within 5 minutes of each other) were decanted and pooled. Each pooled incubate was stored at $-20^{\circ}C$ and assayed for CRF within 7 days (see Storage of CRF in Methods and Results).

16.00 hr

Immediately following removal from the animals at 16.00 hr, groups of 3 hypothalami were incubated for one of the following lengths of time: 2 minutes, 5 minutes, 15 minutes, 20 minutes, 25 minutes, or 30 minutes. At the end of each respective incubation period, the media from 3 hypothalami were decanted and pooled. Each pooled incubate was stored at $-20^{\circ}C$ and assayed for CRF within 7 days (see Storage of CRF in Methods and Results).

In an additional experiment, groups of 3 hypothalami obtained at 16.00 hr were incubated for 5 minutes and the media then decanted and discarded. The media were then replaced with a further 3 ml of KRBG and the hypothalami incubated for a further 15 minutes. At the end of the latter 15 minute incubation period, that is, an incubation lasting between minutes 5 to 20 of the total incubation time of the hypothalami, the media from 6 hypothalami (2 groups of 3 hypothalami incubated consecutively within 5 minutes of each other) were decanted and pooled. The pooled incubate was stored at $-20^{\circ}C$ and assayed for CRF within 7 days (see Storage of CRF in Methods and Results).

6)   Incubation 1 and Incubation 2 Incubation Procedure

Apart from the Time-Course-of-CRF-Release Experiments described above, in all other experiments hypothalamic incubations were performed according to the following procedure (Fig. 5):-

From the foregoing Time-Course-of-CRF-Release Experiments it was evident that there was a clear-cut difference between the CRF output

from the hypothalamus during the first 15 minutes (0 to 15 min) and the second 15 minutes (15 to 30 min) of incubation (see Time Course of CRF Release from the Hypothalamus In Vitro in Results); therefore, these two periods of incubation were used for further studies of the release of CRF from the rat hypothalamus in vitro.

Incubation 1 - First 15 Minute Incubation

Immediately following removal from the animals at the times and under the conditions specified in the following sections, groups of 2 to 3 hypothalami were incubated for 15 minutes, that is, an incubation lasting between minutes 0 and 15 of the total incubation time of the hypothalami. At the end of this period, the media from 2 to 3 hypothalami were decanted and pooled. The pooled media were assayed for CRF immediately, or, if the incubation was performed other than at the time of the CRF assays (13.30 hr - see Assay of CRF in Methods) the pooled media were stored at $-20^{\circ}C$ for assay within 7 days (see Storage of CRF in Methods and Results).

Incubation 2 - Second 15 Minute Incubation

Immediately following Incubation 1, the incubation media of the groups of 2 to 3 hypothalami were replaced with a further 3 ml KRBG, and a second 15 minute incubation performed, starting within 90 seconds of the end of Incubation 1. At the end of this incubation period, that is, an incubation lasting between minutes 15 and 30 of the total incubation time of the hypothalami, the media from 2 to 3 hypothalami were decanted and pooled. The media were assayed for CRF immediately, or, if the incubation was performed other than at the time of the CRF assays (13.30 hr - see Assay of CRF in Methods) the pooled media were stored at $-20^{\circ}C$ for assay within 7 days (see Storage of CRF in Methods and Results).

7)   Circadian Rhythm Experiments

In these experiments CRF release in Incubation 1 and in

Incubation 2 (see Incubation 1 and Incubation 2 Incubation Procedure,

above) was investigated at different times of the day.   Groups of 2

to 3 hypothalami were removed from animals decapitated at 07.00,

08.00, 10.00, 13.30, 16.00, 17.30, 19.00 or 01.00 hr and the

"Incubation 1 and Incubation 2" protocol carried out.   The pooled

media obtained from Incubation 1 or Incubation 2 were stored at $-20^{\circ}C$

and assayed for CRF within 7 days (see Storage of CRF in Methods and

Results).   The data therefore represent the CRF released during two

consecutive 15 minute incubations, Incubation 1 and Incubation 2, at

the times indicated in the 24 hour light/dark cycle.

Samples of trunk blood for determination of plasma corticosterone

(see Measurement of Corticosterone, below) were also collected from six differ-

ent animals at each of the following times: 07.00, 08.00, 10.00, 12.00,

14.00, 16.00, 18.00, 19.00, 20.00, 22.00, 24.00, 01.00, and 04.00 hr.

8)   Reversed Light Cycle Experiments

Animals previously kept for two weeks under a lighting regime of

daylight between 07.20 and 19.20 hr (see Animals, above) were kept for

7 to 10 days on a reversed lighting schedule, that is, the light/dark

cycle was altered in phase by 12 hours.   Animals were kept under the

conditions described under Animals, above, with the exceptions that

they were kept in similar conditions to, but in a separate room from,

the assay animals, and that they were not handled but were disturbed

for changing of soil trays, feeding, and watering at the usual time

in the afternoon (approximately 16.30 hr;   see Animals, above).

Cleaning and feeding was done as quietly as possible and under conditions

of minimal lighting, using light from an adjacent room.   Environmental

cues were therefore reduced to a minimum, except those of the reversed

lighting regime.

7 to 10 days was chosen as the appropriate time to allow any

changes in the H-P-A axis to occur: Since Haus (1964) and Krieger

and Hauser (1978) found that the circadian pattern of plasma corti-

costerone levels inverts within two weeks after reversal of the light

cycle, whilst Morimoto et al (1977) found that the circadian patterns

of plasma corticosterone levels and of feeding invert on the third day

after reversal of the light cycle.

Between 7 and 10 days of reversed lighting, groups of 3 hypo-

thalami were removed from animals decapitated at 07.00 hr or 19.00 hr,

just before "lights off" or just before "lights on" respectively, and

the protocol for Incubation 1 and Incubation 2 was carried out (see

Incubation 1 and Incubation 2 Incubation Procedure and Circadian

Rhythm Experiments, above).   The pooled media obtained from Incubation

1 or Incubation 2 were stored at $-20^{\circ}C$, and assayed for CRF within 7

days (see Storage of CRF in Methods and Results).

In addition, 7 to 10 days after reversal of the light cycle,

samples of trunk blood for determination of plasma corticosterone (see

Measurement of Corticosterone, below) were collected from six animals

at each of the following times: 07.00, 08.00, 10.00, 12.00, 14.00,

16.00, 18.00, 20.00, 22.00, 24.00, and 04.00 hr.

9)   Calcium Free Incubation Medium Experiments

In order to determine whether CRF release is dependent on the

presence of Ca^{2+} ions in the incubation medium, hypothalami were

incubated in media in which Ca^{2+} was removed and replaced by Mg^{2+}.

Normal KRBG consisted of Ca^{2+} 2.51 mM and Mg^{2+} 1.18 mM (see Appendix

B); calcium free media consisted of Ca^{2+} 0mM and Mg^{2+} 3.69 mM (see

Appendix B).

Groups of 3 hypothalami obtained from animals decapitated at

13.30 hr were incubated either in calcium free KRBG or in normal KRBG

for two consecutive 15 minute incubations, that is, Incubation 1 and

Incubation 2 (see Incubation 1 and Incubation 2 Incubation Procedure,

above). When hypothalami were incubated for collection of CRF in

Incubation 2 in calcium free media, the prior 15 minute incubation,

Incubation 1, was also carried out in calcium free media. The pooled

media obtained from Incubation 1 or Incubation 2 were either assayed

for CRF immediately, or stored at $-20^{\circ}C$ and assayed for CRF within 7

days (see Storage of CRF in Methods and Results). The calcium

concentration of the pituitary incubation medium in the CRF assay

(Incubation 3, Fig. 5) was adjusted to 2.51 mM with calcium elevated

KRBG (see Appendix B) when calcium free hypothalamic incubation media

were added in the assay procedure.

10)   Assay of CRF

CRF was assayed by a triple in vitro technique which measures

the output of ACTH from paired hemisected pituitary glands in vitro

when aliquots of hypothalamic incubation media or extracts containing

CRF are added (Thomas and Sadow, 1975a - Fig. 5). All CRF assays

were performed at 13.30 hr in order to avoid any artefacts in the

results due to circadian rhythmicity in the ability of pituitary and

adrenal glands to release ACTH and corticosterone, or in their

sensitivity to CRF and ACTH respectively (see Introduction).

CRF Samples

Two to three animals were decapitated at 13.30 hr and their

FIGURE 5

Schematic diagram of the CRF bioassay.

HA   =   Hypothalamic Area

KRBG   =   Krebs-Ringer Bicarbonate Glucose Solution

MEO   =   Median Eminence Output

T   =   Test dose of CRF measured in Median Eminence
            Output (MEO)

C   =   Controls, unstimulated pituitary halves

(HA) in KRGB

INCUBATION 1 | 15min.37°C

FRESH KRBG

INCUBATION 2 | 15min.

POOLED
INCUBATES

ALIQUOT (MEO FRACTION)

½T   T   C

INCUBATION 3   1 hour

DITTO   DITTO

ALIQUOT

INCUBATION 4   INCUBATION 5

PREINCUBATED   1 hour
ADRENAL
QUARTERS 30min.

CORTICOSTERONE   SPECTROFLUORIMETER
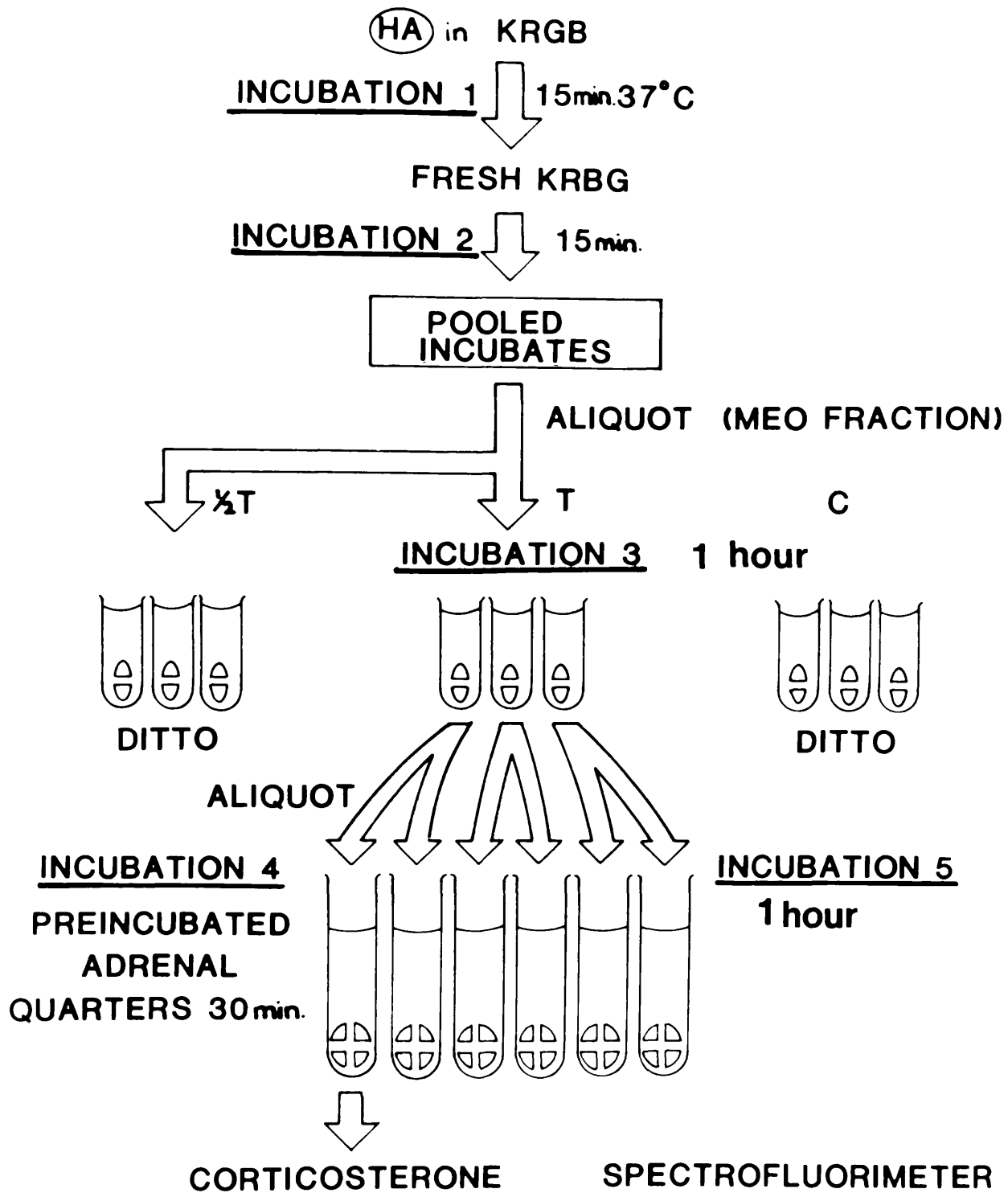
hypothalami removed (see Removal of Hypothalamic Tissue, above). The hypothalami were incubated for two consecutive 15 minute incubation periods, Incubation 1 and Incubation 2. (Fig. 5 - see also Incubation of Hypothalami and Incubation 1 and Incubation 2 Incubation Procedure, above). These samples, Incubation 1 and Incubation 2 at 13.30 hr, served as a standard CRF incubate in all assays. These incubation periods were chosen as standards because, from the Time-Course-of-CRF-Release Experiments (see Results) it was evident that there was a clear-cut difference between the CRF output from the hypothalamus during the first 15 minutes (0 to 15 min) and the second 15 minutes (15 to 30 min) of incubation; thereafter these two periods incubation were used for further studies of the release of CRF from the rat hypothalamus in vitro. The final choice of Incubation 2 at 13.30 hr as a standard CRF preparation in the calculation of relative potencies was also made because a log dose-response curve for this preparation had been established (Thomas, 1977), and the amount of CRF released in Incubation 2 was also found to be less variable than in Incubation 1 (Thomas, personal communication; see also Fig. 16 and Fig. 17 in Results); and because Incubation 1 or Incubation 2 were convenient standard CRF preparations which could be obtained with each assay at the time of the assay (see Expression of Data and Statistical Methods; and Assay of CRF in Results).

While the standard Incubation 1 and Incubation 2 incubations were in progress, a test sample of frozen CRF incubate or extract for assay which had been obtained in an experiment performed within 7 days beforehand (see experiments above and below; and Storage of CRF, below) was taken out of the deep-freeze and placed (in its pyrex container) in the incubation bath (maintained at $37^{\circ}C$) for defrosting and warming to $37^{\circ}C$ before addition to the pituitary tissue and its

media.

When test CRF incubation media for assay were obtained at 13.30 hr it was possible to assay the samples immediately (c.f. the shorter incubation periods in the Time Course of CRF Release Experiments at 13.30 hr, and Calcium Free Incubation Medium Experiments, above; CRF Release Experiments at 13.30 hr and Calcium Free Incubation Medium Experiments, above; Cerebral Cortex Experiments and Effects of Hypothalamic and Cerebral Cortex Incubation Media on the Stimulation of Corticosteroidogenesis in the CRF Assay, below) because the test incubation period ended before 14.00 hr the latest time at which the preparation of pituitary tissues for the assay could be started (see Pituitary Glands, below). In this case, the test incubations were carried out in lieu of the standard Incubation 1 and Incubation 2 procedure, and the stored samples of the standard Incubation 1 and Incubation 2 media, obtained at 13.30 hr in a previous assay (within 7 days; see Storage of CRF) were defrosted and warmed whilst the test incubations were in progress.

Doses of CRF incubate or extract were measured in fractions of the 3 ml medium in which each hypothalamus was incubated or extracted, that is, 1.00 (3 ml) to 0.0042 (0.0125 ml) of a Median Eminence Output, 1 Median Eminence Output (MEO) representing the total volume of medium in which each hypothalamus is prepared.

Pituitary Glands

While Incubation 2 (Fig. 5) was in progress and before 14.00 hr, 9 to 14 animals were removed for sacrifice from cages in a different battery from that used for hypothalamic donor animals (see Animals, above). The decapitated skull was cut open and the brain reflected exposing the pituitary gland in the sella turcica. The pituitary gland was removed with a fine hooked needle having broken the membranes

tethering it in situ. The pituitary glands were rinsed in KRBG, placed on filter paper moistened with KRBG, and hemisected. Pairs of hemipituitaries were randomized and re-paired so that each pair of hemipituitaries consisted of a hemipituitary tissue from two different animals. One such pair was allocated to a test group and the other pair to a control group in the assay (Fig. 5). Pituitary glands in control groups in the assay therefore served as true controls for pituitaries stimulated with test samples of CRF, since of each pair of paired hemipituitaries of the same origin, one was allocated to a test group and the other to a control group. The hemipituitaries were weighed and each pair placed in KRBG in a pyrex tube.

Various test amounts of hypothalamic incubation media or extracts for CRF assay were added to each of 6 to 10 tubes containing pituitary tissue (test groups), and the other tubes were used (1) for doses of the 13.30 hr Incubation 1 or Incubation 2 standard CRF preparation (control group) and (2) for non-stimulated ACTH release from the pituitary tissue (Controls in Fig. 5). The final volume of medium in each tube was made up to 3 ml with KRBG.

The pituitary tissue was then incubated for one hour, Incubation 3 (Fig. 5). The pituitary tissue was not subjected to a preincubation period prior to the 1 hour test incubation, Incubation 3, in order to avoid the possibility of release of ACTH due to any vasopressin possibly present in the hypothalamic incubation media or especially in the hypothalamic extracts. Pituitary glands which are not preincubated do not respond to vasopressin, whereas those that are preincubated for various periods of time do release ACTH in response to vasopressin (Fleischer and Vale, 1968; Sadow and Penn, 1972; Lutz-Bucher et al, 1977), although the reason for this is still the subject of discussion (see

Introduction and Discussion). At the end of Incubation 3 the pituitary tubes were taken from the incubation bath, and the pituitary halves rapidly removed from the media to prevent their being cooled when the tubes were placed in ice. Non-specific release of pituitary hormones has been shown to be associated with cooling of pituitary tissue (Hong and Poisner, 1974).

Adrenal Glands

During the incubation of the pituitary tissue, Incubation 3, between 14.10 and 14.30 hr, a further 9 to 14 rats were removed from a third battery of cages and swiftly decapitated (see Animals, above). The adrenal glands were rapidly removed by the dorsal approach using curved forceps and scissors, and placed on cooled petri dishes ($\sim 6^{\circ}$C) containing filter paper moistened with KRBG. The adrenals were then cleaned of fat and connective tissue and finally quartered. The adrenal quarters were then randomized in groups of 4 adrenals from 4 different animals (Saffran and Schally, 1955). This latter procedure reduces interanimal variation, and by allocating each group of 4 quartered adrenals to a different group in the assay controls are established, since the differences in the final steroid production of each one of the four groups of quartered adrenals may be attributed to differences in the amount of ACTH added because each group of four quartered adrenals consists of quarters of the same origin (Saffran and Schally, 1955). The groups of 4 adrenal quarters were weighed on a torsian balance to the nearest 0.05 mg and placed in 5 ml KRBG in pyrex tubes. The adrenal quarters were then preincubated for 30 minutes, Incubation 4 (Fig. 5), in order to further reduce inter-animal variation and improve the sensitivity of the tissues to ACTH (Saffran and Schally, 1955).

At the end of Incubation 4, the incubation media in the adrenal

tubes were then aspirated, replaced with 2 ml fresh KRBG, and 1 ml

aliquots of the pituitary incubation media, Incubation 3, containing

ACTH, were added to each tube such that the production of ACTH by

each pair of hemipituitaries was assayed in duplicate by two tubes

of adrenal quarters (Fig. 5). The adrenal quarters were then incubated

for 1 hour, Incubation 5 (Fig. 5), starting between 15.30 and 15.45 hr.

At the end of Incubation 5, 30 ml of chilled chloroform was added to

each tube and corticosterone extracted by shaking the tubes for 8

minutes. The adrenal quarters and incubation media were then aspirated

and the chloroform, containing the extracted corticosterone, was

analyzed for corticosterone either immediately, or stored overnight

at $4^{\circ}C$ for corticosterone analysis the next day. Corticosterone was

determined fluorimetrically by the method of Zenker and Bernstein (1958)

(see Measurement of Corticosterone). Blanks and corticosterone

standards (see Measurement of Corticosterone, below) were prepared for

each analysis and extracted at the same time as the adrenal incubation

media.

The raw data are expressed as the steroid production per 100 mg

of adrenal tissue per hour and estimates of the amount of CRF were

calculated by establishing the log dose-response relationship between

the logarithm of the dose of assayed CRF and the response measured as

the steroid production in the assay system (see Expression of Data and

Statistical Methods).

With 3 trained experimenters the nature of the assay permits

the use of a maximum of 14 paired hemipituitaries in each assay. This

is because, whilst the presence of more experimenters would permit more

pituitary glands to be prepared within the time limits essential to

ensure the viability of the tissues, the presence of three or more

experimenters working in the animal laboratory and the use of more

animals would also be more likely to alert the animal colony and cause nefarious results due to an increase in hypothalamo-pituitary-adrenal activity. As a consequence, the design of the assays was restricted to one in which a maximum of twelve pituitary tubes could be used for doses of CRF and two tubes used for control, unstimulated hemi-pituitaries; hence the difficulties in sophisticated statistical treatment. In practice, assays in which a maximum of 12 paired hemi-pituitaries were prepared proved more satisfactory than assays in which 14 paired hemipituitaries were prepared, both because more paired hemi-pituitaries were viable and responsive to CRF when fewer were prepared (owing to temporal limitations), but also because more satisfactory results were obtained with the adrenal tissues when 24 rather than 28 adrenal glands were used for assaying the output of ACTH from the hemi-pituitaries.

Thus in nearly all the assays, 12 paired hemipituitaries and 24 groups of quartered adrenals were prepared. This number of samples permitted, in theory, the use of a 2 by 2 bioassay design in which 2 doses of a test sample of CRF and 2 equally spaced doses of a standard sample of CRF could be assayed, each dose being assayed on 3 pairs of hemipituitaries and 6 groups of adrenal quarters, producing 6 responses for each dose. Again, in practice this proved unsatisfactory, firstly because in each assay sufficient satisfactory responses for each dose of test and standard CRF could not be obtained; and secondly because the inclusion of a control group of unstimulated hemipituitaries was essential for the assessment of the state of the animals prior to the assay (see precautions in above sections). The design of the assays was therefore restricted in most instances to one in which 6 paired hemipituitaries could be allocated to two doses of a test CRF preparation, 3 paired hemipituitaries allocated to a single dose of the standard CRF

preparation, and 3 paired hemipituitaries allocated to the control group of unstimulated hemipituitaries. With this design, data for each CRF preparation were selected from a number of assays for regression analysis (see Expression of Data and Statistical Methods, below). Although this means that some of the variance in the responses is due to inter-assay variance, it also means that the results are less likely to be influenced by variations in the sensitivity of the assay system.

11) Storage of CRF

Experiments were performed in order to determine whether hypothalamic incubation media may be stored at -20°C and then assayed on another occasion without loss of corticotrophin-releasing activity, in particular when the timing of hypothalamic incubations did not allow temporal coincidence with the fixed time of the assays (see Assay of CRF, above).

Groups of 3 hypothalami were incubated at 13.30 hr (see Incubation of Hypothalami, above), and the second fifteen minute incubation media (Incubation 2) decanted and pooled. The total volume, 9 ml, of pooled incubation medium was then divided into 4 samples in pyrex tubes that were stoppered or sealed with aluminium foil. Three of these samples were immediately stored in a freezer at -20°C.

Day 0: The sample that was not frozen was assayed for CRF immediately using a dose of 0.033 MEO.

Days 4, 6 and 7: On each of the days, 4, 6 and 7 following incubation and storage, one of the 3 samples stored at -20°C was removed from the freezer, defrosted in the incubation bath at 37°C with the stopper or aluminium seal left on, and assayed for CRF, again using doses of 0.033 MEO.

The results were then calculated and plotted as the response to

0.033 MEO of the standard incubation medium (Incubation 2 at 13.30 hr) stored at -20°C for 0, 4, 6, or 7 days (see Storage of CRF in Results).

In all instances where hypothalamic incubation media were stored for subsequent assay the same procedure was adopted. That is, the incubation media were pooled and divided into 4 samples, one fresh sample to be used in each assay. The samples, once defrosted before the assay were not refrozen, but discarded. In nearly all instances, samples of frozen hypothalamic incubation media were assayed within 3 days, on some occasions within 5 days, and on rare occasions 7 days.

12) <u>Porcine Hypothalamic CRF Extract Experiments</u>

A partially purified porcine CRF preparation was assayed, and its log dose-response characteristics investigated. The extract, Fraction D5, was a generous gift from Dr D Schulster, and contained in 13.5g material derived from 640 pig hypothalami. The extract had been found to have activity in a dispersed pituitary cell CRF assay system (Cooper, <u>et al</u>, 1976; Synetos <u>et al</u>, 1978; Synetos, personal communication) in doses ranging between $1.0.10^{-3}$ and 10.10^{-3} of the material derived from one pig hypothalamus (Schulster, personal communication to the laboratory); or here, for descriptive parsimony, in doses ranging between $1.0.10^{-3}$ and 10.10^{-3}

<u>Hypothalamic Equivalents (HE)</u>.

The lyophilized extract was kept in its sealed container in a refrigerator at $4^{O}C$ until it was prepared for use.

On use, 0.2109g of the extract was weighed at room temperature and dissolved in 10ml citric acid/sodium hydrogen phosphate buffer solution (pH 4). 1ml samples of this stock solution were then pipetted into pyrex tubes, and the tubes were sealed with aluminium foil and stored frozen at $-20^{O}C$. This procedure was carried out on one occasion only, the extract being sealed in a container and being extremely hygroscopic, only permitting weighing on one occasion (Schulster, personal communication to the laboratory). The samples of the stock solution of D5 extract, kept at $-20^{O}C$, were all used within 6 weeks of preparation.

Just before each assay, one sample of the stock solution of D5 extract was removed from the freezer and defrosted at room temperature. 0.5ml of the stock solution was then diluted in 50ml cold ($\sim4^{O}C$) KRBG (pH 7.4), and warmed to $37^{O}C$ just before use in the assay. Doses of this solution ranging between 0.025ml ($0.25.10^{-3}$ HE) and 1.0ml (10.10^{-3}

HE) were then added to the pituitary tissue in the CRF assay (Incubation 3, Fig 5). In all other respects the assays were carried out as described under Assay of CRF, and the results were analysed as described for CRF in Expression of Data and Statistical Methods (see also Assay of CRF in Results).

13) Cerebral Cortex Experiments

In order to investigate whether the CRF assay measured CRF activity due to a substance specifically released from the hypothalamic/median eminence region or a non-specific corticotrophin-releasing activity which may be released from other neural tissues, the effects of media in which pieces of cerebral cortex were incubated were tested in the assay system.

20mg blocks of cerebral cortex tissue were obtained from groups of 3 animals at 13.30hr in the same manner as that described for hypo-thalamic tissue in the section Removal of Hypothalamic Tissue. The blocks of tissue were incubated for a single period of 15 minutes, Incubation 1. The cerebral cortex incubation media were then pooled for the assay of CRF. The assay was performed either immediately, or after storage at $-20^{\circ}C$ and assay within 7 days (see Storage of CRF). The CRF activity of the cerebral cortex incubation media was assayed using two doses which were added to pituitary tissue in the assay (Incubation 3, Fig 5), either 0.1ml or 0.3ml (analogous to 0.033 MEO or 0.1 MEO of hypothalamic incubation media); in all other respects the assays were performed as described under Assay of CRF for hypo-thalamic incubation media, and the results analysed as described in Expression of Data and Statistical Methods and in the appropriate section of the Results.

14) <u>Effects of Hypothalamic and Cerebral Cortex Incubation Media on</u>
<u>Corticosteroidogenesis in the Adrenal Gland</u>

In order to establish whether hypothalamic or cerebral cortex

incubation media interfered in the assay system either by containing

ACTH activity or by having an ACTH-preserving effect (Uemura <u>et al</u>,

1976 - see <u>Introduction</u>) or an ACTH-inactivating effect (Van Loon and

Kragt, 1970 - see <u>Introduction</u>), hypothalamic and cerebral cortex

incubation media, Incubation 1 at 13.30hr (see <u>Incubation of Hypo-</u>

<u>thalami</u> and <u>Cerebral Cortex Experiments</u>, above) were added in the same

concentrations as those used in the assay to adrenal quarters stimulated

with ACTH.   0.1ml (0.033 MEO) of cerebral cortex or hypothalamic

incubation media, obtained in Incubation 1, which is equivalent to 0.3 ml

(0.1 MEO) added to each pair of pituitary halves in the assay, was added

to each tube of adrenal quarters (Incubation 5, Fig 5; <u>Assay of CRF</u>)

in addition to 1ml of unstimulated pituitary tissue incubation medium

containing ACTH (i.e. Controls Incubation 3, Fig 3; <u>Assay of CRF</u>).

The results were analysed, as described in <u>Expression of Data and</u>

<u>Statistical Methods</u>, for the calculation of responses in the CRF assay,

and are presented as a comparison of the steroid production stimulated

by ACTH alone, versus the steroid production stimulated by ACTH and

added hypothalamic or cerebral cortex incubation media.

15) <u>Cyclic-AMP Experiments</u>

The concentrations of cyclic-AMP in hypothalamic incubation media,

Incubation 1 and Incubation 2 at 13.30hr (see <u>Incubation of Hypothalami</u>),

were assayed by the competitive protein-binding radioreceptor assay of

Brown <u>et al</u> (1972).   This procedure was carried out in order to ensure

that the cyclic-AMP concentrations in Incubations 1 and 2 were not such

that they might represent a non-specific stimulus to the release of

anterior pituitary hormones (see Introduction). The assays were performed in collaboration with, and with the guidance of, Dr S Nahorski, and are described in Appendix C.

In a further experiment, cyclic-AMP was added to pituitary glands in the CRF assay (Incubation 3, Fig 5; see Assay of CRF) in a dose approximately ten times the concentration found in Incubation 1 and Incubation 2, in order to check that any cyclic-AMP present in Incubation 1 or Incubation 2 does not represent a non-specific corticotrophin-releasing agent. A stock solution (1mM) of cyclic-AMP was diluted in KRBG to a concentration of 10 p moles/ml and pituitary glands (Incubation 3, Fig 5 - see Assay of CRF) were incubated in this medium. The results are presented as a comparison of the steroid production in the assay elicited by control pituitary glands incubated in normal KRBG (Controls Incubation 3, Fig 5; see Assay of CRF) versus the steroid production elicited by pituitary glands incubated in 10 p moles/ml cyclic-AMP in KRBG.

16) Effects of Regular Handling of the Donor Animals on the Secretion of ACTH from Pituitary Glands Isolated and Incubated In Vitro

The animals were handled daily at the same time of the day, for 2 weeks prior to sacrifice (see Animals). This procedure involved removing one animal from each cage, stroking it with ungloved hands for about 15 seconds, and then returning the animal to its cage; the second animal from each cage was then handled in the same way. The whole procedure for all the animals in the room took about 40 minutes. The effect of this regime upon the subsequent release of ACTH from pituitaries isolated and incubated in vitro was investigated by measuring the release of ACTH from pituitaries obtained from animals kept on the handling regime for 1 week, 2 weeks or 3 weeks. Following "handling" for 1 week, 2 weeks or 3 weeks, animals were decapitated at 13.30hr.

The pituitary glands were removed, hemisected, and allocated within each respective group of tubes containing Krebs-Ringer bicarbonate glucose solution. The pituitary glands were then incubated for one hour (Incubation 3 Controls, Fig 5 - see Assay of CRF), and the release of ACTH measured by the subsequent release of corticosterone from adrenal quarters incubated in vitro previously derived from animals handled for 2 weeks (Incubation 5, Fig 5 - see Assay of CRF). The results were analysed as described in Statistical Methods and Expression of Data, and are presented as a comparison of the steroid production elicited by pituitary glands obtained from animals handled for 1 week, 2 weeks, or 3 weeks.

17) Measurement of Corticosterone

Stock solutions of standard concentrations of corticosterone were prepared and replaced every 4 weeks, and kept at $4^{O}C$. 100mg corticosterone (11β,21-Dihydroxy-4-pregnene-3,20-dione) was weighed and dissolved in 20ml ethanol at room temperature (Stock Solution A). 1ml of this solution was diluted in 100ml deionized water (Stock Solution B) giving a concentration of 50 μg/ml.

For each corticosterone analysis:-

For adrenal incubation media, 1ml of Solution B was diluted in 10ml deionized water, giving Solution C, 5μg/ml.

For samples of plasma, 1ml of Solution B was diluted in 100ml deionized water giving Solution D, 0.5μg/ml.

Corticosterone blanks and standards were prepared in duplicate by pipetting 0 (blanks), 0.25, 0.5, or 1ml of Solution C (for adrenal incubation media) or Solution D (for plasma samples) into tubes which were then made up to 3ml with deionized water. This gave blanks and standards of:-

0 μg, 1.25 μg, 2.5 μg, and 5 μg for the analysis of adrenal

incubation media (3ml samples - Incubation 5, see Assay of

CRF)

or, 0 μg/100ml, 12.5 μg/100ml, 25 μg/100ml and 50 μg/100ml

for the analysis of samples of plasma (1ml aliquots diluted in

2ml deionized water, see below).

The blanks and standards were prepared before the cortico-

sterone extraction procedure was carried out in each case.

Preparation of blood samples for determination of corticosterone

Trunk blood from decapitated animals was collected in pyrex

tubes prepared with heparin. The tubes were centrifuged for 7 minutes,

and the plasma collected from each tube and stored at -20°C. The

samples were kept for not longer than 4 weeks before determination of

corticosterone levels. On the day of analysis, the plasma samples

were thawed, coagulated proteins removed, and a 1ml aliquot of each

sample added to 2ml of deionized water. Corticosterone blanks and

standards were prepared at the same time (see above). Corticosterone

was extracted from the samples, blanks, and standards by adding 30ml

chloroform to each tube and shaking the tubes for 8 minutes. The

plasma/deionized water phase was then aspirated from each tube, and

the chloroform analysed for corticosterone (see below).

Preparation of adrenal incubation media for determination of cortico-
sterone - see Assay of CRF, Adrenal Glands

Determination of Corticosterone in the Chloroform Extracts (Zenker and
Bernstein, 1958)

Fluorescence reagent was prepared by titrating 2.4 volumes conc.

H_2SO_4 into 1 volume chilled 50% ethanol (especially purified for the

fluorimetric determination of corticosteroids).

The chloroform extracts were washed by adding 5ml 0.1N NaOH to

each of groups of 4 tubes; the tubes were shaken for 20 seconds, and centrifuged at 1200 rpm for 1 minute. The NaOH phase was then removed from each tube within 30 seconds. 15ml aliquots of the washed chloroform extracts were then added to tubes each containing 3ml fresh fluorescence reagent, which were then shaken for 10 minutes.

The chloroform was then aspirated from each tube and discarded, and the tubes, containing fluorescence reagent, allowed to stand for 2 hours at room temperature to allow fluorescence to develop. The samples of fluorescence reagent were then transferred to microcuvettes, and fluorescence was measured on a Hitachi Perkin Elmer Model 204 spectro-fluorimeter using an exciter wavelength of 470nm and an analyzer wave-length of 525nm. The spectrofluorimeter was calibrated with the blanks and standard samples so that it gave a linear relationship between the corticosterone concentration and the fluorescence readings, using the 0 to 100 scale of the spectrofluorimeter so that the highest concen-tration of standard gave a full scale deflection reading of 100. All the readings were completed within 35 minutes, and the standards were read again at the end of this period to check that there had been no decay of the fluorescence.

Knowing the concentrations of corticosterone standards with which the spectrofluorimeter had been calibrated (see above), the results were calculated as:-

For adrenal incubation media,

$$\frac{Reading \times 5 \mu g}{100} \times \frac{100}{mg \text{ adrenal tissue}}$$

$$= \frac{Reading \times 5}{m \text{ g adrenal tissue}} \qquad \mu g \text{ Corticosterone/100mg adrenal tissue/hr}$$

For samples of plasma,

$$\frac{\text{Reading}}{100} \times 50 \, \mu g/100ml$$

$$= \frac{\text{Reading}}{2} \, \mu g \text{ Corticosterone}/100ml \text{ plasma}$$

The method is specific for corticosterone to the extent that only cortisol and estradiol cause significant fluorescence (19.8% and 14.2%, respectively, of the fluorescence produced by corticosterone - Zenker and Bernstein, 1958). This specificity is achieved by using the appropriate dilution of sulphuric acid in ethanol to produce fluorescence with corticosterone, by allowing the correct amount of time for corticosterone fluorescence to develop, and by using the exciter and analyser wavelengths in the spectrofluorimeter corresponding to the fluorescence peak of corticosterone. Further, since in the rat corticosterone is the main corticosteroid secreted by the adrenal gland (Bush, 1953), the method is suitable for work with this species. The method is also highly sensitive and detects, in plasma, an amount as little as 0.069 μg (Zenker and Bernstein, 1958) or concentrations as low as 5 $\mu g/100ml$ (Stockham, 1964). Corrections are made in the determinations for reagent blanks (see above), but not, however, for residual fluorescence due to cholesterol and other non-specific factors (Flack and Stockham, 1966). Although residual fluorescence may contribute as much as "4 $\mu g/100ml$" to the levels of plasma corticosterone, the index of corticosterone production by adrenal glands <u>in</u> <u>vitro</u> used here, $\mu g/100$ mg adrenal tissue/hour (i.e. μg corticosterone/ mg adrenal tissue/hour - Bakker and De Wied, 1961), is unaffected by residual fluorescence (Flack and Stockham, 1966). All the reagents used in the studies were of analytical grade (BDH or Fisons), except for NaOH, H_2SO_4 and $CHCl_3$ which were of standard laboratory reagent grade; H_2SO_4 and $CHCl_3$ were changed to analytical grade following batches which produced large amounts of non-specific fluorescence.

18)   Expression of Data and Statistical Methods

In the section describing the assay of CRF, it was noted that
a limited number of samples could be processed in any single assay and
that the amount of data obtainable from a single assay was not sufficient
to perform a two by two bioassay protocol.   The procedures adopted to
assess the data are explained in the following sections.

(a)   Calculation of Responses in the Assay System and of Plasma
       Corticosterone Levels

As the methodology depends upon the ultimate physico-chemical
determination of corticosterone concentrations, the data is initially
expressed either as μ g corticosterone produced per 100 mg adrenal
tissue per hour measured fluorimetrically in the adrenal test incubation,
Incubation 5 (see Assay of CRF, above; and Fig. 5), or as μ g cortico-
sterone per 100 ml plasma in samples of trunk blood.   As described
above (Measurement of Corticosterone) corticosterone concentrations in
adrenal incubation media and in samples of plasma were determined
fluorimetrically by the method of Zenker and Bernstein (1958) using
reagent blanks and 3 standard concentrations of corticosterone for
calibration of the spectrofluorimeter.   From the readings and the prior
calibration of the spectrofluorimeter with the appropriate standard
solutions, the corticosterone concentrations in the samples were
calculated (see Measurement of Corticosterone).

Each measurement is an observation in statistical terminology,
and the number of observations included in any statistical analysis is
denoted by the small letter n.   The mean corticosterone concentration
(μ g/100 ml) or corticosterone production (μ g/100 mg adrenal tissue/hr)
of a number of samples (usually, n = 6) was then calculated, as was the
standard deviation and standard error of the mean (SEM) (Bailey, 1959;
Campbell, 1967), for samples within each experimental condition (see

also Assessment of CRF Assay Data for Dose-Response Relationships, below).
The latter data therefore constituted the simplest basic calculation
from which the effects of different experimental variables could be
assessed using the statistics of approximation to the normal distribution
for small numbers of samples (Bailey, 1959). Tests of significance were
performed using students t test. Variance ratio tests (F tests) were
carried out to check that the variances of the compared means were equal
(Bailey, 1959); when necessary, the correct number of degrees of freedom
were calculated from the variances (Bailey, 1959). In all statistical
evaluations differences were assumed not to be significant at a level
of probability greater than 0.05.

Responses calculated in the CRF assay were not transformed from
production of corticosterone at the end-point of the assay into ng or
μU of ACTH released by pituitary tissues, because although a laboratory
standard curve relating corticosterone production to amount of synthetic
ACTH 1-24 exists (see Assay of CRF in Results), there was no equivalent
curve relating corticosterone production to the amount of a naturally
occurring rat ACTH molecule nor is the nature of the ACTH molecule(s)
released by rat pituitary glands in response to CRF entirely known (see
Introduction). Further, because a limited number of samples could be
run in each assay (see Assay of CRF, above) it was not possible to make
a standard ACTH dose-response relation in each assay. The results
were therefore calculated in the form of a measure of the total ACTH
bioactivity released by CRF, that is, the production of corticosterone
by adrenal glands isolated and incubated in vitro. Thus, in this
assay system doses of CRF are related directly to the production of
corticosterone by adrenal tissues; and the overall rationale of the
assay is one of measurement of the total bioactivity both of the CRF
molecules released by the hypothalamus and of the ACTH molecules
released by the pituitary gland. A reason for this, as already

surmised (see Introduction), is that at present, of the hypothalamo-
pituitary-adrenal hormones, only the corticosteroids may be measured
by physico-chemical methods with some confidence.

b)   Assessment of Data from the Assay of CRF: Dose-Response Relation-
        ships

        Data previously obtained in this laboratory established the

existence of a log dose-response relation between the amount of CRF

obtained in a standard incubation of 15 minutes (Incubation 2 at 13.30

hr) and the production of corticosterone from the triple stage in vitro

assay system (Thomas, 1977). This relation is shown in Fig.6 of the

Results and is discussed in the section Assay of CRF in the Results.

Individual assays were first assessed from the raw data obtained with

each dose of CRF and from the basal release of ACTH in the assay

(pituitary-adrenal controls, Fig. 5; see Assay of CRF, above). Each

assay included pituitary-adrenal control tissues and a dose of

Incubation 2 at 13.30 hr (see Calculation of Relative Potency of CRF

Preparations, below) which produced significant responses in the assay

(see Assay of CRF in Results). Assays which showed conclusive evidence

either (i) of stressed assay animals as indicated by a large basal release

of ACTH from control pituitary tissues, or abnormally incremented

responses to control doses of the standard production of CRF in Incubation

2 at 13.30 hr compared with data derived over a long period (see Assay of

CRF in Results, and Thomas, 1977), or (ii) of unresponsive tissues as

indicated by a low basal release of ACTH from pituitary tissues or a poor

response to a control dose of standard CRF released in Incubation 2 at

13.30 hr (see Assay of CRF in Results, and Thomas, 1977), were excluded

from the results. Assays which, on the other hand, indicated only that

some of the tissues obtained from assay animals (see description of

randomization and allocation of hemipituitaries and adrenal quarters in

Assay of CRF, above) were obtained from stressed animals or were not viable or unresponsive, were included in the results but the data obtained with the tissues that showed either a single stressed donor animal or a single unresponsive tissue were excluded. Further, the results obtained with the tissues concerned were checked in both control and test groups in the assays, since tissues of common origin were allocated to both control and test groups (see description of randomization and allocation of hemipituitaries and adrenal quarters in Assay of CRF, above), and when necessary results obtained from the same tissues were excluded from both control and test groups. Invariably, however, the discrepant results were obtained from a single pair of hemipituitaries and not their controls, since both duplicate estimates (see Assay of CRF, above) of the ACTH release of one pair of hemipituitaries were unsatisfactory whilst the duplicate estimates of the ACTH release from the other, control, pair of hemipituitaries were satisfactory, indicating also that the adrenal tissues were satisfactory since each of the duplicates essentially agreed in their responses to aliquots of pituitary incubation medium. Data excluded from further analysis consisted, in nearly all instances, either of results obtained with individual pituitary tubes (Incubation 3, Fig. 5) in the assays, or whole assays which indicated unsatisfactory assay animals in these instances. All raw data in the individual assays were then used to calculate the mean, standard deviation, and standard error of the mean of the responses to each dose of CRF and the mean, standard deviation, and standard error of the mean of the basal release of ACTH in the assay (see Calculation of Responses in the Assay System and of Plasma Cortico-sterone Levels, above). The mean response to each dose of CRF was then compared within each assay (two doses of an unknown CRF preparation were used in each assay - see Assay of CRF, above)

(i)   to examine the existence of a response to either of the doses
        of the unknown sample of CRF,

(ii)   to examine the existence of a dose-response relation for the
        unknown sample of CRF,

(iii)   to compare the responses to doses of the unknown sample of CRF
        and the dose of Incubation 2 at 13.30 hr.

When the responses to doses of the unknown CRF samples were not evident
or were small, one of two hypotheses were made: either (i) the potency
of the unknown CRF sample was low, and the doses of the unknown CRF
preparation were therefore increased in the subsequent assay.   This
method of successive approximation allowed the relative potency (see
Calculation of Relative Potency of CRF Preparations, below) of the
unknown samples of CRF to be determined once doses which produced
responses in the linear, ascending, part of the CRF log dose-response
curve were found (see Assay of CRF in Results, and Fig. 6), or (ii) the
potency of the unknown CRF sample was high, and the doses were in the
'supramaximal' range of the CRF dose-response relation (see Assay of
CRF in Results, and Fig. 6) producing submaximal responses.   Evidence
of this was invariably obtained when the larger of two doses of a CRF
preparation produced smaller responses than the smaller of the two
doses.   Doses of the unknown CRF preparation were therefore decreased
in the subsequent assay. Again, by successive approximations, doses
were found which produced responses in the ascending part of the log
dose-response curve (see Assay of CRF in Results, and Fig. 6).

Thus after a number of assays it was possible to determine whether
a dose-response relation existed for each unknown preparation of CRF.
Raw data from a number of assays were later pooled for linear regression
analysis if a set of doses and responses were established to bear an
ascending relation between log dose of CRF and response (by examination

of the mean responses in each assay as described above). Doses and
responses which appeared to be subthreshold or supramaximal in the
assay (see above) were excluded from the linear regression analysis.

c)   Calculation of the Linear Regression Function of CRF Assay Data

Once data from a number of assays had established the existence
of a dose-response relation for each unknown CRF preparation, as
described in the previous section, it was possible to make preliminary
estimates of relative potency by comparing equivalent doses (i.e. doses
which produced equal responses) of the unknown CRF preparation in each
assay and of the standard CRF preparation, Incubation 2 at 13.30 hr,
which was allocated a potency of 1.0, using a log dose-response relation
for CRF in Incubation 2 at 13.30 hr previously established in the
laboratory (see Assay of CRF in Results, and Fig. 6). This method of
calculation (see Calculation of Relative Potency of CRF Preparations,
below) of relative potency is essentially graphical and was used in
previous publications of some of the data (Thomas, 1977; Kamstra et al,
1978). However, with sufficient data available, linear regression
analysis of the results obtained with the unknown preparations of CRF
was then made. Although calculating linear regression functions with
data collected from a number of assays entails combining inter-assay
variance with intra-assay variance (thereby treating the data as if it
were derived from one assay), it is preferable for a number of reasons.
Firstly, in a simple graphical comparison of the mean responses obtained
in single assays with a standard CRF log dose-response curve (Incubation
2 at 13.30 hr) the exact dose-response relation of the unknown CRF
preparation is not known, and as a consequence parallelism between the
log dose-response relations of the unknown CRF preparation and of the
standard CRF preparation is not assessed; this in turn can lead to
variations in relative potency estimates with the level of response if

the log dose-response relations are not parallel. Secondly, it
follows that even if the log dose-response relations of the unknown
and standard CRF preparations are not parallel, since exact numerical
parallelism is improbable and only non-significant differences in
slope may be found, it is preferable to find the equivalent doses of
unknown and standard CRF preparations at a single level of response
rather than comparing equivalent doses at a number of levels of
response. Thirdly, it also follows that in order to find the
equivalent doses of the unknown CRF preparation and the standard CRF
preparation at a single level of response the regression function for
each unknown CRF preparation must be found. Fourthly, because a
linear regression function is calculated for the CRF preparations from
a number of assays, variations in the response to doses of the CRF
preparation from one assay to the next no longer affect the estimate
of relative potency since the linear regression analysis calculates
the mean of all the responses in all the assays to each dose of CRF.
The resulting regression function is therefore the best description of
the linear relation between log dose and response for each CRF prepar-
ation when data must be collected from different assays. Fifthly, by
calculating the regression function of the standard CRF preparation
from Incubation 2 at 13.30 hr (see Calculation of Relative Potency of
CRF Preparations, below) from control, test doses of Incubation 2 at
13.30 hr in each of the experiments (see Assay of CRF, above), unknown
CRF preparations were compared with data obtained for the standard CRF
(Incubation 2 at 13.30 hr) preparation in the same experiments. This
was preferable to comparing test data to laboratory data obtained for
the CRF in Incubation 2 at 13.30 hr in an earlier, different, series
of experiments (Thomas, 1977).

For the reasons enumerated above the statistics used in calcul-
ating the potency of CRF preparations were based on linear regression

analysis (see Calculation of Relative Potency of CRF Preparations, below). Although two by two bioassays and treatment of data by an analysis of variance procedure would have been preferable, for the reasons outlined in the section Assay of CRF (above), this was not possible. Regression analysis of collected data was considered to be the next best possible alternative, and with sufficient data having been obtained, was used in preference to the original graphical methods.

Data were collected for linear regression analysis on a pre-programmed calculator according to the criteria described above in the section Assessment of CRF Assay Data for Dose-Response Relationships. Data which was subjected to linear regression analysis was therefore data which, by previous assessment, clearly showed some dose-response relation, and which did not display dose-related responses simply because of artefacts caused by variations in the assays' sensitivity. The calculations performed in the programmed linear regression analysis were described in the manufacturer's manual (Texas Instruments) and corresponded to the formulae described in the literature for linear regression analysis (Bailey, 1959; Campbell, 1967). The quantities derived by the calculator were the following:-

Intercept of the regression function, α

Slope of the regression function, b

giving the regression function

$y = \alpha + b x$, where y is the response and x is the log dose

Correlation coefficient   r

Number of data points   n

Sum of responses $\sum y$

Sum of squared responses $\sum y^2$

Sum of log doses $\sum x$

Sum of squared log doses $\sum x^2$

Sum of products $\sum xy$

From the above the following were calculated (Bailey, 1959; Campbell, 1967):-

1.   The corrected sum of squares of responses

$$Syy = \sum y^2 - \frac{(\sum y)^2}{n}$$

2.   The corrected sum of squares of log doses

$$Sxx = \sum x^2 - \frac{(\sum x)^2}{n}$$

3.   The corrected sum of products

$$Sxy = \sum xy - \frac{(\sum x)(\sum y)}{n}$$

From the above three quantities the following were calculated (Bailey, 1959; Campbell, 1967):-

1.   The residual variance

$$s^2 = \frac{1}{n-2} \left(Syy - \frac{Sxy^2}{Sxx} \right)$$

2.   The estimate of the variance of the slope

$$= \frac{s^2}{Sxx}$$

3.   The estimate of the variance of the mean response

$$= \frac{s^2}{n}$$

or, for a point estimate of a response the variance

$$= \frac{s^2}{n} + s^2 \frac{(x - \bar{x})^2}{Sxx}$$

where x is the specified log dose, $\bar{x}$ the mean log dose, and Sxx is defined above.

From the above quantities the following were calculated:-

1. The interval estimate of the slope

$$b \pm t \sqrt{\frac{s^2}{Sxx}}$$

where t is students t taken with (n - 2) degrees of freedom

at the 5% level of probability.

2. The index of precision

$$\lambda = \frac{\sqrt{\frac{s^2}{n}}}{b}$$

using the estimate of the variance of the mean response.

Thus, from the linear regression analysis for each CRF preparation the following were found:-

The linear regression function

$$y = \alpha + bx$$

The correlation coefficient   r.

The interval estimate of the slope

$$\pm t \sqrt{\frac{s^2}{Sxx}}$$

The index of precision

$$\lambda = \frac{\sqrt{\frac{s^2}{n}}}{b}$$

The correlation coefficient was checked for significance, using (n - 2) degrees of freedom, from tables (Bailey, 1959), the 5% probability level being chosen as significant. Lack of significant correlation between responses and log doses, however, was not taken as an indication of lack of a relation between responses and log doses since the correlation co-efficient is an invalid test when data do not conform to bivariate normality (Bailey, 1959) - this is obviously the case for assay data

because, whilst responses can be assumed to follow a normal distribution, log doses are a discontinuous variable with a number of selected values. The correlation coefficient as a test of significance of assay data is therefore fundamentally invalid, especially when the number of different doses used is low and unevenly distributed, and was therefore disregarded. The other tests of significance performed with the regression analyses were:-

1.   In instances where possible, the significance of the difference of the slope, b, from zero (Bailey, 1959):-

Students t was calculated as

$$t = \frac{b}{\sqrt{\dfrac{s^2}{Sxx}}}$$

and the significance of t was checked with (n - 2) degrees of freedom at the 5% level of probability. Again, this test is affected by the lack of an adequate description of the variance of the log doses (Sxx) (see also next section).

2.   Comparison of the slope, b, with the slope of the standard CRF preparation, Incubation 2 at 13.30 hr. Although this is a poor substitute for a test of parallelism in an analysis of variance of a two by two bioassay, this test is important in establishing the validity of making a potency estimate (see Calculation of Relative Potency of CRF Preparations, below):-

Students t was calculated as

$$t = \frac{bu - bs}{\sqrt{\dfrac{Su^2}{Sxxu} + \dfrac{Ss^2}{Sxxs}}}$$

where the subscripts u and S denote the unknown and standard CRF preparations respectively. The significance of the value of t was assessed at the 5% level of probability with $(n_u + n_s - 4)$

degrees of freedom if the variance ratio $(F = \frac{Su^2}{Ss^2}$; Bailey,
1959) was not significant, or with the calculated number of
degrees of freedom given by formulae in Bailey (1959) if the
variance ratio was significant.

The regression functions of the test CRF preparations and the
standard CRF preparation (Incubation 2 at 13.30 hr) were then compared
for estimation of the relative potency of the test CRF preparations
(see next section).

d)   Calculation of Relative Potency of CRF Preparations

The responses obtained in the assay to various doses of different
CRF preparations were calculated into regression functions and relative
potency estimates for a number of reasons:-

1.   Simple comparison of the mean response obtained with similar
       doses of test and standard (control) CRF preparations is not
       justified if the log dose-response relations for the test and
       standard CRF preparations are not similar.   The assay system
       must show similar log dose-response characteristics to test and
       standard CRF preparations before the test and standard CRF
       preparations can be assumed to be comparable.

2.   Even if the log dose-response relation for two CRF preparations
       is identical, straightforward comparison of responses does not
       give any realistic indication of the relative amounts of CRF
       activity in the two CRF preparations.   This is because responses
       are not directly proportional to doses in a function of the type

       'response = k. dose'

where k is a constant, but are related by a function of the type

       'response = α + k' log dose'

where α and k' are constants.

By comparing responses to two CRF preparations a comparison of the two doses is intended, and increments or decrements in responses are proportional to a function of the dose,k' log dose. Without a knowledge of the constants α and k' straightforward responses cannot give a true indication of the relative amounts of CRF present, except in the most general terms of 'more CRF' or 'less CRF' and gradations of these terms. More succinctly, comparison of responses in bioassay systems is <u>not</u> a valid estimate of the relative potency or relative amounts of a substance in two preparations (Finney, 1964, p. 61) since the relation between response and dose can only be made linear by a function of the logarithmic transformation of the dose. However, by first finding the relation between dose and response accurately for each CRF preparation, a comparison of equivalent doses which produce equal responses can give estimates of the relative amounts of CRF present.

In essence, relative potency is defined as the antilogarithm of the distance, M, on the log dose axis between parallel log dose-response lines (Finney, 1964). That is, for all equal responses the product of the dose (x) and potency (R) is equal for each preparation,

i.e. $\quad X_u R_u = X_s R_s$

where X_u and R_u are the dose and potency of the unknown preparation, and X_s and R_s are the dose and potency of the standard preparation which produce an equal response. From this, the <u>relative potency</u> of the unknown preparation is $\dfrac{Ru}{Rs}$

and $\quad \dfrac{Ru}{Rs} = \dfrac{Xs}{Xu} \qquad = $ antilog $(\log X_s - \log X_u)$

$\qquad\qquad\qquad\qquad\quad = $ antilog distance between parallel log dose-response curves on the dose parameter

$\qquad\qquad\qquad\qquad\quad = $ antilog M

Rather than making comparisons on the y (response) axis of the
regression function, then, comparisons are made on the X (log dose)
axis. This is a more direct method of comparing the amounts of a
substance present, and in a sense disregards the regression function
altogether. However, the regression function is necessary as a pre-
requisite before making any comparisons, for the reasons given above
and in the previous section. Also, the regression function is
necessary to calculate M, the distance between the regression functions
on the X (log dose) axis.

In the method of equivalent doses, therefore, the logarithm of
the relative potency, M, is simply

$$M = \log X_s - \log X_u$$

(Finney, 1964, p. 523). In calculations with preliminary data of some
of the results relative potency was calculated as

$$\text{Relative potency} = \frac{Ru}{Rs} = \frac{Xs}{Xu}$$

by comparing the dose of unknown CRF which produced a certain mean
response in an assay with the dose of standard CRF Incubation 2 at 13.30
hr which produced the same response as read from the laboratory standard
CRF preparation (Incubation 2 at 13.30 hr) log dose-response curve. A
number of estimates of relative potency were then collected from mean
responses calculated in several assays and the mean and standard error
of the mean of the estimates calculated (Thomas, 1977; Kamstra et al,
1978). The limitations of this method have been described in the
previous section and summarised and reiterated above.

In the present methods, regression functions were calculated from
the data collected from a number of assays with each CRF preparation (see

previous section). A regression function was also obtained for the standard CRF preparation (Incubation 2 at 13.30 hr) from data obtained within the same assays which assayed the unknown CRF preparations (see Assay of CRF, above). Incubation 2 at 13.30 hr was selected as a CRF assay standard for the following reasons:

1. Data for the log dose-response relationship of Incubation 2 at 13.30 hr had previously been established (Thomas, 1977); further, this incubation had shown the least variation between tissues and produced the lowest of all the responses then measured in the assay. The laboratory then took that standard as 1.0 (relative potency) against which to measure the relative potency of other preparations.

2. Incubation 2 at 13.30 hr was a convenient standard because it could be prepared for all the assays at the same time as the assays (see Assay of CRF, above).

3. As Incubation 2 at 13.30 hr had previously been used as the standard, it was essential to use the same preparation as a standard to ensure consistency in the standard used for the calculation of the results so that results may be compared. The relative potencies of the unknown CRF preparations were therefore calculated using the potency of Incubation 2 at 13.30 hr as 1.0. The amount of CRF present in each unknown CRF preparation was in each case compared to the amount present in Incubation 2 at 13.30 hr, and variations in the amount of CRF present in each incubation are variations relative to the amount present in Incubation 2 at 13.30 hr.

Although relative potency is the antilogarithm of the distance between parallel log dose-response curves, parallel log dose-response relationships were not always obtained; however, none of the log dose-

response relations differed significantly in slope from the standard
CRF preparation (Incubation 2 at 13.30 hr). Since the log dose-
response relations were not exactly parallel the definition of the
distance between log dose-response lines becomes dependent on the
level of response chosen to compare the log dose-response lines and
equivalent doses (see discussion above). This does not occur if
log dose-response lines are arbitrarily drawn parallel, but on the
other hand the correct choice of parallel log dose-response lines can
only be determined by the analysis of variance of data obtained from
a two by two bioassay. This being the case, the procedure adopted
was to select a single level of response with which all log dose-
responses lines could be compared. The response level chosen was
7.0 μg corticosterone/100 mg adrenal tissue/hour. This response is
significantly different from the response due to the unstimulated
release of ACTH from pituitary tissue in the assays; and the raw data
obtained for the assay of most of the CRF preparations included responses
in this part of the dose-response curve (see Assay of CRF in Results,
and Fig. 6). The log dose (x_s) of CRF produced during Incubation 2
at 13.30 hr initiating this response was then calculated from its
regression function, and the log dose (x_u) of each unknown CRF prepar-
ation producing this response was calculated from each associated
regression function. The relative potency was then calculated as

$$\text{antilog } M = \text{antilog } (\log x_s - \log x_u)$$

where, in this instance, $\log x_u$ and $\log x_s$ are the doses of unknown
and standard CRF preparations, respectively, which produce a response
of 7.0 μg corticosterone/100 mg adrenal tissue/hour. The variance
of M is the sum of the variances of the log equivalent doses,

i.e. $V_M = V_u + V_s$

where V_M is the variance of M, V_u is the variance of $\log x_u$, and V_s

is the variance of $\log x_s$ (Finney, 1964). The variances V_u and V_s
were calculated from the formulae

$$V_u = \frac{0.564 \, \sigma_u \, d}{n_u}$$

and

$$V_s = \frac{0.564 \, \sigma_s \, d}{n_s}$$

(Gaddum; given by Finney, 1964), where σ_u and σ_s are the standard
deviations of the log doses (i.e. $\sqrt{\frac{Sxx}{(n-1)}}$ in previous section), n_u and
n_s are the number of log doses entered in the regression analysis, and
d is the interval between successive log doses (in these experiments,
log 2). The confidence interval of the logarithm of the potency
estimate was then calculated as

$$M \pm t \sqrt{V_M}$$

with the value of t taken at the 5% level of probability and $(n_u + n_s - 4)$
degrees of freedom if V_u and V_s were assumed to be equal (F test;
Bailey, 1959), or with the number of degrees of freedom calculated from
V_u and V_s if V_u and V_s were not assumed to be equal (Bailey, 1959).
The relative potency and its 95% confidence interval is therefore anti-
log M, antilog $(M + t \sqrt{V_M})$, antilog $(M - t \sqrt{V_M})$. The relative
potencies of different CRF preparations were therefore compared using
the confidence intervals of the logarithms of the potencies. Using
students t test, t was calculated as

$$t = \frac{M_1 - M_2}{\sqrt{V_{M_1} + V_{M_2}}}$$

where the subscripts 1 and 2 denote the two preparations compared. The
significance of the value of t was assessed at the 5% level of probability,
with the sum of the number of degrees of freedom used in finding the
confidence interval for each preparation as the number of degrees of

freedom; if V_{M_1} and V_{M_2} were unequal (F test; Bailey, 1959) the number of degrees of freedom was calculated according to formulae given by Bailey (1959).

RESULTS

1)   Assay of CRF

In the absence of a conventional international standard CRF
preparation, CRF is measured by weight of a laboratory CRF extract
preparation (e.g. Saffran et al, 1955; Guillemin et al, 1957; De Wied,
1961b); by weight of protein contained in a CRF extract (e.g. Seelig
and Sayers, 1977; Vale and Rivier, 1977); by fractions (or equivalents)
of the CRF material derived from one stalk-median eminence (SME) extract
(e.g. Arimura et al, 1967; Gillies and Lowry, 1978), hypothalamic-
median eminence (HME or HE) extract (e.g. Portanova et al, 1970;
Takebe et al, 1975; Lutz-Bucher et al, 1977), or median eminence extract
(e.g. Hiroshige et al, 1968; Chan et al, 1969a); or by volume of the
medium into which CRF is released from the hypothalamus in vitro
(Bradbury et al, 1974; Jones et al, 1976). In the present assay system,
CRF is measured by fractions of the total volume of medium in which each
individual hypothalamus is incubated, the total volume being equivalent
to 1 Median Eminence Output (MEO) (Thomas and Sadow, 1975a). CRF
activity is expressed in terms of the corticosterone or ACTH production
in the assay system, or in terms of the percentage stimulation elicited by
the CRF preparation by reference to the unstimulated production of
corticosterone or ACTH in the assay system.

A log dose-response relationship was established between the
dose, measured in MEO, of CRF released in Incubation 2 at 13.30 hr and
response measured in μg corticosterone/100 mg adrenal tissue/hour and
is shown graphically in Fig. 6 and Fig. 7. It is noted from previous
work in this laboratory (Thomas, 1977) that the dose-response curve is
not a straight line with doses of CRF greater than 0.1 MEO, the responses
becoming submaximal with increasing doses of CRF. Responses elicited for
this series of experiments is shown in Fig. 6 and Fig. 7 with the
statistical analysis; however, only the shape of the curve is shown and

FIGURE 6

Log dose-response curve of CRF, obtained from Incubation 2 at 13.30 hr; log dose-response curve of synthetic ACTH 1-24. The doses of CRF incubation medium are measured in Median Eminence Output (MEO) (see text). The line drawn through the responses to increasing doses of ACTH is a curve drawn through the points as judged by eye. Data for this curve has been derived from previous work in this laboratory. The ascending line drawn through the responses to increasing doses of CRF (Incubation 2 at 13.30 hr) is a linear regression function for doses of CRF less than and including 0.1 MEO (see text). Doses greater than 0.1 MEO CRF Incubation 2 at 13.30 hr produce sub-maximal responses in the assay system; data for this part of the curve, which was excluded from the regression analysis, has also been derived from previously published work from this laboratory and is shown in the descending part of the log dose-response curve for CRF, Incubation 2 at 13.30 hr. The broken line (——) indicates the non-stimulated release of ACTH in the CRF assays. The standard error of the mean unstimulated pituitary release of ACTH is not shown as this is too small to be shown clearly in the figure. The vertical bars indicate the standard error of the means for each point in the figure.

ACTH 1-24:-   n = 6 for each dose (1 assay)

CRF Incubation 2 13.30 hr:-   n = 6 for each dose (12 assays)

Non-stimulated (Basal) Pituitary ACTH Release:-   n = 12 (12 assays)

The following increasing doses of CRF (Incubation 2 at 13.30 hr) produced significantly different responses in the assays (t test):-

Non-Stimulated (Basal) Pituitary ACTH Release V. 0.0167 MEO CRF $p < 0.05$

0.0067 MEO CRF   V. 0.033 MEO CRF $p < 0.01$

0.01 MEO CRF   V. 0.033 MEO CRF $p < 0.05$

0.0167 MEO CRF   V. 0.1 MEO CRF $p < 0.02$

0.1 MEO CRF   V. 0.33 MEO CRF $p < 0.001$

0.1 MEO CRF   V. 0.5 MEO CRF $p < 0.002$
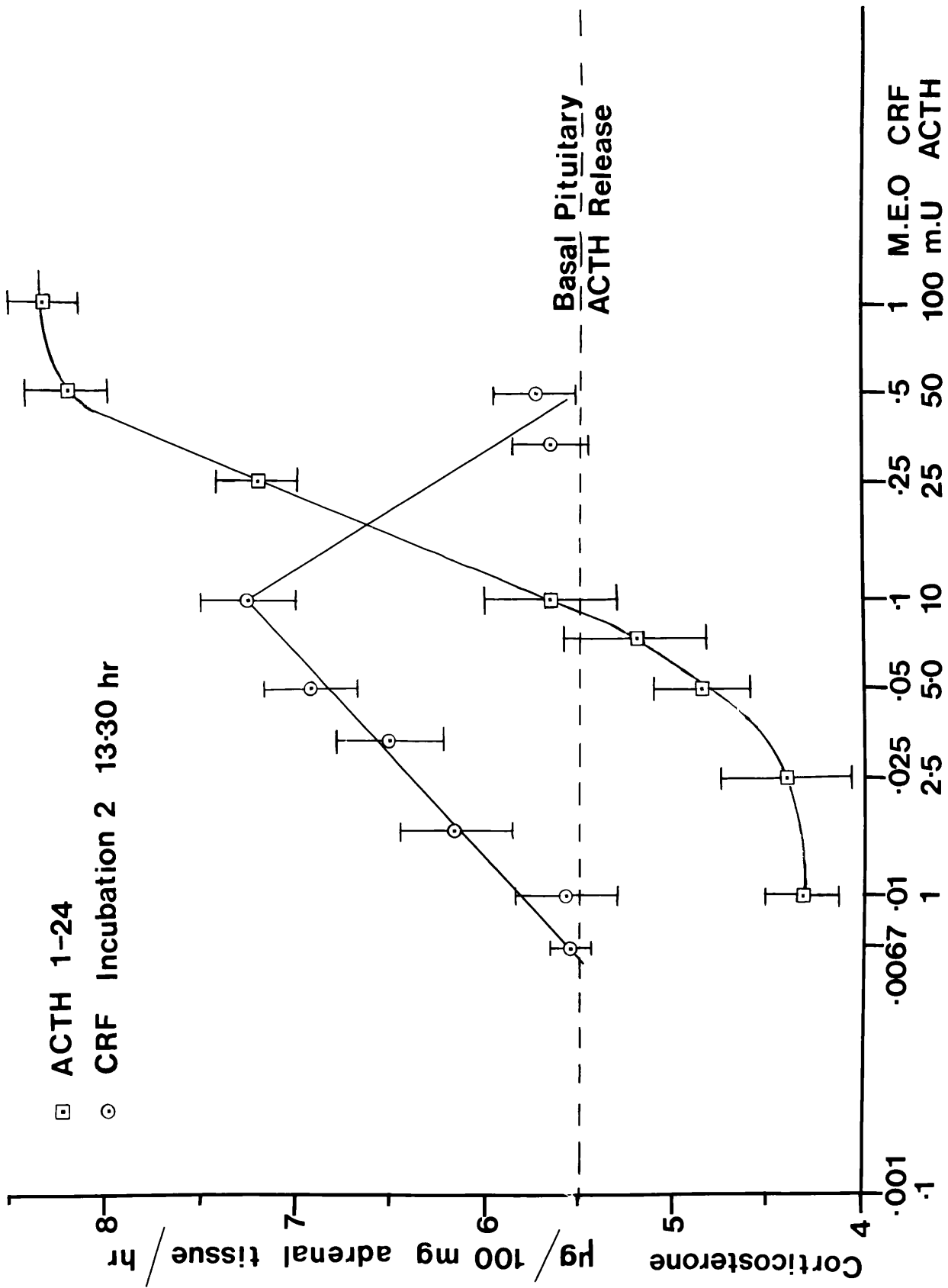
data obtained with doses of CRF greater than 0.1 MEO has been derived
from previous work in this laboratory (Thomas, 1977). These results,
however, are important since data obtained in the descending part of
the curve may give rise to ambiguity in estimates of the amount of CRF
present in hypothalamic incubates because similar responses may be
obtained with the assay system with dissimilar doses owing to the
'bell' shape of the dose-response curve. Therefore, in obtaining data
for estimates of the amount of CRF released under different conditions
care was taken to verify that the data corresponded to the ascending,
working range of the dose-response curve for CRF (see Expression of
Data and Statistical Methods).

Regression analysis of the ascending part of the log dose-
response relation obtained in the experiments illustrated in Fig. 6
with doses of CRF up to and including 0.1 MEO, has a slope of 1.434
$\pm$ 0.06 (Slope $\pm$ standard deviation; Thomas, 1977) significantly
different from 0 (p < 0.001) and an index of precision of 0.178 (Thomas,
1977). A log dose-response curve for the effects of ACTH 1-24
(Cortrosyn-Organon) on adrenal glands in the CRF assay is also shown
in Fig. 6 (Sadow, laboratory data), and in the linear range has a slope
of 3.67 which is significantly different from that for CRF in the assay
system (p < 0.02). The reasons for this non-parallelism are that

(i) It can be seen from Fig. 6 that the relation between
dose of CRF and amount of ACTH released from pituitary glands is not
necessarily one to one; since, for arguments' sake, by assuming the
dose-response curve for ACTH released from rat pituitary glands in
response to CRF is the same as that for ACTH 1-24, an approximately six-
fold increase in the dose of CRF is apparently necessary to achieve a
two-fold increase in the amount of ACTH.

(ii) It is unlikely that the dose-response curve for ACTH

released from rat pituitary glands in response to CRF is the same as that for synthetic ACTH 1-24; this is because a heterogeneous variety of ACTH molecules is released from the pituitary gland (see Introduction), so that, strictly speaking, the dose-response curve for ACTH 1-24 is not comparable to that for CRF, the ACTH molecules released being much more complex in nature and variety.

The CRF activity of a hypothalamic extract (Extract D5, a gift from Dr D Schulster) was tested in the CRF assay system and a preliminary attempt was made to investigate its dose-response characteristics. The results are shown graphically in Fig. 7 together with those obtained with the CRF activity found in Incubation 2 at 13.30 hr. A log dose-response relation was found to exist between the dose of D5 extract, measured in HE (Hypothalamic Equivalents) and the response in μg corticosterone/100 mg adrenal tissue/hour for doses of the extract up to 2.10^{-3}HE. Thereafter, increasing doses appeared to produce no significant change from the maximal response elicited with 2.10^{-3}HE. Regression analysis of the doses and responses with doses of the extract between $0.25.10^{-3}$ and 2.10^{-3}HE gave a slope of 1.348 ± 0.356 (slope $\pm$ standard deviation) significantly different from 0 (p $<$ 0.001) and an index of precision of 0.074 (see Appendix D). Regression analysis of the doses and responses with doses of the extract between 2.10^{-3} and 10.10^{-3} HE, however, gave a slope of 0.226 ± 0.382 (slope $\pm$ standard deviation) not significantly different from 0 (see Appendix D) and this part of the curve is therefore drawn with a slope of 0 in Fig. 7. The slopes of the ascending part of the dose-response curve for CRF in Incubation 2 and for the CRF extract are not significantly different (p $>$ 0.1). This indicates the assay responds similarly to CRF activity derived from both sources, although it is by no means established that the CRF activity of the two preparations is identical. However, the responses to supramaximal doses of Incubation 2 and CRF extract are

FIGURE 7

Log dose-response relations of CRF in Incubation 2 at 13.30 hr, and of D5 extract, a porcine hypothalamic CRF extract obtained from another laboratory (see below). The doses of CRF incubation medium are measured in Median Eminence Output, MEO (see text), and the doses of D5 extract are measured in Hypothalamic Equivalents, HE (see text). For details of the lines drawn through the responses to CRF in Incubation 2 at 13.30 hr see Fig. 6 and text. The ascending line drawn through the responses to increasing doses of D5 extract is a linear regression function for doses of D5 extract less than and including 2.10^{-3} HE (see text). The line is not drawn below the response to 0.25. 10^{-3} HE since this is the lowest dose of D5 extract for which data were obtained. The broken line extending from doses of D5 extract greater than 2.10^{-3} indicates a slope not significantly different from zero in the regression analysis of data obtained with doses of D5 extract greater than 2.10^{-3} HE (see text), and is dotted to indicate uncertainty about the shape of this part of the curve. The broken line (-.-.-) indicates the basal release of ACTH in the D5 extract assays, and the broken line (——) indicates the basal release of ACTH in the CRF Incubation 2 at 13.30 hr assays. The standard errors of the means for the basal release of ACTH in each set of assays are not shown as they are too small to be drawn clearly in the figure. The vertical bars indicate the standard error of the means for all other points.

CRF Incubation 2 13.30 hr:-   n = 6 for each dose (12 assays)

D5 CRF Extract:-   n = 6 for each dose, except n = 4 for $0.25.10^{-3}$ HE, and n = 5 for $1.25.10^{-3}$ HE (8 assays)

Basal Pituitary ACTH Release in the CRF Incubation 2 at 13.30 hr assays:-   n = 12 (12 assays)

Basal Pituitary ACTH Release in the D5 extract assays:-   n = 12 (8 assays)

The mean basal release of ACTH in the CRF Incubation 2 at 13.30 hr assays and in the D5 CRF Extract assays is not significantly different (t test, $p > 0.1$). The significantly different responses to CRF Incubation 2 at 13.30 hr are noted in the legend to Fig. 6. The following increasing doses of D5 CRF extract produced significantly different responses in the assays (t test):-

Basal Pituitary ACTH Release   V. $0.5.10^{-3}$ HE $p < 0.01$

$0.25.10^{-3}$ HE   V. 2.10^{-3} HE $p < 0.05$

$0.5.10^{-3}$ HE   V. 2.10^{-3} HE $p < 0.05$

$0.25.10^{-3}$ HE   V. 10.10^{-3} HE $p < 0.05$

The D5 CRF extract was a kind gift from Dr D Schulster.
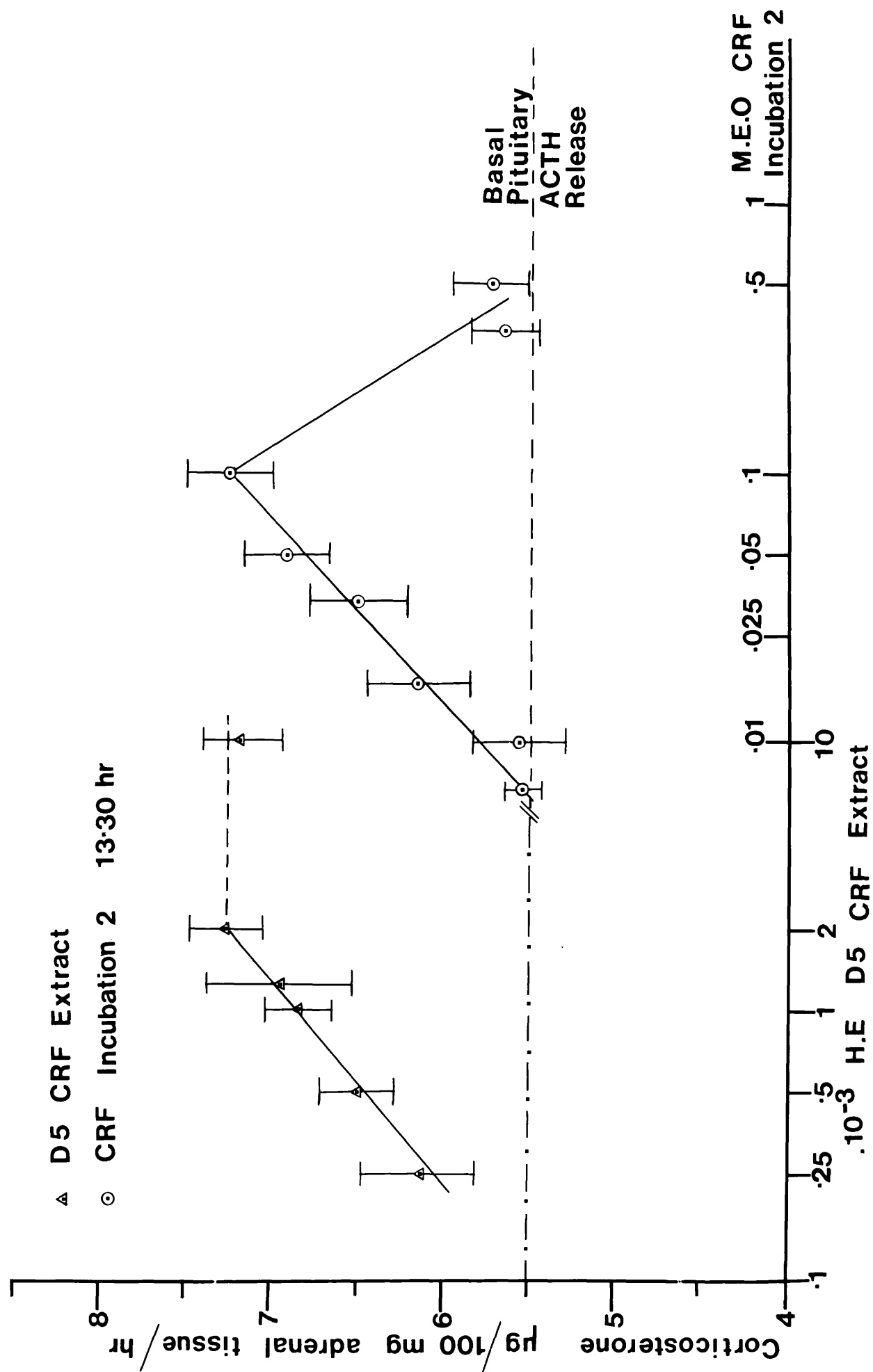
dissimilar, there being apparently little or no change in response with the CRF extract but a decrease in response with Incubation 2. The reason for this is unclear, but the variety of peptide material obtained in Incubation 2 may be dissimilar from that in the CRF extract, and it has been suggested that under certain conditions a corticotrophin inhibiting factor (CIF) may be released from the hypothalamus in vitro (Thomas, 1977).

CRF assays were carried out accompanied by a test dose of the CRF released in Incubation 2 at 13.30 hr. This was done in order to establish control data for CRF in each assay from which the relative potency of assayed preparations may be calculated using the potency of Incubation 2 at 13.30 hr as 1.0 (see Assay of CRF and Expression of Data and Statistical Methods in Methods). The doses of Incubation 2 (or Incubation 1 in some assays - see Assay of CRF in Methods) were chosen in the linear, ascending part of the dose-response relation established earlier and shown in Fig. 6. Regression analyses of this data, which was used as the standard for the calculation of relative potencies, gave a slope of 1.411 ± 0.103 (slope $\pm$ standard deviation) significantly different from 0 ($p < 0.001$) and an index of precision of 0.033 for Incubation 1 at 13.30 hr; and a slope of 1.478 ± 0.607 (slope $\pm$ standard deviation) significantly different from 0 ($p < 0.02$) and an index of precision of 0.058 for Incubation 2 at 13.30 hr (see Appendix D). The slopes of Incubation 1 and Incubation 2 did not differ significantly from the previously established data for Incubation 2 at 13.30 hr (see above; Fig. 6). However, the position of the linear regression function for Incubation 1 and Incubation 2 from the assay experiments differed from that in the earlier data for Incubation 2 at 13.30 hr. Thus the doses of Incubation 1 and Incubation 2 from the assay experiments which produced a response of 6.5 μg corticosterone/

100 mg adrenal tissue/hour were 0.013 and 0.016 MEO respectively, whereas for Incubation 2 in the earlier experiments the dose was 0.031 MEO. The reason for this apparent two-fold difference in amount of CRF is unclear. However since Incubation 1 and Incubation 2 in the assay experiments were found to have released approximately similar amounts of CRF (relative potencies of 1.211 and 1.0 respectively, see Appendix D) this cannot be attributed to a difference in the behaviour of the hypothalamus incubated in vitro at 13.30 hr since the media obtained from Incubation 1 and Incubation 2 at 13.30 hr had been previously found to contain approximately the same amounts of CRF (Thomas, 1977). Further, the amount of CRF released in Incubation 1 and Incubation 2 in the assay experiments was consistent from experiment to experiment, a finding also previously established (Thomas, 1977). However, a number of factors may have contributed to the fact that more CRF was released in control hypothalamic incubations at 13.30 hr in the assay experiments than in the earlier investigations of the dose-response characteristics of CRF released in Incubation 2. Firstly, analysis of the data using different criteria (see Expression of Data and Statistical Methods) may be suspected - this, however, is unlikely since simple examination of the responses obtained with Incubation 1 and Incubation 2 after sufficient data had been obtained in assay experiments revealed a clear difference with the earlier dose-response curve. Secondly, although the condition of the experimental animals may be suspected as a reason for the difference it is unlikely since the basal output of ACTH from pituitary glands obtained from assay animals (Controls, Fig. 5 - see Assay of CRF in Methods) was consistent during the course of the studies. Further, the log dose-response relations for CRF in Incubation 2 do not differ, suggesting a difference in the performance of the assay system was not the cause of the difference. Thirdly, in the original experiments the amount of hypothalamic tissue removed for

incubation included the median eminence but not the whole of the medio-
basal hypothalamus (Thomas and Sadow, 1975a; Thomas, 1977). The
larger amount of tissue removed in later experiments (see Removal of
Hypothalamic Tissue in Methods) may be expected to contribute to a
greater release of CRF in Incubation 1 and Incubation 2. This point
is important because although CRF is present in the highest concen-
trations in the median eminence, it is also present in high concentrat-
ions in other parts of the mediobasal hypothalamus (see Introduction).
Therefore, it seems most likely that the extent of the hypothalamic
tissue block removed for incubation contributed to a change in the
amount of CRF released but not in the dose-response characteristics of
CRF in the assay system.

Although the data obtained for Incubation 1 appeared to provide
a better standard for the calculation of relative potency of CRF
preparations (see Expression of Data and Statistical Methods) than did
the data obtained for Incubation 2 (see Appendix D), Incubation 2 was
retained as the standard because it had been previously used for
calculations of relative potency (see Expression of Data and Statistical
Methods) and an alteration of the standard incubation used for the
calculation of relative potency would affect the relation between the
amounts of CRF released under different conditions.

The results obtained with the CRF assays were therefore
calculated into estimates of the amount of CRF released using Incubation
2 at 13.30 hr as the standard (see Expression of Data and Statistical
Methods). Although the calculations performed relate to the calcula-
tion of relative potency the term amount of CRF is used, because the
term potency implies the possibility of changes in the nature of the
CRF substances being measured rather than in the amount; whilst the
latter may be preferable, the log dose-response relations for the CRF

preparations were found not to be significantly different in their slope and the use of the term amount, inferring changes in the concentration of CRF in the incubation media, is better justified.

The regression analyses of the assay data for Incubation 1 and Incubation 2 at 13.30 hr and for the D5 extract are presented in Appendix D, together with the relevant statistical analyses. Data derived from the regression analyses for the other CRF preparations in the Results are presented in Tables 2, 3, 4 and 5 in the appropriate sections of the Results, below. Combining all the data obtained in the regression analyses of 46 CRF preparations, the slopes of the regression functions have a mean value of 1.446 ± 0.081 ($\pm$ standard deviation), and a mean index of precision of 0.13 ± 0.062 ($\pm$ standard deviation); these values do not differ significantly from those obtained previously with the assay (see above and Fig. 6; and Thomas, 1977) and this indicates that the performance of the assay was consistent throughout the course of a prolonged period of studies.

2)   Storage of CRF

Samples of the incubation media obtained in Incubation 2 at
13.30 hr were assayed for CRF activity immediately, and on days 4, 6
and 7 following incubation and storage of the samples at $-20^{o}C$. Doses
of 0.033 MEO were used to assess the activity of each sample.   The
responses produced are shown in Fig. 8.

No significant change in CRF activity occurred during 7 days
of storage of the incubation media at $-20^{o}C$.   These results suggest
that hypothalamic incubation media may be kept at $-20^{o}C$ for up to 7 days
at least, without any loss of CRF activity.   Therefore, in all the
experiments in which it was necessary to store samples of hypothalamic
incubation media at $-20^{o}C$ for CRF assay, the samples were assayed on
most occasions within 3 days, on some occasions within 5 days, and
exceptionally on 7 days.

FIGURE 8

Replicate assays of the CRF activity of samples of hypothalamic incubation media (Incubation 2 at 13.30 hr) stored at -20°C for various lengths of time. The dose of each sample used was 0.033 MEO. The horizontal bars indicate the standard error of the mean for each column. n = 6 for each column.

DAYS STORAGE AT-20°C

μg CORTICOSTERONE/100mg ADRENAL TISSUE/HOUR
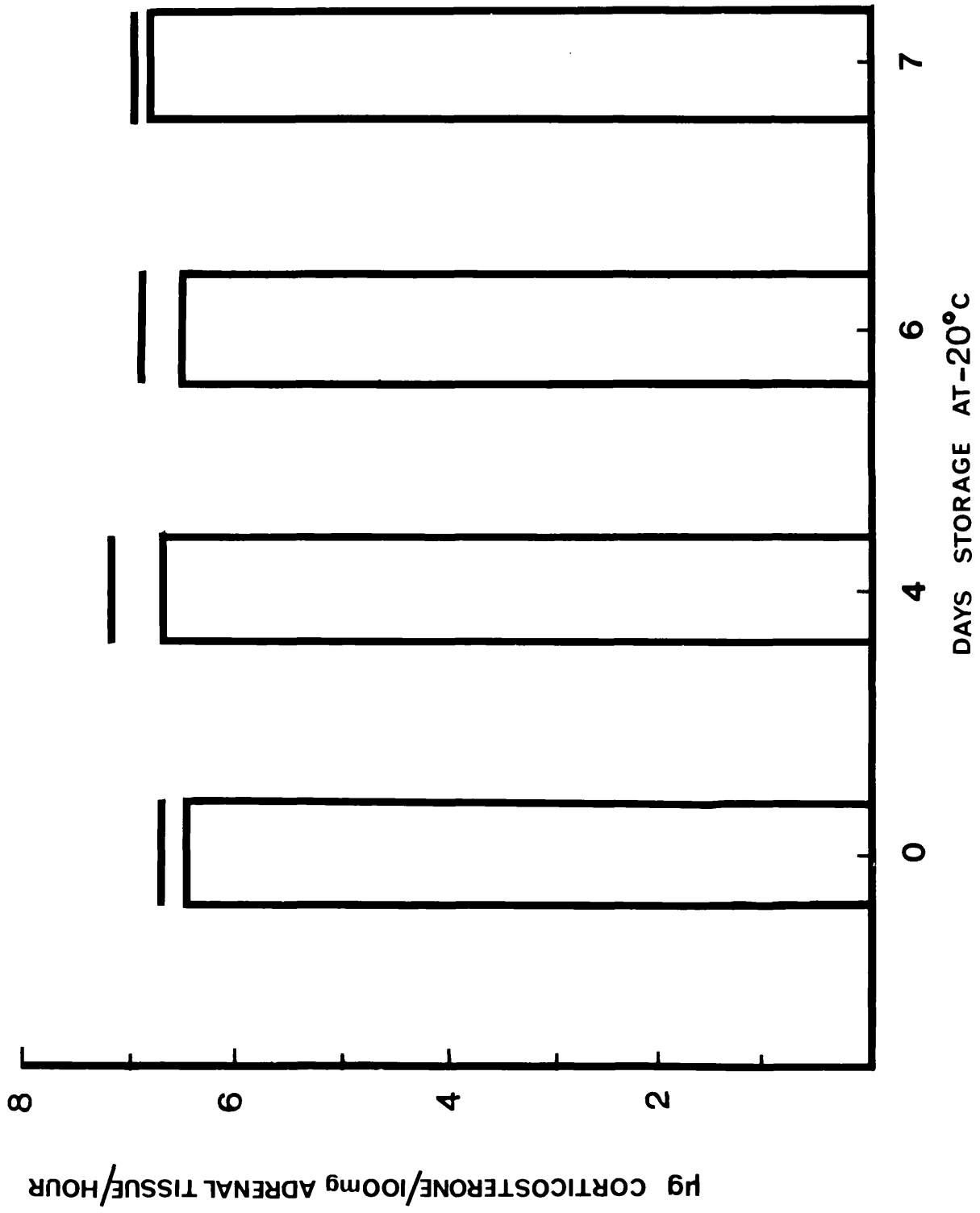
3)   Measurement of Cyclic-AMP in Hypothalamic Incubation Media

The concentrations of cyclic adenosine 3',5'-monophosphate
(cyclic-AMP) in the media obtained from Incubations 1 and 2 at 13.30
hr were assayed by the competitive protein-binding assay of Brown et
al (1972) (see Appendix C). The concentration of cyclic-AMP in
Incubation 1 was 1.12 p mole/ml, and in Incubation 2 1.05 p mole/ml.
These concentrations of cyclic-AMP are less than (by about 10^{-6}) the
concentrations of dibutyryl cyclic-AMP which elicit an increase in
ACTH release from pituitary segments in a static incubation system
(mM concentrations; Fleischer et al, 1969; Zimmerman and Fleischer,
1970), but are within the concentrations of dibutyryl cyclic-AMP which
elicit an increase in ACTH release from pituitary segments in a peri-
fusion system (4 p mole/ml; Koch et al, 1979). In order to verify
that the concentrations of cyclic-AMP in Incubation 1 and Incubation 2
do not interfere in the assay by causing the release of ACTH, cyclic-
AMP was added to hemipituitaries in the assay system (Incubation 3 -
Fig. 5) and the release of ACTH compared to that of unstimulated hemi-
pituitaries. No phosphodiesterase inhibitors were added since this
would not make the experiment comparable to the normal assay conditions.

The addition of 10 p mole/ml cyclic-AMP to the hemipituitaries
in vitro had no significant effect on the release of ACTH, as shown in
Table 1:-

Table 1   Lack of effect of 10 p mole/ml cyclic-AMP on ACTH release
            in vitro

Controls: 5.59 $\pm$ 0.3 μg corticosterone/100 mg adrenal tissue/hr

10 p mole/ml Cyclic-AMP: 5.59 $\pm$ 0.16 μg corticosterone/100 mg adrenal

tissue/hr

Mean $\pm$ Standard error of mean;   n = 6.

This result makes it unlikely that any cyclic-AMP in the hypo-
thalamic incubation media interferes in the measurement of CRF by the
assay method described.

4)   Effects of Handling Animals on ACTH Release From Pituitaries

         Isolated and Incubated In Vitro

Pituitary donor rats were handled daily for 1, 2 or 3 weeks,

and the subsequent release of ACTH from unstimulated hemipituitaries

was measured (Fig. 9).   The release of ACTH from pituitaries

obtained from animals handled for 2 weeks is significantly less than

that released from pituitaries from animals handled for 1 week only

(p < 0.05), or for 3 weeks (p < 0.0005).   Handling of animals for 2

weeks also results in a decreased variance in ACTH output in vitro

by comparison to pituitaries obtained from animals handled for 1 week

only (p < 0.01).   Handling of animals for 2 weeks therefore decreases

the basal release of ACTH in vitro and improves the precision of the

assay, and is therefore effective as a pretreatment of assay animals

for CRF bioassay.

Handling of animals has been shown to cause a rapid activation

(within 15 minutes) of the H-P-A axis (Barrett and Stockham, 1963;

Ader et al, 1967; Ader and Friedman, 1968).   However, when this

procedure is carried out repeatedly in infant rats, the H-P-A response

to handling in adult animals is diminished (Sieck and Ramaley, 1975;

Thomas, 1977).   Repeated handling, or "taming", of mature animals

also has an effect on H-P-A activity, as the results in Fig. 9

demonstrate.   The duration of the taming period appears to be critical

since animals handled for 1 week or 3 weeks were found to be more

reactive to handling than animals handled for 2 weeks, both with

respect to their H-P-A activity and their behavioural responses to

handling.   Thus, animals which had been handled for 1 week, although

less "nervous" or active than animals not yet handled, still showed

tense muscular tone.   This relative lack of effect on H-P-A reactivity

of handling animals for one week only in relation to the effect

FIGURE 9

The effects of handling pituitary donor animals for different

lengths of time on the subsequent release of ACTH from hemi-

sected pituitaries isolated and incubated in vitro, expressed

as μg corticosterone/100 mg adrenal tissue/hour.   The

horizontal bars indicate the standard error of the mean for

each column.

n = 20 for 2 and 3 weeks of handling.

n = 7 for 1 week of handling.

The release of ACTH from pituitary glands isolated and incubated

in vitro obtained from animals handled for 2 weeks is significant-

ly less than that from pituitary glands from animals handled for

1 week ($p < 0.05$) or for 3 weeks ($p < 0.0005$).   The release of ACTH

from pituitary glands isolated and incubated in vitro from animals

handled for 1 or 3 weeks is not significantly different.
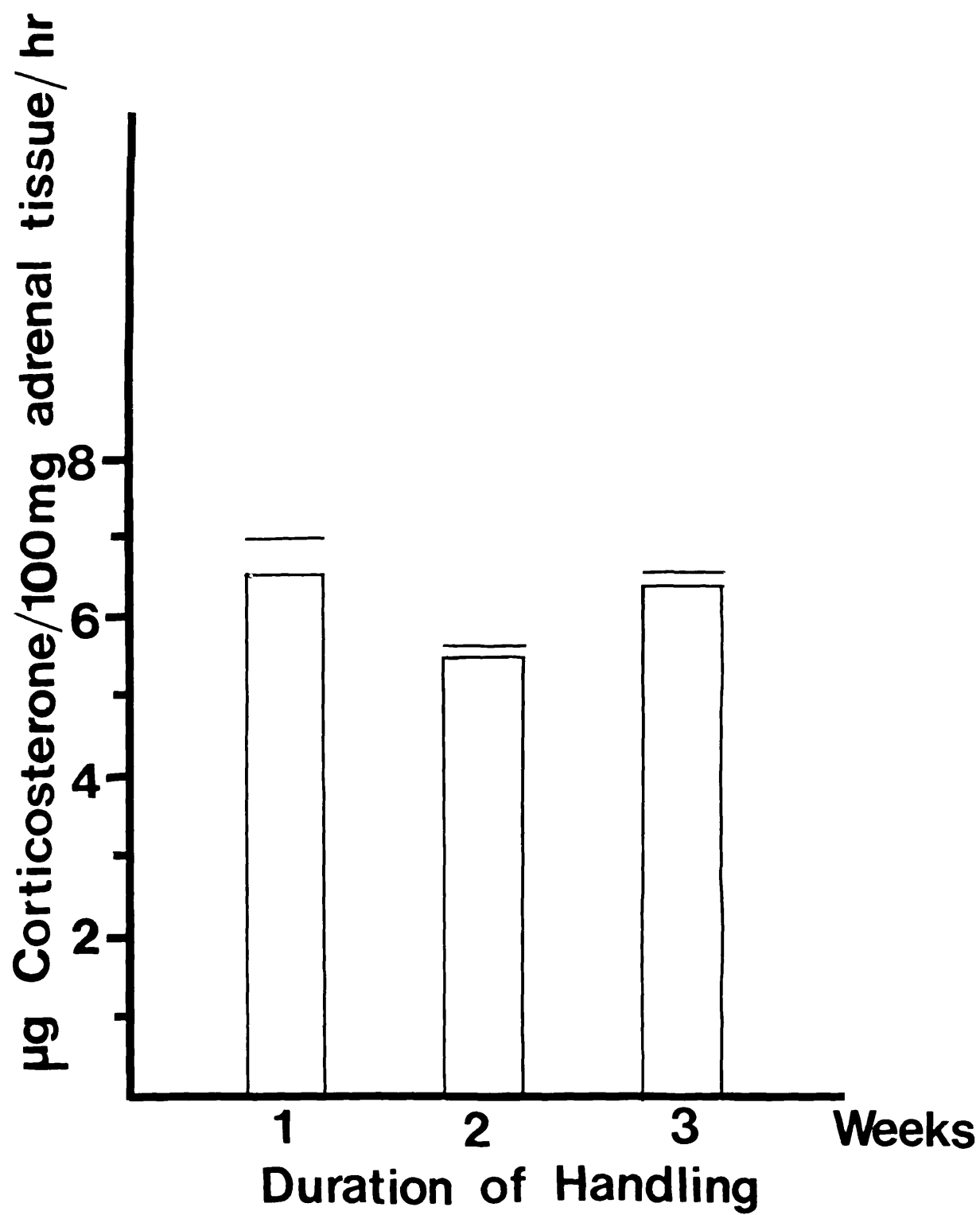
produced by handling animals for 2 weeks is evident in Fig. 9; and also in the results of Hodges and Mitchley (1970a) who found that handling animals for one week only was ineffective in preventing an increase in plasma corticosterone levels in response to injection stress.

Animals handled for two weeks, however, approached the experimenter readily and showed relaxed muscular tone; animals handled for this length of time show reduced H-P-A reactivity as may be seen in Fig. 9. Animals handled for three weeks or more, although still easy to handle and willing to approach the experimenter, are more active and show a greater amount of exploratory behaviour than animals handled for two weeks; animals handled for three weeks show an increased H-P-A activity over animals handled for two weeks, but still show less variability in the activity of their pituitary glands isolated and incubated in vitro than animals handled for 1 week only (Fig. 9). Thus, animals handled for more than two weeks, although less desirable than animals handled for two weeks exactly, are still useful for experimentation provided adequate controls are provided within the assay system.

5)   <u>Effects of Cerebral Cortex Tissue Incubation Media in the</u>
            <u>CRF Assay</u>

20 mg pieces of cerebral cortex tissue were incubated in KRBG for 15 minutes (Incubation 1), and the incubation media decanted and tested for CRF activity by adding doses of 0.1 ml and 0.3 ml (analogous to 0.033 MEO and 0.1 MEO of hypothalamic incubation media) to hemipituitaries in the assay system. The responses produced by the cerebral cortex incubation media and 0.033 MEO (0.1 ml) hypothalamic Incubation 1 are compared in Fig. 10. The cerebral cortex incubation media produced a significant response in the assay system, doses of 0.1 ml and 0.3 ml eliciting a greater effect over and above the basal, non-stimulated, release of ACTH from control hemipituitaries ($p < 0.05$). However, the response to 0.1 ml (0.033 MEO) hypothalamic Incubation 1 at 13.30 hr is significantly greater than that to either 0.1 ml or 0.3 ml of cerebral cortex incubation medium ($p < 0.05$), and the responses to 0.1 ml and 0.3 ml of cerebral cortex incubation medium are not significantly different from each other. These results suggest that the media obtained from incubation of cerebral cortex tissue do have some corticotrophin-releasing activity, which, although less potent than hypothalamic incubation media in these experiments, may be as potent as hypothalamic incubation media if it is remembered that the weight of hypothalamic tissue used was approximately 30 mg and that of cerebral cortex tissue was 20 mg (see <u>Methods</u>), a consideration which may be important since a doubling of the weight of hypothalamic tissue used (15 to 30 mg) produces a considerable difference in the results (see <u>Assay of CRF</u>, above). However, the fact that no dose-response relation existed for the doses of cerebral cortex incubation media used, 0.1 ml and 0.3 ml, suggests that the CRF activity of the cerebral cortex incubation media is dissimilar to that of hypothalamic incubation media - on the other hand, this conclusion must be qualified by the

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FIGURE 10

The effects of cerebral cortex incubation media when added to hemipituitaries in the CRF assay (striped columns); comparison with the basal, non-stimulated, release of ACTH from hemipituitaries (clear column), and with the effect of hypothalamic incubation media (Incubation 1 at 13.30 hr) (cross-hatched column). The doses of cerebral cortex and hypothalamic incubation media added to hemipituitaries is indicated beneath each column. The horizontal bars indicate the standard error of the mean for each column. $n = 6$ for each column.
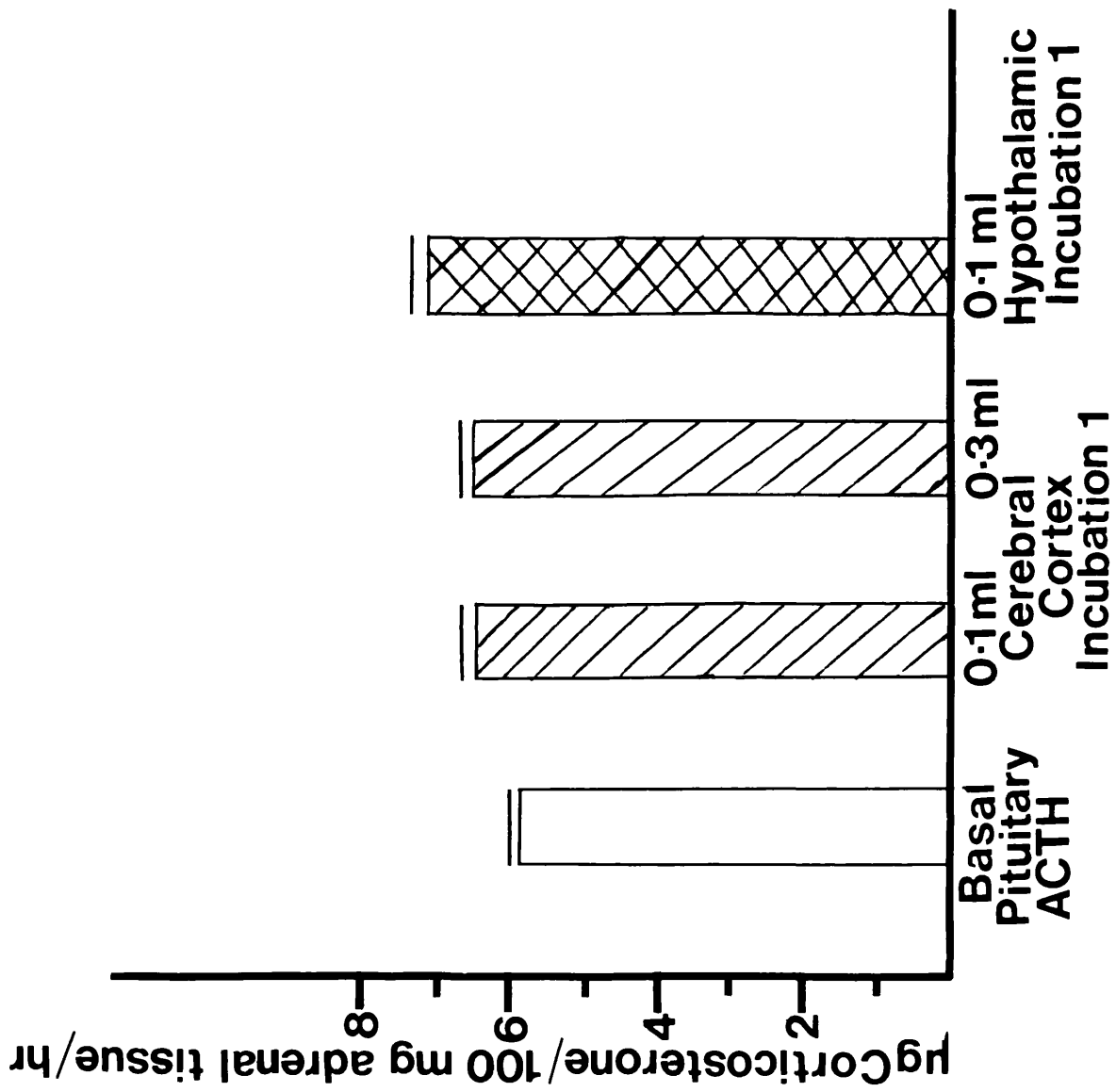
proviso that only two doses of cerebral cortex incubation media were investigated, doses which may not have been in the range optimal for producing a dose-response effect. The exact nature of the activity contained in the cerebral cortex incubation media is therefore unclear, but it may also be commented here that the cerebral cortex incubation media do have some (but insignificant) ACTH-preserving or ACTH-potentiating activity in the assay system (see next section), an effect which is clearly not due to true CRF bioactivity.

6)   <u>Effects of Cerebral Cortex and Hypothalamic Incubation Media</u>
<u>on the Stimulation of Corticosteroidogenesis in the CRF Assay</u>

Cerebral cortex and hypothalamic incubation media were added
in doses of 0.1 ml to the incubation medium in which quartered adrenal
glands <u>in vitro</u> (Incubation 5, Fig. 5) were stimulated with the ACTH
previously released from basal, non-stimulated hemipituitaries isolated
and incubated <u>in vitro</u> (Incubation 3, Fig. 5). The effects of ACTH
alone, ACTH and cerebral cortex incubation media, and ACTH and hypo-
thalamic incubation media were compared by measurement of the production
of corticosterone by quartered adrenal glands isolated and incubated
<u>in vitro</u>. The results are shown in Fig. 11. Although the addition of
cerebral cortex incubation media resulted in a slight increase in
corticosteroidogenesis, neither cerebral cortex nor hypothalamic
incubation media have any significant effect on the corticosteroido-
genesis stimulated by ACTH in the assay. Cerebral cortex and hypo-
thalamic incubation media, therefore, do not have any significant ACTH-
preserving effect (Uemura <u>et al</u>, 1976) or ACTH-inactivating effect (Van
Loon and Kragt, 1970) in the CRF assay described here. Further, the
results suggest that cerebral cortex and hypothalamic incubation media
also have no significant ACTH activity or ACTH-potentiating activity in
the assay.

FIGURE 11

The effects of cerebral cortex (striped column) and hypothalamic
(cross-hatched column) incubation media when added in doses of
0.1 ml to quartered adrenal glands isolated and incubated in
vitro and stimulated with ACTH (controls - clear column). The
horizontal bars indicate the standard error of the mean for each
column.

n = 6 for controls and hypothalamic incubation media.

n = 5 for cerebral cortex incubation media.
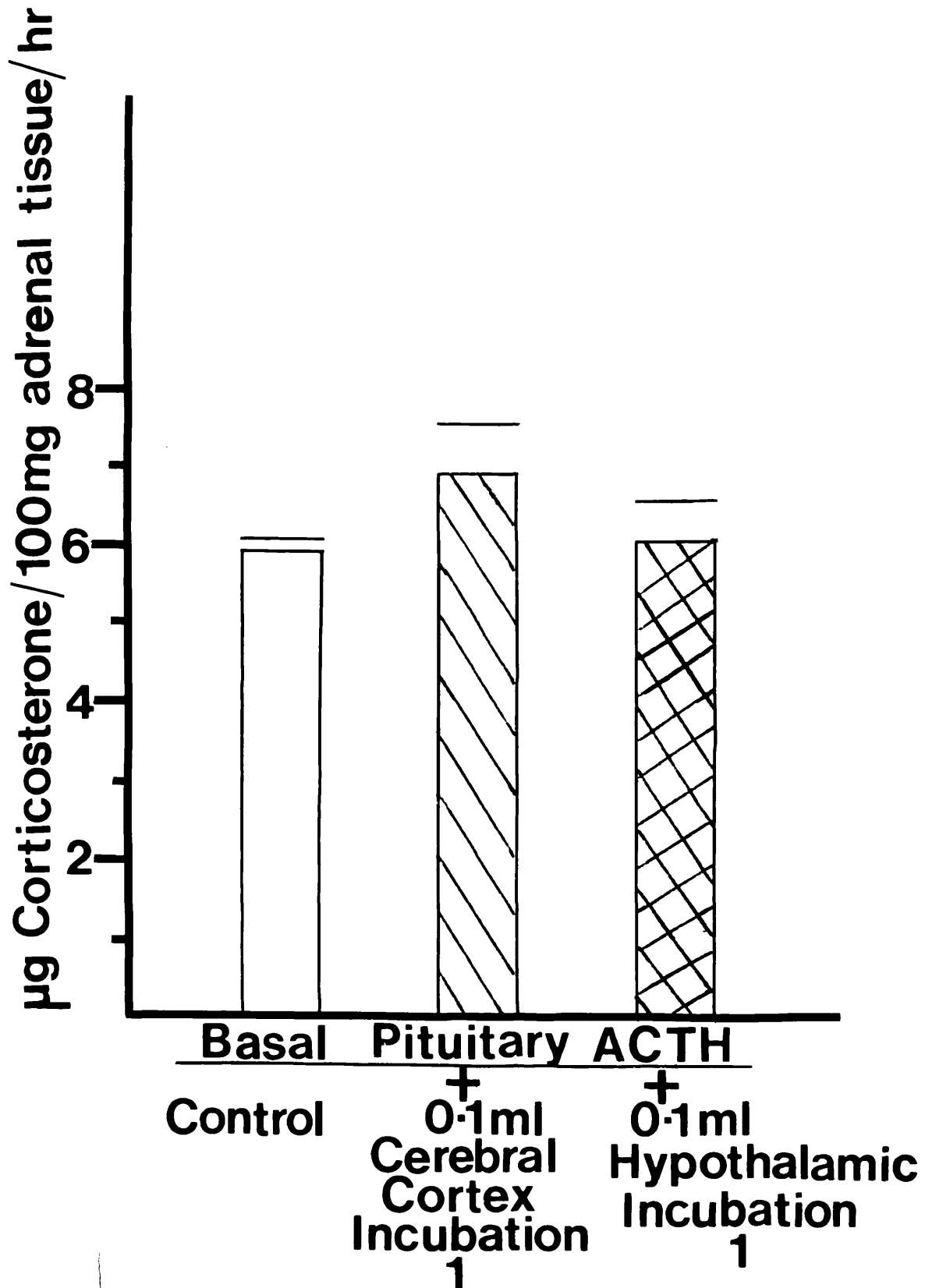
7)   Effects of Incubating Hypothalami in Calcium-Free Media

Hypothalami were incubated for two consecutive 15 minute
incubation periods, Incubation 1 and Incubation 2, at 13.30 hr in the
presence and absence of calcium ions in the incubation media.   The
incubation media were then assayed for CRF and the relative potency
calculated with reference to the potency of the standard Incubation 2
at 13.30 hr, allocated a potency of 1.0 (see Methods and Assay of CRF).
The results, shown in Fig. 12 and Table 2, clearly indicate that the
release of CRF both in Incubation 1 and Incubation 2 is dependent on
extracellular calcium, the amounts of CRF released in calcium-free media
being significantly less than in 2.5 mM calcium media ($p < 0.001$,
Incubation 1 and Incubation 2).   The sensitivity of the CRF secretion
process to extracellular calcium concentrations is further implied by
the fact that no calcium-depleting procedures in calcium-free media
were carried out prior to Incubation 1.   It may also be noted that in
these particular experiments the amounts of CRF in Incubation 1 when
hypothalami were incubated in normal, 2.5 mM calcium, media were less
than the amounts found in other experiments (compare Inc. 1 2.5 mM
Calcium in Table 2 with Inc. 1 at 13.30 hr in Table 3; $p < 0.001$).
This discrepancy may be due to a difference in the conditions in which
the two sets of experiments were performed, in particular the condition
of the animals - this possibility seems more likely than any difference
in the execution of the experiments or performance of the CRF assay
since the amounts of CRF in Incubation 2 when hypothalami were incubated
in normal, 2.5 mM calcium, media were not significantly different from
those found in other experiments (compare Inc. 2 2.5 mM Calcium in
Table 2 with Inc. 2 at 13.30 hr in Table 3).   Nevertheless, the fact
that the amounts of CRF in Incubation 1 in 2.5 mM calcium media were
unusually low in the experiments presented in Fig. 12 and Table 2,

FIGURE 12

The amounts of CRF in Incubation 1 and Incubation 2 at 13.30 hr when isolated hypothalami are incubated in normal

(clear columns) and in calcium-free (striped columns) incubation media. The horizontal bars indicate the 95%

fiducial limits of the CRF estimates. The number of observations and hypothalami included in each column are

indicated in Table 2, together with the relevant numerical and statistical data.

Inc. 1 = Incubation 1. Inc. 2 = Incubation 2.

Figure. Amount CRF – Incubation 2 13·30 hr = 1·0, plotted against incubations under 2·5 mM Calcium (Inc.1, Inc.2) and Calcium Free (Inc.1, Inc.2).
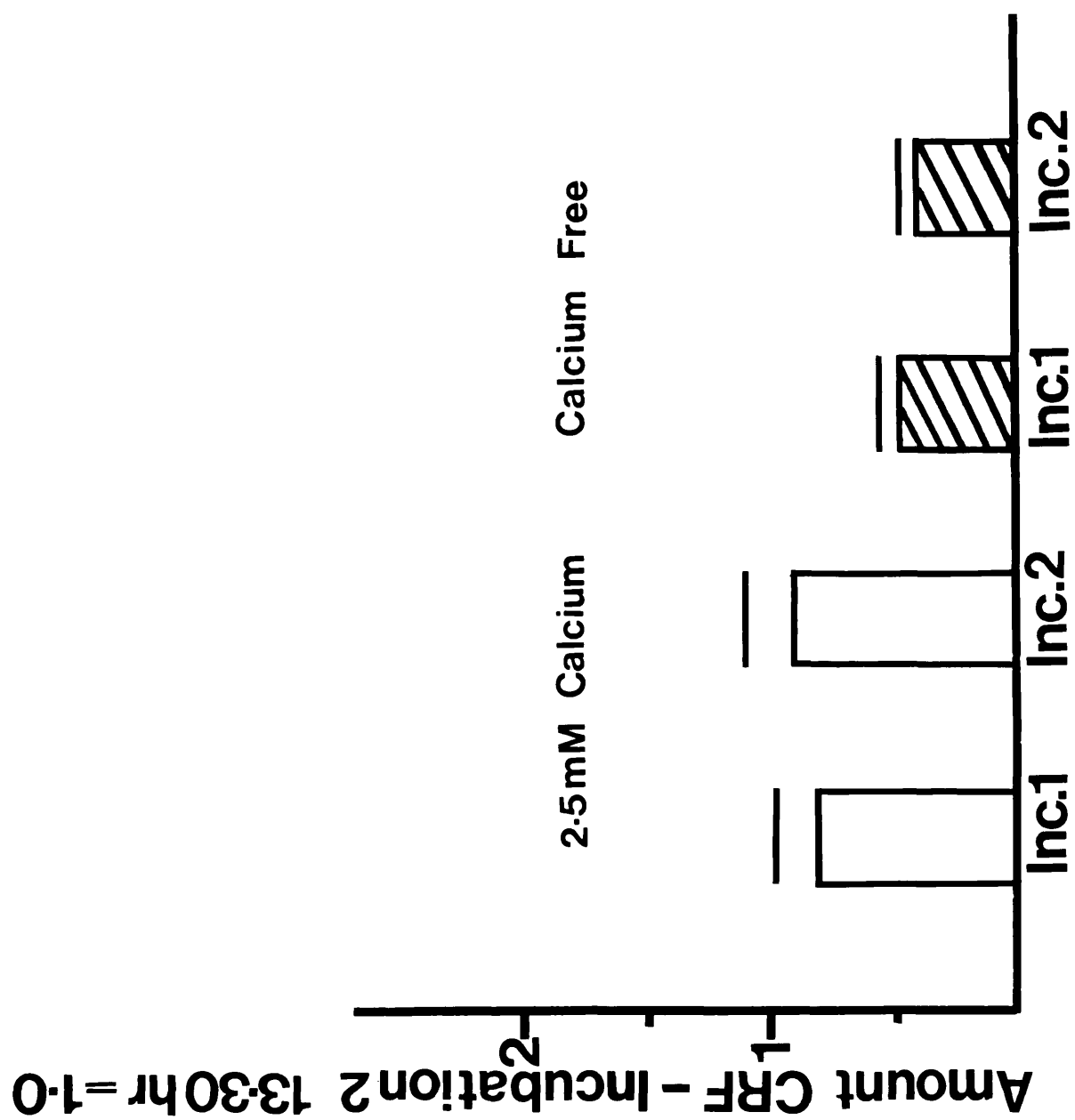
Table 2   Numerical and Statistical Data Pertaining to Figure 12

Incubation Medium	Period of Incubation	Number of Observations in Regression Analysis n	Number of Hypothalami Used in Assays Combined for Regression Analysis	Equivalent Dose of Standard Incubation 2 13.30 hr. MEO.	Equivalent Dose of Unknown Incubation Medium. MEO.	Log Potency ± Standard Deviation	Amount CRF (Potency) and 95% fiducial limits
2.5 mM Calcium	Inc. 1	31	33	0.034	0.043	−0.095 ± 0.047	0.802, 0.992, 0.649
	Inc. 2	33	18	"	0.037	−0.038 ± 0.046	0.917, 1.128, 0.745
Calcium-Free	Inc. 1	48	18	"	0.070	−0.311 ± 0.034	0.488, 0.569, 0.419
	Inc. 2	40	9	"	0.084	−0.387 ± 0.036	0.411, 0.484, 0.349

Inc. 1 = Incubation 1.   Inc. 2 = Incubation 2.   MEO = Dose of CRF, Median Eminence Output.

further emphasises the reduced amounts of CRF in Incubation 1 when hypothalami were incubated in calcium-free incubation media.

In conclusion, these results suggest that the release of CRF both in Incubation 1 and Incubation 2 does not represent the 'passive leakage' or 'diffusion' of CRF from damaged neurons, but is a process sensitive to extracellular calcium concentrations. The results also suggest that the mechanism(s) underlying the secretion of CRF in Incubation 1 and in Incubation 2 cannot be separated by calcium ion-dependence.

8)   Time Course of CRF Release from the Hypothalamus Isolated and
     Incubated In Vitro at 13.30 hr and 16.00 hr - Comparison of
     the Amounts of CRF Released Investigated Over Short and Longer
     Periods of Incubation

Hypothalami were removed from animals at 13.30 hr and 16.00 hr,
and the secretion of CRF assayed at the end of various periods of
incubation.   The relative potency was calculated with reference to
the potency of Incubation 2 at 13.30 hr which was allocated a potency
of 1.0 (see Methods and Assay of CRF).   13.30 hr was chosen as a time
at which to investigate the time course of CRF release because data
for the secretion of CRF during two consecutive 15 minute incubation
periods, Incubation 1 and Incubation 2 at 13.30 hr, had previously been
obtained in the laboratory - the objective of the experiments was there-
fore to permit a closer examination of the dynamics of CRF secretion at
13.30 hr.   16.00 hr was chosen as the other time at which to investigate
the time course of CRF release because preliminary data obtained with
Incubation 1 and Incubation 2 at different times in the day indicated
that the secretion of CRF from the hypothalamus isolated and incubated
in vitro is increased at 16.00 hr (Thomas, 1977;   Kamstra et al, 1978;
see Circadian Rhythmicity in CRF Secretion from the Hypothalamus Isolated
and Incubated In Vitro, below).   The results of these time-course-of-
CRF release experiments at 13.30 hr and 16.00 hr are presented in Fig.
13, and the relevant statistical data are presented in Table 3.

The secretion of CRF both at 13.30 hr and 16.00 hr would appear
from Fig. 13 to be phasic, the accumulation of CRF in the incubation
medium with increasing durations of incubation showing a series of
increases;   between 0 and 1 ($p < 0.001$), 5 and 15 ($p < 0.001$), and 60
and 120 ($p < 0.001$) minutes of incubation at 13.30 hr;   and between 0
and 2 (although this is uncertain since the amount of CRF at 0 minutes
at 16.00 hr was not determined), 5 and 20 ($p < 0.001$), and 25 and 30

FIGURE 13

The amounts of CRF present after incubating hypothalami for incubation periods of varying duration at 13.30 hr and 16.00 hr. The figure represents the amount of CRF in the incubation media at the end of incubations of varying duration, and therefore describes the accumulation of CRF secreted at the end of various time intervals.

The vertical bars indicate the 95% fiducial limits of the CRF estimates. The line through the points at 16.00 hr is not drawn to the origin (O minutes) since no experiments were performed for this incubation (wash in KRBG) at 16.00 hr. The number of observations and hypothalami included for each point are indicated in Table 3, together with the relevant numerical and statistical data.
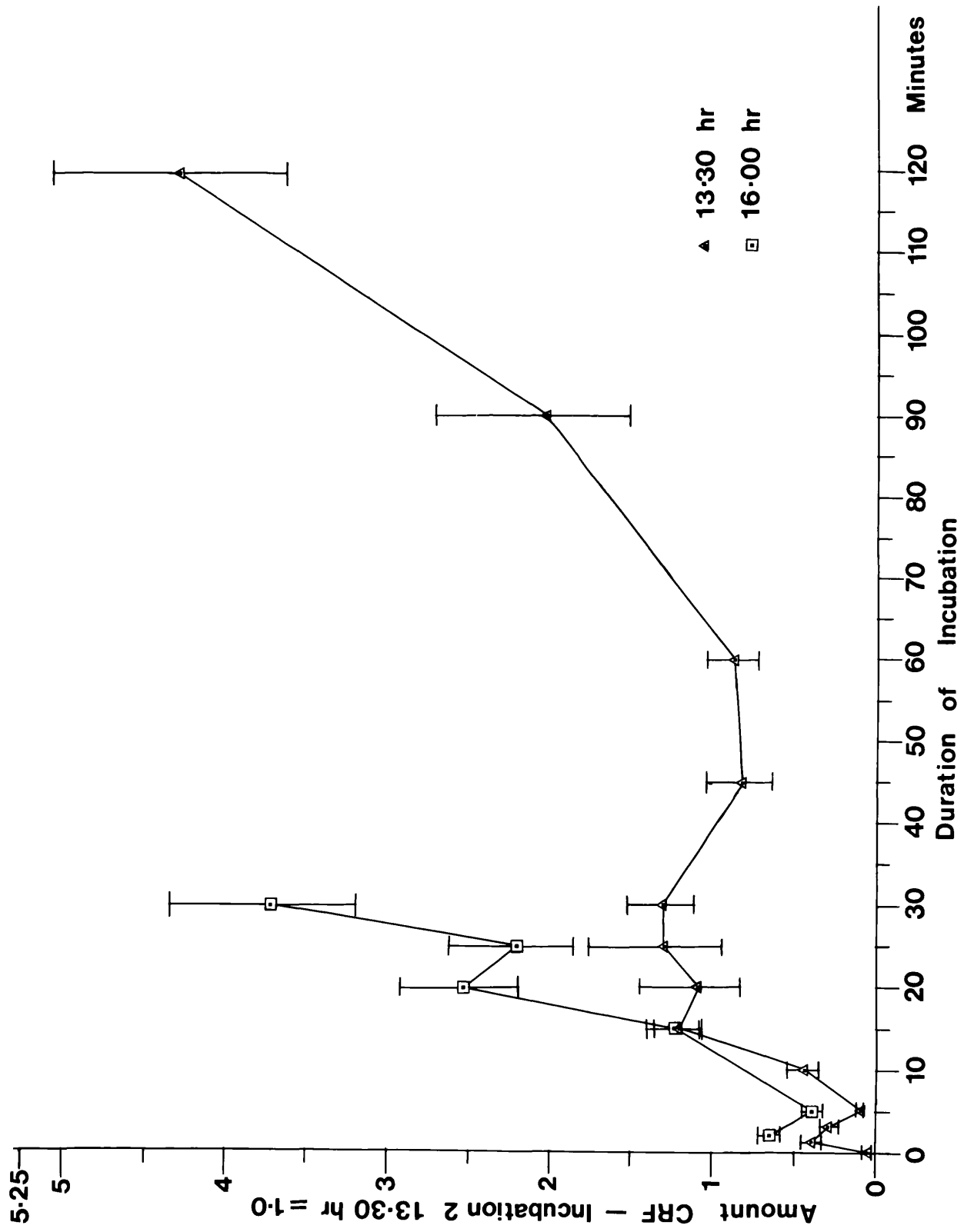
Table 3   Numerical and Statistical Data Pertaining to Figure 13 and Figure 14

Time hr.	Period of Incubation Min.	Number of Observations in Regression Analysis n	Number of Hypothalami Used in Assays Combined for Regression Analysis	Equivalent Dose of Standard Incubation 2 13.30 hr. MEO	Equivalent Dose of Unknown Incubation Medium. MEO	Log Potency ± Standard Deviation	Amount CRF (Potency) and 95% fiducial limits
	O (Wash)	10	4	0.034	0.723	−1.323 ± 0.078	0.048, 0.071, 0.032
	O-1	32	12	"	0.088	−0.407 ± 0.039	0.392, 0.467, 0.328
	O-3	15	4	"	0.122	−0.552 ± 0.045	0.280, 0.348, 0.226
	O-5	17	5	"	0.374	−1.037 ± 0.053	0.092, 0.119, 0.071
	O-10	16	4	"	0.077	−0.350 ± 0.043	0.446, 0.55, 0.362
13.30	O-15 (Inc. 1)	208	48	"	0.028	0.083 ± 0.026	1.211, 1.363, 1.077
	O-20	12	9	"	0.031	0.038 ± 0.056	1.092, 1.449, 0.823
	O-25	13	7	"	0.027	0.111 ± 0.062	1.293, 1.76, 0.95
	O-30	45	12	"	0.026	0.117 ± 0.034	1.309, 1.528, 1.121
	15-30 (Inc. 2)	76	51	"	0.034	0 ± 0.018	1.0, 1.083, 0.923
	O-45	10	6	"	0.042	−0.086 ± 0.049	0.820, 1.049, 0.642
	O-60	21	9	"	0.04	−0.059 ± 0.039	0.874, 1.049, 0.728
	O-90	9	4	"	0.017	0.306 ± 0.056	2.024, 2.71, 1.511
	O-120	24	6	"	0.008	0.633 ± 0.037	4.296, 5.08, 3.633
	O-2	34	9	"	0.052	−0.179 ± 0.02	0.663, 0.725, 0.606
	O-5	35	6	"	0.088	−0.408 ± 0.035	0.391, 0.458, 0.33
16.00	O-15 (Inc. 1)	47	15	"	0.028	0.089 ± 0.031	1.226, 1.407, 1.069
	O-20	36	6	"	0.014	0.404 ± 0.032	2.535, 2.928, 2.195
	5-20	22	6	"	0.018	0.271 ± 0.038	1.866, 2.218, 1.569
	O-25	22	6	"	0.016	0.344 ± 0.038	2.207, 2.624, 1.856
	O-30	30	6	"	0.009	0.571 ± 0.034	3.723, 4.342, 3.192
	15-30 (Inc. 2)	50	12	"	0.044	−0.106 ± 0.031	0.783, 0.898, 0.682

Inc. 1 = Incubation 1.   Inc. 2 = Incubation 2.   MEO = Dose of CRF, Median Eminence Output.

(p$<$0.001) minutes of incubation at 16.00 hr. Since for each

incubation period the medium from a number of hypothalami was pooled

for assay (see Methods) the phasic pattern of CRF secretion in Fig.

13 represents the average phasic secretion of CRF by a number of

hypothalami isolated and incubated in vitro (see Table 3 and Methods),

and this pattern may not necessarily be identical with the pattern of

secretion from an individual hypothalamus isolated and incubated in

vitro. However, the results do imply that there is a reproducible

pattern of phasic CRF secretion from such hypothalami. Fig. 13 also

suggests that there are periods of reduced CRF secretion, that is,

between 1 and 5, 15 and 30, and 30 and 60 minutes of incubation at

13.30 hr, and between 2 and 5 and 20 and 25 minutes of incubation at

16.00 hr. Further, since Fig. 13 represents the cumulative amount

of CRF present at the end of various periods of incubation, the

decreases in the amounts of CRF when the secretion of CRF is reduced

(that is, between 1 and 5 (p $<$ 0.001) and 30 and 60 (p $<$ 0.001)

minutes of incubation at 13.30 hr and between 2 and 5 (p$<$ 0.001)

minutes at 16.00 hr) imply that CRF may also be inactivated or

destroyed within the incubation system. The decreases in the amount

of CRF present of various times of incubation may also be due, however,

to differences in the phasic pattern of CRF secretion in different

groups of hypothalami; this explanation is possible since most of the

points in Fig. 13 represent data obtained with the pooled incubation

media from groups of 2 to 3 hypothalami incubated in two separate

experiments (see Table 3 and Methods). On the other hand, the facts

that the amount of CRF at both 3 and 5 minutes and both at 45 and 60

minutes is reduced at 13.30 hr, and that there is a sharp decrease in

the amount of CRF between 1 minute and 5 minutes at 13.30 hr and

similarly between 2 minutes and 5 minutes at 16.00 hr, together suggest

the decreases of CRF activity are not simply due to variability in the secretory activity of the hypothalamus _in vitro_, but are due to reproducible phases of reduced CRF secretion combined with a process for the inactivation for CRF.

Overall examination of the time course of CRF release at 13.30 hr and 16.00 hr therefore suggests a number of conclusions: Firstly, the pattern of CRF secretion from the hypothalamus isolated and incubated _in vitro_ is phasic. Hypotheses concerning the processes possibly underlying the pattern of CRF secretion from the hypothalamus isolated and incubated _in vitro_ are considered further in the _Discussion_. Secondly, the results in Fig. 13 imply that CRF may be to some extent destroyed or inactivated _in vitro_. It is possible that a proteolytic enzyme may exist in the tissue and incubation medium which destroys or inactivates CRF. The possibility that a proteolytic enzyme is involved is suggested in preference to the possibility that CRF may simply be unstable in KRBG at $37^{O}C$ because, as Fig. 8 (see section above) demonstrates, CRF bioactivity is not lost by storing it in KRBG at $-20^{O}C$ for up to 7 days and then warming it to $37^{O}C$ for assay. If the former hypothesis is correct then estimates of the amount of CRF released by the hypothalamus may be influenced by the activity of a proteolytic enzyme(s) in the hypothalamus; therefore in interpreting the results of the present studies caution should be exercised since estimates of the CRF activity in the incubation media may well be less than the amount of CRF actually released by the hypothalamus. However, the CRF measured was reproducible from assay to assay and fairly consistent in a given set of conditions, so whatever the decrements due to possible inactivation, the amount of CRF measured is likely to be a constant fraction of what may have been present. This is also an argument against such a variable phenomenon as instability of the

molecule but does not of itself exclude scaled amounts of a proteolytic enzyme. It also follows that the addition of a proteolytic enzyme inhibitor to the incubation medium would be a useful experiment to perform in order to investigate this problem.

The reduced rate of CRF secretion seen at 13.30 hr after 15 minutes of incubation (Fig. 13) is maintained until 60 minutes of incubation (however, it is possible that phases of CRF secretion may have been missed between 30 and 45 and between 45 and 60 minutes of incubation). Following 60 minutes of incubation the secretion of CRF at 13.30 hr (actual time 14.30 hr, since the incubations commenced at 13.30 hr) increases; thus, the amounts of CRF present at 90 minutes of incubation are greater than those present at 60 minutes (p < 0.001), and the amounts of CRF at 120 minutes are greater than those at 90 minutes (p < 0.001). These results demonstrate that the hypothalamus isolated and incubated in vitro is viable and continues to secrete CRF for prolonged periods of incubation - further, the fact that the amounts of CRF secreted at the end of 120 minutes of incubation are greater than the estimated total hypothalamic content of CRF at 13.30 hr (p < 0.001) (see Fig. 18 and Table 4 in next section) implies that CRF is synthesised de novo by the hypothalamus isolated and incubated in vitro.

Comparison of the time-course of CRF release at 13.30 hr and at 16.00 hr during the first 30 minutes of incubation in Fig. 13 reveals a number of differences in the pattern of CRF secretion from hypothalami incubated at the two different times. The pattern of CRF secretion during the first 15 minutes of incubation at 13.30 hr and 16.00 hr is similar in that in the first 5 minutes of incubation there is a phase of rapid secretion of CRF, which is greater at 16.00 hr than at 13.30 hr. This phase is then followed by a second phase of CRF secretion

lasting between 5 minutes and 15 minutes of the incubation at 13.30 hr, but from 5 minutes to 20 minutes of the incubation at 16.00 hr. The amount of CRF present at 15 minutes of incubation at 13.30 hr is not significantly different from that at 15 minutes of incubation at 16.00 hr. Thus, during the first 15 minutes of incubation the pattern of CRF secretion at 16.00 hr is very similar to that at 13.00 hr, and this finding is summarised by the lack of a significant difference in the amount of CRF present in Incubation 1 at 13.30 hr and 16.00 hr shown in Fig. 14. The results presented in Fig. 14, taken from Fig. 13 and from the results in Fig. 17 in the next section, demonstrate the following:-

Consider Time 13.30 hr:

1. During the first 15 minutes of incubation, 0-15 minutes (Incubation 1), there is an output of CRF of 1.211 relative to Incubation 2 at 13.30 hr.

2. No further output of CRF is seen over the next 15 minutes of incubation as shown by comparison of the 0-15 minutes and 0-30 minutes incubation periods.

3. However, if the incubation medium is removed and replaced at 15 minutes of incubation, further production of CRF takes place from the same tissue between 15-30 minutes (Incubation 2).

4. Therefore, during 0-30 minutes there is some form of "local inhibition" of CRF output - this could be due to the accumulation of metabolites or of some other interfering factor, or to the possibility that CRF on itself may influence its own production.

Consider Time 16.00 hr:

FIGURE 14

The amounts of CRF present after incubating hypothalami during various periods of incubation at 13.30 hr and 16.00 hr.

The figure is derived partly from data in Fig. 13 and from data in Fig. 16 and Fig. 17, and represents the amount of CRF present at the end of incubations lasting between 0 and 15 minutes (0 - 15; i.e. Incubation 1), 0 and 30 minutes (0 - 30), and 15 and 30 minutes (15 - 30; i.e. Incubation 2, following the 15 minute Incubation 1) at 13.30 hr and at 16.00 hr. In addition, at 16.00 hr, hypothalami were incubated between 5 and 20 minutes (5 - 20; i.e. a 15 minute incubation following a 5 minute preincubation).

The horizontal bars indicate the 95% fiducial limits of the CRF estimates. The number of observations and hypothalami included for each column are indicated in Table 3, together with the relevant numerical and statistical data.

Inc. 1 = Incubation 1. Inc. 2 = Incubation 2.
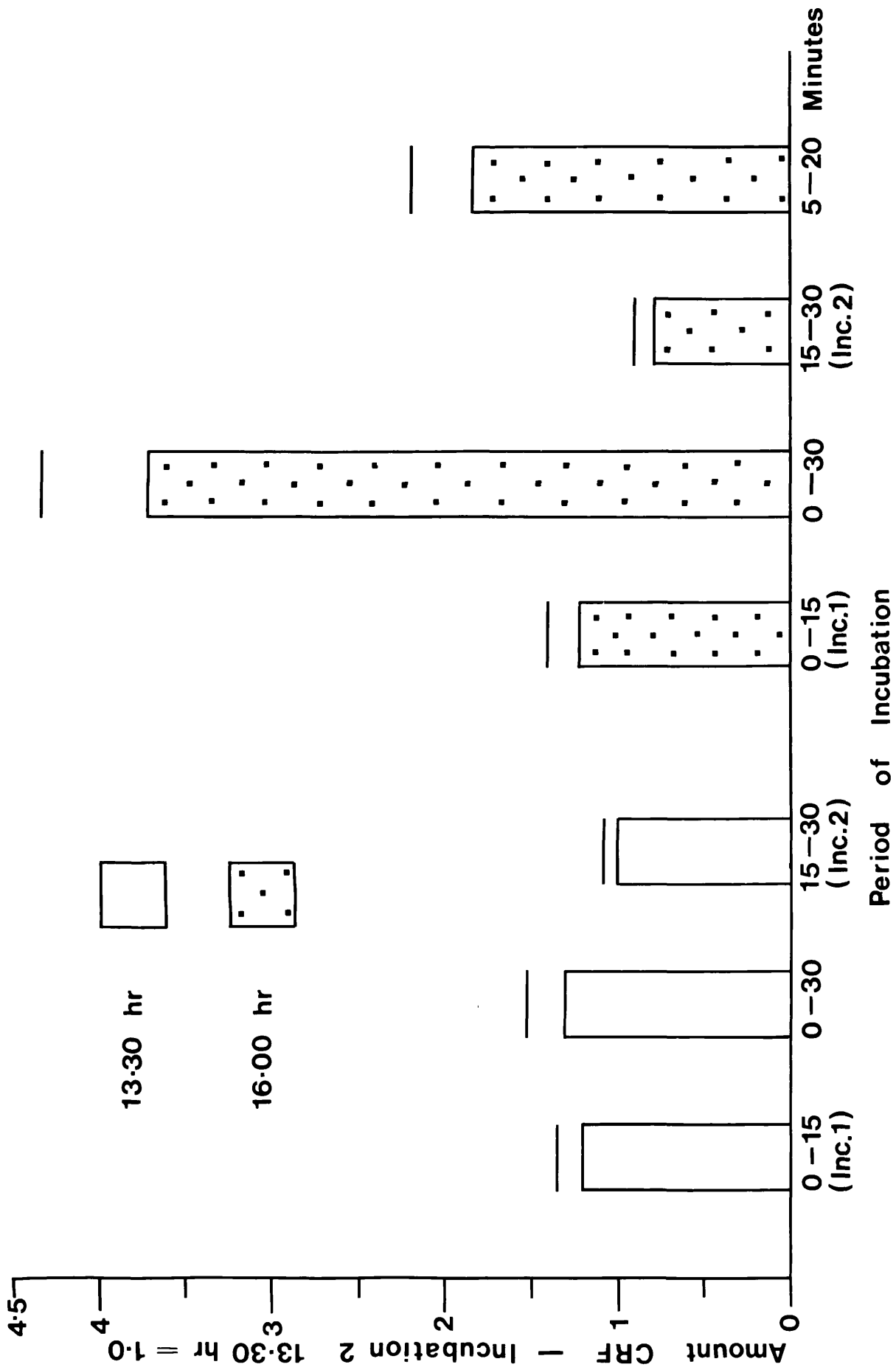
5. During the first 15 minutes of incubation, 0-15 minutes (Incubation 1), the output of CRF is similar to that at 13.30 hr during 0-15 minutes (Incubation 1).

6. During 15-30 minutes (Incubation 2) the output of CRF, although significantly less than during 15-30 minutes (Incubation 2) at 13.30 hr ($p < 0.01$), produces a similar result to the output of CRF during 15-30 minutes (Incubation 2) at 13.30 hr - that is, more CRF is produced during the second incubation.

7. The inhibition of CRF output seen during 0-30 minutes incubation at 13.30 hr is not seen at 16.00 hr. If the amounts of CRF secreted during Incubation 1 are added to the amounts secreted during Incubation 2 and this is compared to the output of CRF in the prolonged incubation 0-30 minutes, all at 16.00 hr, then there appears to be a significant augmentation in the output of CRF at 16.00 hr during the 0-30 minute incubation period ($p < 0.001$).

8. When the short 5-20 minutes incubation is considered with the longer 0-30 minutes incubation at 16.00 hr, it can be seen that the increase in the output of CRF is already present by 15-20 minutes of incubation. Further, the process of augmentation is increased by the increased incubation to 30 minutes.

9. Changes of the incubation media at the critical time of 15 minutes cannot on their own be considered to have disturbed the physiological output of CRF since the changes in output seen at this time are not consistently altered by changes of the media; that is, changes at 13.30 hr at 15 minutes of incubation consistently do not

produce the same results as at 16.00 hr.   It is therefore

concluded that these changes are not due to technique.

10.   Since it can be shown from Fig. 18 that the hormones of

the brain-pituitary-adrenal axis are in a state of flux

at 16.00 hr (compared with 13.30 hr), then it may be

tentatively concluded that whatever the nature of the

inhibition at 13.30 hr it can be "overridden" at 16.00 hr.

11.   It may be seen, therefore, that the output of CRF at 13.30

hr and 16.00 hr during the 15-30 minutes incubation

(Incubation 2, carried out following a change of the

incubation medium) is unaffected by the changes in CRF

output evident during continuous incubations exceeding 15

minutes (i.e. 0-30 minutes incubation).

It may also be seen from the results presented in the next section
that the secretion of CRF in Incubation 2 is also less affected by the
changes in the secretion of CRF evident in Incubation 1 at different
times of the day; that is, when the secretion of CRF during Incubation
1 and Incubation 2 is examined at a series of times in the 24-hour cycle
(see following section and Figures 16, 17 and 18) it may be seen that
whilst there is a 3.8-fold variation in the secretion of CRF in
Incubation 1 (see Fig. 16 and Table 4) over the 24-hour cycle, there
is only a 2-fold variation in the secretion of CRF in Incubation 2 (see
Fig. 17 and Table 4); further, when the secretion of CRF in Incubation
1 and Incubation 2 is examined under reversed lighting conditions (see
below and Fig. 19), whilst there is a significant reversed morning/
evening difference in the secretion of CRF in Incubation 1, there is no
significant morning/evening difference in the secretion of CRF in
Incubation 2.   The reason for this "stability" in the secretion of

CRF in Incubation 2 is unclear. However, as implied in the enumerated points above, the results would suggest the possibility that during prolonged incubations, substances which interfere with the secretion of CRF may be accumulated in the incubation medium; alternatively, CRF released into the media may influence its own further output. Removing and replacing the incubation medium prior to the second 15 minutes of incubation (Incubation 2) may therefore prevent these interfering effects seen during prolonged incubations without disturbing the physiological output of CRF, as explained in the points enumerated above. These conclusions are considered further in the Discussion.

9) Circadian Rhythmicity in CRF Secretion from the Hypothalamus
    Isolated and Incubated In Vitro

Plasma corticosterone rhythmicity over the 24-hour cycle was
investigated by taking trunk blood from decapitated rats killed at
specific times over the cycle. Estimates of corticosterone were made
fluorimetrically as described in the methods. The results obtained
from rats kept in "normal" lighting conditions (lights on between 07.20
hr and 19.20 hr) are presented in Fig. 15 (solid line). A significant
(p < 0.01), 2.5-fold peak-to-trough, circadian rhythm in plasma cortico-
sterone levels was established in these animals. Plasma corticosterone
concentrations increased steadily between 14.00 hr and 19.00 hr,
reaching a peak of 29.4 μ g/100 ml plasma at 19.00 hr, significantly
different from the value at 14.00 hr (p < 0.001). Plasma corticosterone
levels then fell between 19.00 hr and 22.00 hr (p < 0.01). This trough
was then followed by a second, but insignificant, peak between 24.00 hr
and 01.00 hr, corticosterone levels then falling again (p < 0.001) slowly
during the rest of the dark period to reach a value of 12.4 μg/100 ml
plasma at 04.00 hr.

In order to examine the circadian rhythm in plasma corticosterone
levels by a statistical curve-fitting procedure, a cosine function
(period, τ, = 24 hr) was fitted to the mean plasma corticosterone
concentration at each of the times indicated by least squares partial
linear regression analysis (see Appendix E). This procedure was per-
formed principally in order to determine the times of the peak and trough
plasma corticosterone levels from the fitted cosine function; thus, by
this method, the peak of the circadian rhythm in plasma corticosterone
levels is at 20.58 hr and the trough is at 08.58 hr. This result is
in agreement with what may be ascertained by visually fitting a curve
to the points in Fig. 15; however, the peak in the circadian rhythm
in Fig. 15 quite obviously occurs before 20.58 hr, at 19.00 hr. The

FIGURE 15

Plasma corticosterone levels in rats at different times in the 24-hour cycle.

Solid line = animals kept under normal lighting conditions, with lights on between 07.20 hr and 19.20 hr.

Broken line = animals kept under a reversed lighting schedule, with lights on between 19.20 hr and 07.20 hr.

The vertical bars indicate the standard error of the mean for each point.

n = 6 animals at each time.
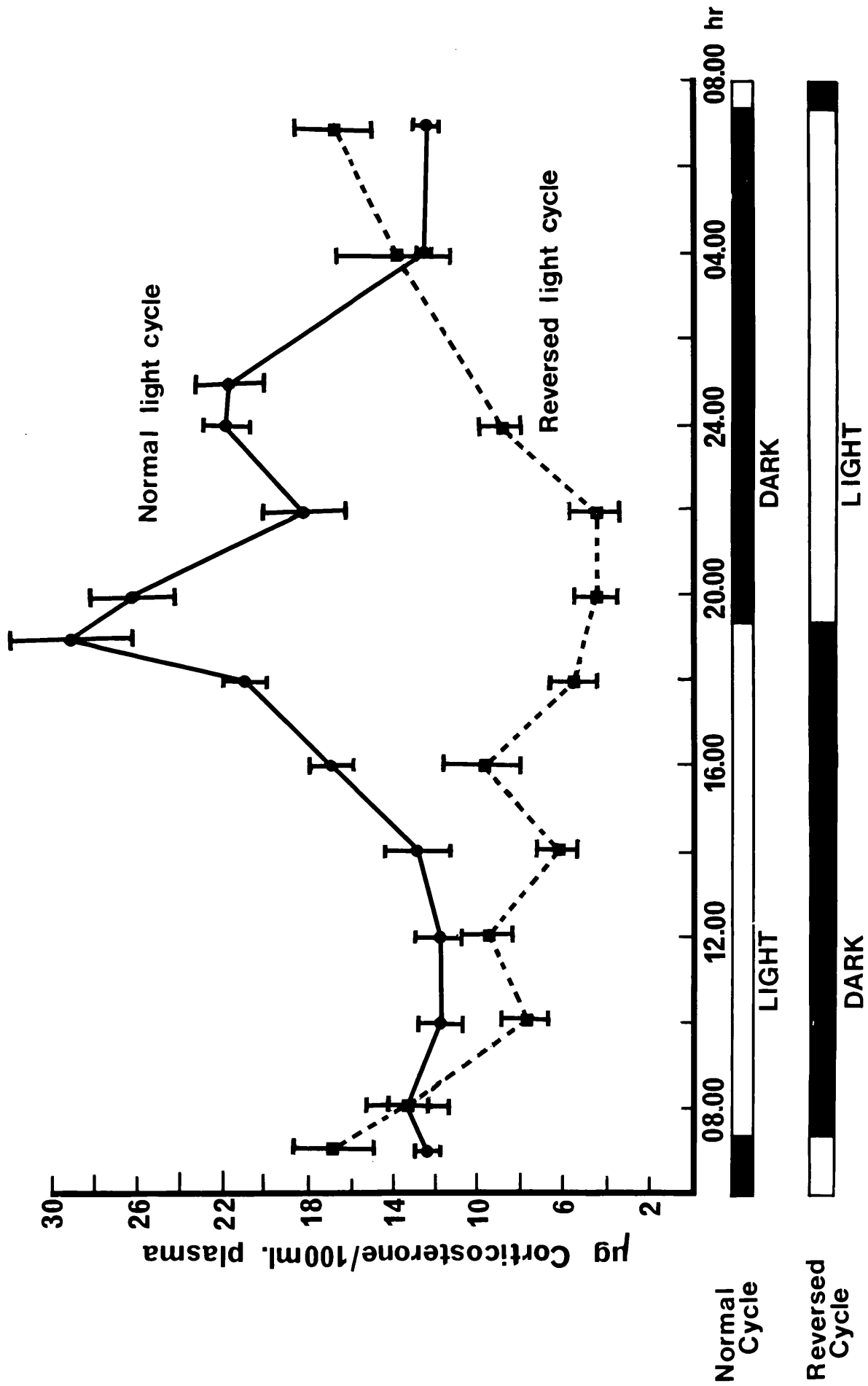
latter further emphasizes the sharp rise and then fall in plasma
corticosterone levels between 18.00 hr and 22.00 hr (see Appendix E).

Animals kept under the same conditions as those used for the determination of plasma corticosterone concentrations were used for investigation of the secretion of CRF from hypothalami. Hypothalami from these animals were removed and incubated at specific times of the 24-hour cycle. The incubation media thus obtained from two consecutive 15 minute incubations, Incubation 1 and Incubation 2, at 07.00, 08.00, 10.00, 13.30, 16.00, 17.30, 19.00 and 01.00 hr were assayed for CRF activity at 13.30 hr on the next convenient experimental day (within, at most, 7 days - see Storage of CRF, above) and the relative potencies calculated with reference to the potency of Incubation 2 at 13.30 hr (see Methods and Assay of CRF, above).

The circadian variation in CRF secretion obtained during Incubation 1 at different times in the day is shown in Fig. 16, and the relevant statistical data are presented in Table 4. There is a significant, 3.8-fold, circadian variation in CRF secretion over the 24-hour cycle demonstrated from Incubation 1; less CRF is released at 07.00 hr than at 19.00 hr (p < 0.001). The amount of CRF released increased gradually during the daylight period, significant increases occurring between 08.00 hr and 10.00 hr, and between 17.30 hr and 19.00 hr (p < 0.001). The peak in CRF secretion demonstrated by Incubation 1 is at 19.00 hr and is later followed by significant decreases between 19.00 hr and 01.00 hr, and between 01.00 hr and 07.00 hr (p < 0.001). The trough in CRF release demonstrated by Incubation 1 occurs between 07.00 hr and 08.00 hr.

FIGURE 16

The amounts of CRF in Incubation 1 when hypothalami are incubated at different times of the day. The vertical bars indicate the 95% fiducial limits of the CRF estimates. The number of observations and hypo-thalami included for each point are indicated in Table 4, together with the relevant numerical and statistical data.
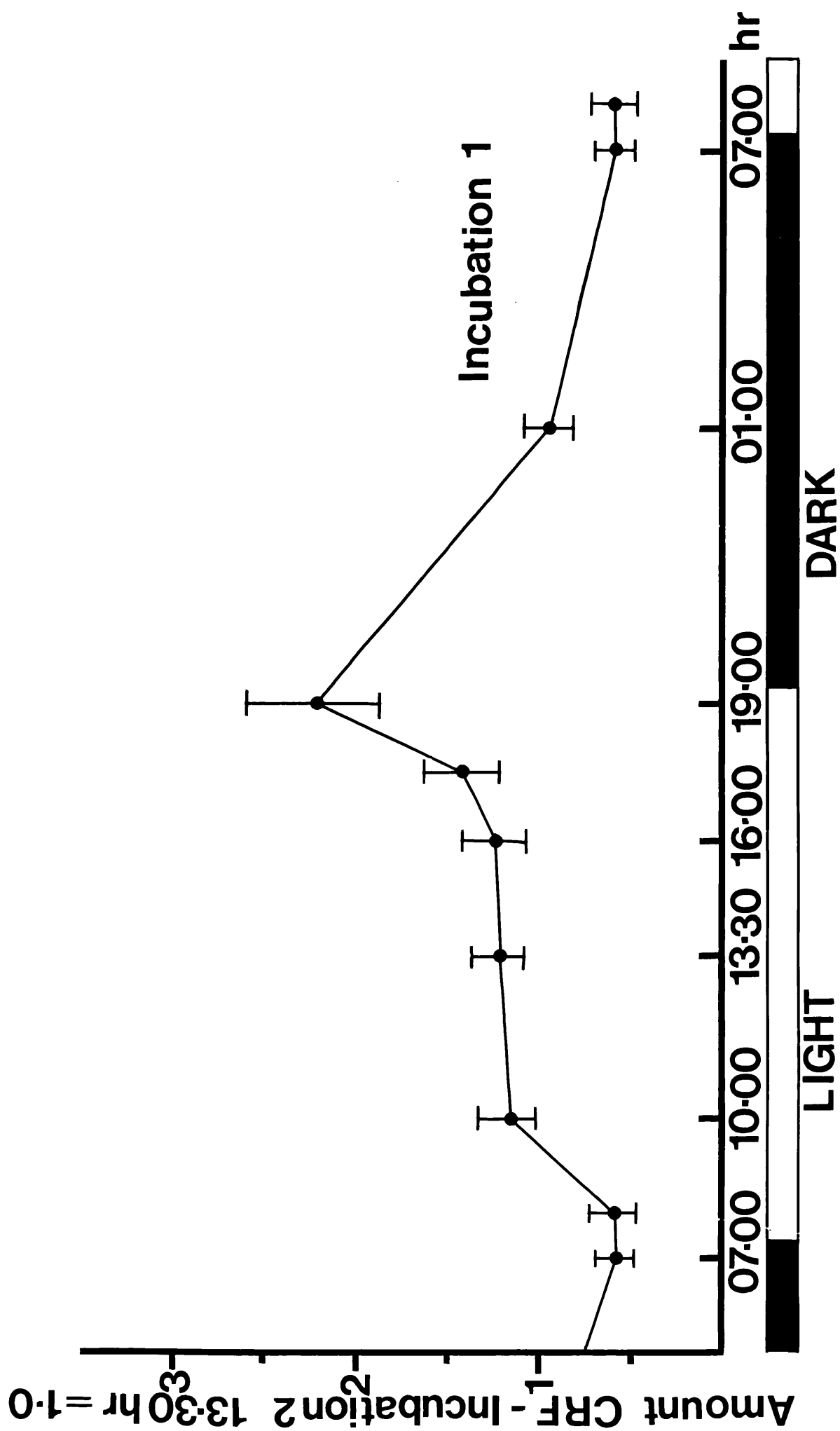
Table 4   Numerical and Statistical Data Pertaining to Figures 16, 17 and 18

Time hr.	Period of Incubation	Number of Observations in Regression Analysis n	Number of Hypothalami Used in Assays Combined for Regression Analysis	Equivalent Dose of Standard Incubation 2 13.30 hr. MEO	Equivalent Dose of Unknown Incubation Medium. MEO	Log Potency ± Standard Deviation	Amount CRF (Potency) and 95% fiducial limits
07.00	Inc. 1	54	18	0.034	0.06	-0.239 ± 0.040	0.576, 0.690, 0.481
	Inc. 2	31	9	"	0.048	-0.151 ± 0.037	0.707, 0.835, 0.598
08.00	Inc. 1	24	12	"	0.059	-0.237 ± 0.045	0.58, 0.718, 0.468
	Inc. 2	17	6	"	0.05	-0.167 ± 0.060	0.681, 0.910, 0.509
10.00	Inc. 1	52	15	"	0.03	0.065 ± 0.029	1.162, 1.323, 1.020
	Inc. 2	45	12	"	0.03	0.063 ± 0.03	1.157, 1.323, 1.012
13.30	Inc. 1	208	48	"	0.028	0.083 ± 0.026	1.211, 1.363, 1.077
	Inc. 2	76	51	"	0.034	0 ± 0.018	1.0, 1.083, 0.923
16.00	Inc. 1	47	15	"	0.028	0.089 ± 0.031	1.226, 1.407, 1.069
	Inc. 2	50	12	"	0.044	-0.106 ± 0.031	0.783, 0.898, 0.682
17.30	Inc. 1	33	15	"	0.024	0.148 ± 0.033	1.405, 1.628, 1.212
	Inc. 2	37	15	"	0.025	0.131 ± 0.034	1.352, 1.573, 1.162
19.00	Inc. 1	27	9	"	0.016	0.343 ± 0.036	2.205, 2.597, 1.872
	Inc. 2	39	12	"	0.027	0.107 ± 0.033	1.281, 1.487, 1.103
01.00	Inc. 1	64	15	"	0.037	-0.03 ± 0.032	0.934, 1.079, 0.809
	Inc. 2	50	12	"	0.065	-0.278 ± 0.030	0.528, 0.605, 0.460
07.00	Content	8	3	"	0.015	0.358 ± 0.058	2.28, 3.134, 1.659
13.30	Content	32	9	"	0.014	0.391 ± 0.033	2.461, 2.86, 2.118
16.00	Content	22	6	"	0.008	0.642 ± 0.046	4.389, 5.448, 3.535

Inc. 1 = Incubation 1.   Inc. 2 = Incubation 2.   MEO = Dose of CRF, Median Eminence Output.

The circadian variation in CRF secretion demonstrated by Incubation 2 performed at specific times in the 24-hour cycle is shown in Fig. 17, and the relevant statistical data are presented in Table 4. A significant, 2-fold, circadian variation occurs in CRF secretion during Incubation 2; less CRF is released at 07.00 hr than at 17.30 hr or 19.00 hr (p < 0.001). CRF secretion in Incubation 2 increased between 08.00 hr and 10.00 hr (p < 0.01), and then decreased between 10.00 hr and 16.00 hr (p < 0.001). This is followed by a second increase between 16.00 and 17.30 hr (p < 0.001). The peak in CRF secretion during Incubation 2 occurs at 17.30 hr and is still apparent at 19.00 hr. This is followed by a decrease between 19.00 hr and 01.00 hr (p < 0.001), the trough being followed by an increase between 01.00 hr and 07.00 hr (p < 0.01).

FIGURE 17

The amounts of CRF in Incubation 2 when hypothalami are incubated at different times of the day. The vertical bars indicate the 95% fiducial limits of the CRF estimates. The number of observations and hypothalami included for each point are indicated in Table 4, together with the relevant numerical and statistical data.
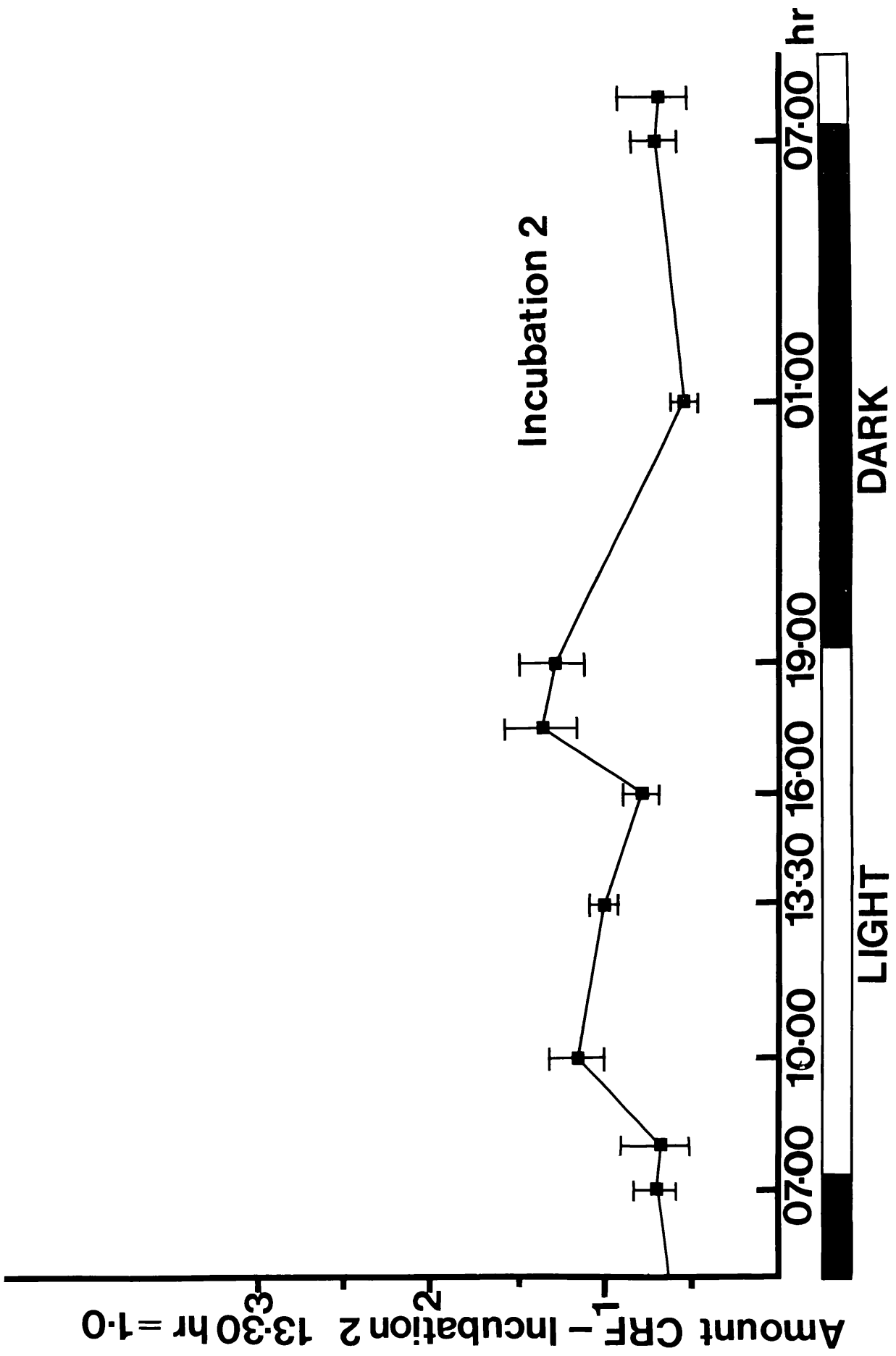
The circadian variations in plasma corticosterone levels and
CRF secretion in Incubation 1 and Incubation 2 are plotted together in
Fig. 18.   The hypothalamic content of CRF was also determined at
07.00 hr, 13.30 hr and 16.00 hr;   the preliminary data are shown in
Fig. 18 and the statistical data are presented in Table 4.   CRF
secretion in Incubation 1 and Incubation 2 parallel each other during
most of the 24-hour cycle.   However, during the time of maximal hypo-
thalamo-pituitary-adrenal activity, during the early afternoon and
evening, there are some differences.   CRF release in Incubation 2 is
less than in Incubation 1 at 13.30 hr $(p < 0.01)$, 16.00 hr $(p < 0.001)$,
19.00 hr $(p < 0.001)$ and 01.00 hr $(p < 0.001)$, but not at 17.30 hr.   The
peak of CRF secretion in Incubation 2 is at 17.30 hr, and is in phase-
advance of the peak in Incubation 1 at 19.00 hr;   however, this peak
is not significantly different from the value at 10.00 hr.   In contrast,
the rise in CRF secretion in Incubation 1 between 17.30 hr and 19.00 hr
$(p < 0.001)$ is conspicuous and is in phase with the maximal rise in plasma
corticosterone levels.   The decrease in CRF release in Incubation 1 is
more steep than that in Incubation 2 and is also more closely in phase
with the decrease in plasma corticosterone levels.   These results may
suggest that CRF secretion in Incubation 1 is more closely linked with
the circadian rhythm of plasma corticosterone levels than is CRF
secretion in Incubation 2;   however, although CRF secretion in Incubation
2 is subject to a smaller variation over the 24-hour cycle, this does
necessarily mean that it is less related to the circadian rhythm in
plasma corticosterone levels.   Further, notwithstanding the fact that
the circadian variations in the secretion of CRF in Incubation 1 and
Incubation 2 do not parallel each other exactly over the 24-hour cycle,
there is a significant association between the changes in the secretion
of CRF in Incubation 1 and Incubation 2 over the day (Table 4;   Spearman

FIGURE 18

Plasma corticosterone levels and the amounts of CRF in Incubation 1 and Incubation 2 at different times in the 24-hour cycle; hypothalamic CRF content shown at 07.00, 13.30 and 16.00 hr.

The standard error of the means for the plasma corticosterone levels are shown in Fig. 15. The 95% fiducial limits of the CRF estimates for Incubation 1 and Incubation 2 are shown in Fig. 16 and Fig. 17 respectively, and in Table 4. The vertical bars indicate the 95% fiducial limits of the estimates of CRF content; the number of observations and hypothalami included in each point are indicated in Table 4, together with the relevant numerical and statistical data.
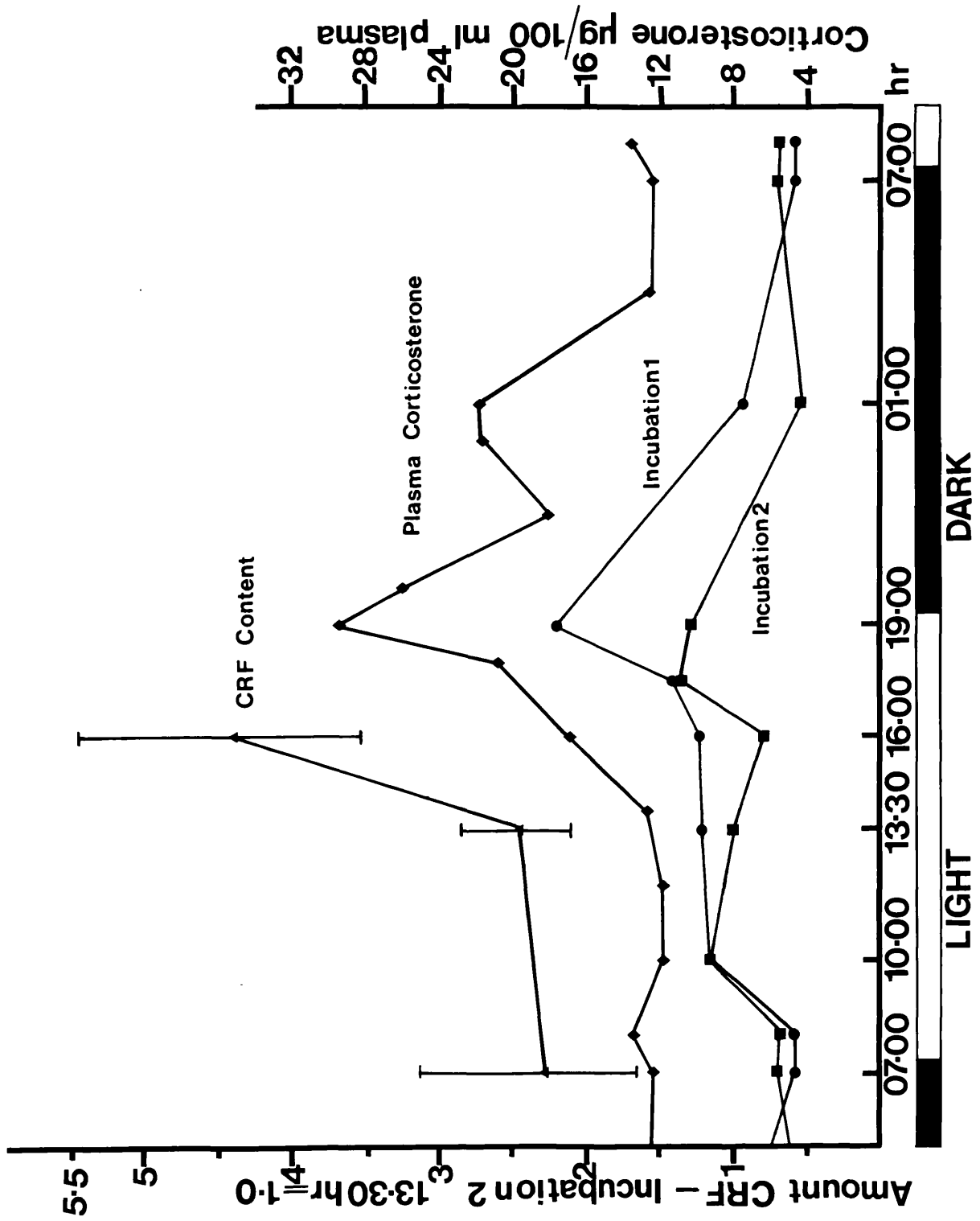
rank correlation coefficient (Siegel, 1956): $r_s = 0.786$, $p < 0.05$,

N = 8). The relation between Incubation 2 and Incubation 1, and an

interpretation of what the results obtained with each incubation may

represent is discussed in more detail in the Discussion.

Although the hypothalamic CRF content data are preliminary and

do not provide sufficient information about the possible changes in

CRF content between 13.30 hr and 19.00 hr, it is apparent that CRF

content increases between 13.30 hr and 16.00 hr ($p < 0.001$), whereas

CRF release in Incubation 1 is not significantly altered and that in

Incubation 2 is decreased ($p < 0.01$). The rise in CRF content there-

fore occurs before the rise in CRF secretion in both Incubation 1 and

Incubation 2. Since tissue hormone content is determined by the

balance between hormone synthesis and release, this suggests CRF syn-

thesis increases in advance of an increase in CRF secretion in Incubation

1 and Incubation 2. In the previous section it was noted that the

secretion of CRF at 16.00 hr is increased (Fig. 13) but only after 15

minutes of incubation (Fig. 13 and Fig. 14); this increase in secretion

is not apparent in Incubation 2 at 16.00 hr in the data presented here,

but was apparent in earlier data from this laboratory (Thomas, 1977;

Kamstra et al, 1978). Thus whilst Figures 16, 17 and 18 show no

increase in CRF secretion at 16.00 hr, when the hypothalamus is incubated

continuously for 30 minutes an increase in CRF secretion is seen to

exist at 16.00 hr (Fig. 13 and Fig. 14). Thus an increase in CRF

secretion may occur in parallel with and following an increase in CRF

content. It is also clear that, because an increase in CRF secretion

is not apparent at 16.00 hr in Incubation 1 or Incubation 2 whilst under

a continuous 30 minute incubation an increase in secretion does exist,

the simplified (in comparison to the time-course-of-CRF-release

experiments) Incubation 1 and Incubation 2 protocol may fail to detect

some of the changes in the phasic secretion of CRF from the hypothalamus isolated and incubated in vitro. This could occur because the two separate 15 minute incubations may obscure changes in the phasic pattern of CRF secretion evident in Fig. 13.

The significance of the increase in CRF secretion both in Incubation 1 and Incubation 2 between 08.00 hr and 10.00 hr is unclear, especially as this is not accompanied by an increase in plasma cortico-sterone levels; however, it is also true that the sensitivity of the pituitary and adrenal glands to CRF and ACTH respectively is at its lowest at this time (see Introduction). It is possible that the increase may be related to the rise in plasma corticosterone levels during the early afternoon; that is, 5 hours after the increase in CRF secretion. Further, and more likely, the increase in CRF secretion may have a "priming" function in increasing the sensitivity of the pituitary gland to CRF, in agreement with the finding that the sensitivity of the pituitary gland to CRF increases during the day (see Introduction). The sharp rise in CRF secretion in Incubation 1 between 17.30 hr and 19.00 hr may then be associated with the rise in plasma corticosterone levels between 18.00 hr and 19.00 hr (p < 0.05). As far as the exact phase relations between CRF secretion and plasma corticosterone levels is concerned the following may be said: Although the increase in CRF secretion in Incubation 1 and Incubation 2 appear from Fig. 18 to be in phase with the rise in plasma corticosterone concentrations, the increase in CRF secretion in both incubations is in phase-advance of the "peak" of the circadian rhythm in plasma corticosterone levels when the circadian rhythm in plasma corticosterone levels is regarded as a cosine function (see above, and Appendix E). Thus, the peak in CRF secretion in Incubation 1 is at 19.00 hr, approximately 2 hours in advance of the "peak" in plasma corticosterone

levels; and the peak in CRF secretion in Incubation 2 is at 17.30 hr, approximately 3½ hours in advance of the "peak" in plasma cortico- sterone levels. However, it is clear that the cosine function does not provide an appropriate description of the peak of the circadian rhythm in plasma corticosterone levels, as stated above, and the observed data do in fact demonstrate that CRF secretion in Incubation 1 and Incubation 2 increases in phase-advance of the sharp rise in plasma corticosterone levels between 18.00 hr and 19.00 hr. The rise in CRF secretion at 16.00 hr during a 30 minute incubation (Fig. 13 and Fig. 14), however, is clearly in phase-advance of the steep rise in plasma corticosterone levels.

As discussed in the previous section, the amount of CRF present in any incubation may not necessarily represent the total amount of CRF actually secreted by the hypothalamus in vitro since CRF may be destroyed or inactivated in vitro - therefore, it is possible that Figures 16, 17 and 18 and Table 4 may not represent the actual total variation in the amount of CRF secreted at different times in the day, but certainly the major proportion of it, and the apparent 3.8-fold variation in CRF secretion in Incubation 1 and 2-fold variation in Incubation 2 may not be a complete estimate but a proportion of it. Further, as noted above, the Incubation 1 and Incubation 2 incubation procedure may fail to detect some changes in the phasic pattern of CRF secretion. However, it is also true that the incubation conditions used in the present studies were standardized, the incubation protocol used at each time being identical; further, as discussed in the previous section, the pattern of CRF secretion at different times does not vary from one group of hypothalami to the next. Thus, given the standardized incubation conditions and the reproducibility of the pattern of CRF secretion at different times, it may be concluded that

the data in Figures 16, 17 and 18 and Table 4 represent circadian variations in CRF secretion which were obtained under standardized conditions. It may also be noted that the results presented in Figures 16, 17 and 18 and Table 4 differ in some respects from the earlier results obtained previously in this laboratory (Thomas, 1977; Kamstra et al, 1978); all the results, both from the preliminary work and from the later work, are combined in their final calculated form in Figures 16, 17 and 18 and Table 4. Taking into account the 1.97-fold difference in the Incubation 2 at 13.30 hr standard used for the calculation of the results (see Expression of Data and Statistical Methods, Appendix D, Assay of CRF (above), and CRF and Its Bioassay (in the Discussion)), the main points to be noted are:-

For Incubation 1:

1) Smaller amounts of CRF were found in Incubation 1 at 07.00 hr 08.00 hr than in the earlier work (in the earlier presentations the results at 07.00 hr and 08.00 hr were combined); however, the earlier results were ambiguous at these times, there being two groupings of results one indicating a high output of CRF at 07.00 hr and another indicating a low output. This may have been due to some animals continuing their nocturnal activity later into the early hours of the morning than other animals. Whatever the reason for the ambiguity in the early results, further experimentation provided no further evidence of the higher output of CRF at 07.00 hr or 08.00 hr; rather, the results confirmed the earlier data demonstrating a low output of CRF at 07.00 hr. The completed results therefore confirm the earlier data showing a low output of CRF at 07.00 hr, since the earlier data demon-strated a relative potency of 0.9 and the results in Table 4 demonstrate relative potencies of 0.58 at 07.00 hr and 08.00 hr (remembering the approximately 2-fold difference in the Incubation 2 at 13.30 hr standard,

as stated above).

2) The rise in CRF secretion between 08.00 hr and 10.00 hr is not different in the results here from the earlier work, and the plateau from 10.00 hr to 13.30 hr was also confirmed.

3) During the afternoon (the period of maximal hypothalamo-pituitary-adrenal activity as evidenced by the plasma corticosterone levels) differences between the completed results and the earlier work are most evident. Between 13.30 hr and 16.00 hr there is a fall in the output of CRF in Incubation 1 in the earlier results if the new standard Incubation 2 at 13.30 hr is used (this may be done since the standard in <u>Appendix D</u> was calculated including results obtained in the earlier experiments); this is not evident in the finalized results, there being no change in CRF secretion in Incubation 1 between 13.30 hr and 16.00 hr. Following this, there is a sharp increase in CRF output between 16.00 hr and 17.30 hr in the earlier work, but not until between 17.30 hr and 19.00 hr in Fig. 16 and Table 4; however, taking into account the differences in the Incubation 2 at 13.30 hr standard, the increases in the secretion of CRF in Incubation 1 in the afternoon are approximately the same, since at 17.30 hr in the earlier results the relative potency is 3.6 and at 19.00 hr in the present results the relative potency is 2.2 (i.e. 1.8 compared to 2.2). Further, the peak in CRF secretion at 19.00 hr is evident both from the preliminary work and from the completed results. The main difference in CRF secretion in Incubation 1 during the afternoon is therefore only in the time at which there is a rise in CRF secretion.

4) Whereas CRF secretion in Incubation 1 does not appear to fall between 19.00 hr and 01.00 hr from the earlier work, in Fig. 16 and Table 4 a fall is evident in CRF secretion between these times. As already mentioned above, this may be due to differences in the activity

of the animals at 01.00 hr. In any event, the difference in CRF
secretion in Incubation 1 at 01.00 hr is less than 2-fold (taking the
relevant Incubation 2 standard into account) and as Table 4 demon-
strates, a considerable amount of data was collected at this time in
order to verify the results since the initial data could not be
confirmed.

5)   Thus, in Incubation 1 the results here differ from the earlier
data only at 16.00 hr, 17.30 hr, and 01.00 hr; that is, in the rising
and in the descending phases of the peak in the circadian rhythm. The
differences in the afternoon are not, in the main, quantitative, but
rather in the time at which CRF secretion increases in Incubation 1.
This may be due to differences in the animal colony during the course
of the studies, since they were performed over 2 years using a new
colony of animals every 2 weeks, derived from the same genetic pool
however (see Methods). Although environmental changes may also be a
cause for this difference this is uncertain since the room temperature
was constantly monitored, and checks were consistently made on noise
levels, humidity, and on the clock mechanism governing the light cycle.
Variations in the synchronization of the animal colony to the light
cycle may also have caused differences. Although the latter may explain
the difference in the secretion of CRF at 01.00 hr, it is more probable
that it was produced by some phenomenon associated with the feeding
activity of the animals during the night since the peak in the plasma
corticosterone levels at 01.00 hr (Fig. 15) may be related to feeding
activity (see Discussion). Thus, changes in the animal colony may
contribute to the differences between the results presented earlier and
the results presented here.

        For Incubation 2:

6)   The pattern of CRF secretion in Incubation 2 over the 24-hour

cycle is not substantially different in the finalized results from

the preliminary work; this may be expected from the results and

conclusions in the previous section of the Results. Having taken

into account the difference in the Incubation 2 at 13.30 hr standard,

only 2 differences with the earlier work may be ascertained:

Although an increase in CRF secretion in Incubation 2 between 08.00 hr

and 10.00 hr was evident in the earlier work, it is slightly larger

in these results; this difference however is small, since in the

earlier work the relative potency at 10.00 hr was 1.42 and in the

present results 1.16 (i.e. 0.7 compared to 1.16 using the same

Incubation 2 at 13.30 hr standard). The only other difference in the

secretion of CRF in Incubation 2 is at 16.00 hr; thus, whereas in the

earlier work CRF secretion appeared to increase at 16.00 hr, further

experimentation could not substantiate this. However, the results at

17.30 hr and 19.00 hr are not different. The results for Incubation

2 at 16.00 hr were checked in all details (see Expression of Data and

Statistical Methods) at least three times since they also provided an

apparent anomaly with the time-course-of-CRF-release experiments in

Fig. 13; this has been commented upon in the previous section and in

Fig. 14. The results obtained with Incubation 2 at 16.00 hr may be

affected by the increased hypothalamo-pituitary-adrenal activity at

16.00 hr as evidenced by the rising plasma corticosterone levels (Fig.

15) and by the increased secretion of CRF during 30 minute incubations

(Figures 13 and 14). Further, it is also possible, as mentioned above,

that the division of Incubation 1 and Incubation 2 into 2 incubations

may miss a phase of increased CRF output at 16.00 hr, in particular the

rapid release of CRF between 15 and 20 minutes of incubation at 16.00 hr

evident in the timed output of CRF studies shown in Fig. 13, and in the

5-20 minutes incubation shown in Fig. 14. Thus, the results obtained

with Incubation 2 over the 24-hour cycle are unchanged when the

Incubation 2 at 13.30 hr standard is taken into account, the only

important difference occurring at 16.00 hr for the reasons given

above. This, in addition to the evidence considered in the previous

section, would suggest that CRF secretion in Incubation 2 is a stable

phenomenon which is not readily changed by extraneous factors.

In addition to the above-mentioned facts that

1)   the Incubation 2 at 13.30 hr standard used in the results

        presented here was a relevant standard which was obtained

        using the same techniques as those used for the investigation

        of CRF secretion at other times of the day (see Expression of

        Data and Statistical Methods, Assay of CRF, CRF and Its Bioassay,

        Discussion and Appendix D);

2)   the circadian variation in CRF secretion in Incubation 1 may,

        perhaps, to some extent have been influenced by seasonal and

        other changes in the animals prior to delivery, during the

        course of the studies

the differences between the earlier presentations and the results

presented here are attributable to the facts that

3)   a larger number of results were combined for final analysis;

        the number of observations and hypothalami used for each point

        is detailed in Table 4, and it may be seen that the number of

        observations included for each incubation is greater than the

        number which were included in the earlier presentations (see

        Thomas, 1977);

4)   the methods of data analysis were modified as detailed in

        Expression of Data and Statistical Methods; thus, using linear

regression analyses the statistical analysis is less subjective and has a number of advantages over the earlier method of making comparisons with small subgroups of data (see Expression of Data and Statistical Methods).

Thus, the circadian variation in CRF secretion in Incubation 1 and Incubation 2 evident in the earlier results (Thomas, 1977; Kamstra et al, 1978) is confirmed by the data presented here. Whilst the pattern of the circadian rhythm in CRF secretion in Incubation 1 during the afternoon and night was not entirely confirmed, taking the difference in the Incubation 2 at 13.30 hr standard into account, the difference is mainly in the times at which CRF secretion rises in the afternoon and falls in the early hours of the morning; possible reasons for this have been detailed above. The pattern of the circadian rhythm in CRF secretion in Incubation 2 evident in the earlier results is confirmed by the present data when the difference in the Incubation 2 at 13.30 hr standard is taken into account, with the single main difference that CRF secretion in Incubation 2 at 16.00 hr is not increased over that at 13.30 hr; the possible reasons for the latter have been detailed above in conjunction with the results presented in the previous section in Figures 13 and 14. In view of the points made above, the Discussion will consider the results presented here in Figures 16, 17 and 18 and Table 4 as described above, and not the earlier work (Thomas, 1977; Kamstra et al, 1978).

10)   Circadian Rhythmicity in CRF Secretion from the Hypothalamus
       Isolated and Incubated In Vitro - Hypothalami Derived from
       Animals Kept Under Reversed Lighting Conditions

Rats were kept under conditions of reversed lighting, that is,

lights on between 19.20 hr and 07.20 hr, and their plasma corticosterone

levels determined after 7 to 10 days.   A significant (p < 0.001)

circadian rhythm was evident in these levels, with a reversed pattern

of peaks and troughs.   Peak plasma corticosterone levels appeared at

07.00 hr and declined to a trough at 20.00 hr.   These results are

shown in Fig. 15.   The pattern of the circadian rhythm in plasma

corticosterone levels in animals kept under reversed lighting conditions

is different from that in animals kept under normal lighting conditions

in a number of respects.   Firstly, the rise in plasma corticosterone

levels between 22.00 hr and 07.00 hr (p<0.001) in animals kept in

reversed lighting is more gradual than that in animals kept in normal

lighting conditions where the rise occurs in 7 hours or less, between

12.00 hr and 19.00 hr;   and there is not the sharp increase in plasma

corticosterone concentrations at the peak in the circadian rhythm evident

in animals kept under normal lighting conditions.   Secondly, when a

cosine function (period, Υ = 24 hr) is fitted to the mean plasma

corticosterone concentration at each time under reversed lighting

conditions (see previous section and Appendix E), the peak and trough

of the circadian rhythm are found to be at 06.22 hr and 18.22 hr respec-

tively.   When these times are compared to the fitted peak, and trough

of the circadian rhythm under normal lighting conditions, that is 20.58

hr and 08.58 hr respectively (see Appendix E), it is evident that the

circadian rhythm under reversed lighting has not fully inverted by 12

hours, but that the peak and trough have been subject to a phase-delay

of approximately 9½ hours.   Thus, it is possible that by 7 to 10 days

of reversed lighting conditions the animals may not have fully reversed their circadian rhythm. However, as suggested in the previous section it is debatable whether cosine functions represent an appropriate description of observed data, and it is clear that the observed peaks and troughs in plasma corticosterone levels are reversed in phase under reversed lighting conditions (see Fig. 15). Thirdly, the plasma corticosterone levels over the 24-hour cycle are lower in animals kept under reversed lighting conditions than in animals kept in normal lighting conditions (see also Haus, 1964) when the 90° phase values are compared ($p < 0.001$; see Appendix E; this is better than comparing mean values since there may be sampling differences under the two conditions), a fact which is also evident from Fig. 15. This difference may be due to differences in the environmental conditions in the two groups of animals (other than lighting schedules; for examples noise cues), or to the possibility that the animals were still adapting to the reversed lighting schedule at 7 to 10 days, as mentioned above. It is also true, however, that circadian rhythms under reversed lighting conditions are not necessarily identical in shape, mean 24-hour level, and amplitude with circadian rhythms under normal lighting conditions (Perkoff et al, 1959; Haus, 1964; Krieger and Hauser, 1978; Reinberg et al, 1978); this is considered further in the Discussion. Fourthly, it can be seen from Fig. 15 (and from Appendix E) that there is a small rise in plasma corticosterone levels during the trough under reversed lighting conditions, between 14.00 hr and 16.00 hr - this rise, however, is insignificant, and the possibility that it is associated with any disturbance caused by cleaning and replenishment of food and water containers at this time (see Methods) may be discounted.

Animals kept in the same reversed lighting conditions were used for investigation of CRF secretion from hypothalami, at 07.00 hr and

19.00 hr, 7 to 10 days following reversal of the light cycle. Hypo-
thalami obtained at 07.00 hr and 19.00 hr were incubated for two
consecutive 15 minute incubation periods, Incubation 1 and Incubation
2, and the media assayed for CRF activity at 13.30 hr and the relative
potency calculated with reference to Incubation 2 at 13.30 hr,
allocated a potency of 1.0 (see Methods and Assay of CRF). The
results obtained were at a preliminary stage, but nevertheless are
presented alongside those obtained under normal lighting conditions
(see previous section, Fig. 18 and Table 4) in Fig. 19 and in Table 5.
Bearing in mind the preliminary nature of the results, the following
may tentatively be said: A significant difference exists in CRF
secretion in Incubation 1 between 07.00 hr and 19.00 hr (p < 0.01), more
CRF being released at 07.00 hr than at 19.00 hr under reversed lighting,
in contrast to the pattern in normal lighting conditions where more CRF
is released in Incubation 1 at 19.00 hr than at 07.00 hr (p < 0.001).
However, CRF secretion in Incubation 2 under reversed lighting conditions
is not significantly different at 07.00 hr and 19.00 hr, whereas under
normal lighting conditions there is a significant circadian variation
in CRF secretion in Incubation 2 (p < 0.001). It is probable that, as
only two time points in the 24-hour cycle were examined under reversed
lighting, a peak in CRF secretion in Incubation 2 may have been missed.
Further, as the data are limited in number, it is possible that further
experimentation would reveal a circadian variation in CRF release in
Incubation 2. Another, speculative, explanation also exists. That is,
since under normal lighting conditions the peak in plasma corticosterone
levels is estimated to occur at 20.58 hr by a curve-fitting procedure
(see previous section and Appendix E) and under reversed lighting
conditions the peak is estimated to occur at 06.20 hr (see above and
Appendix E), as mentioned above, the circadian rhythm under reversed

FIGURE 19

The amounts of CRF in Incubation 1 and Incubation 2 when hypothalami are incubated at 07.00 hr and 19.00 hr; the animals were kept either in "normal" lighting conditions (open columns), or in "reversed" lighting conditions for 7 to 10 days (striped columns). The horizontal bars indicate the 95% fiducial limits of the CRF estimates.

The number of observations and hypothalami included for each column are indicated in Table 4 for "normal" lighting conditions, and in Table 5 for "reversed" lighting conditions, together with the relevant numerical and statistical data.

Inc. 1 = Incubation 1.   Inc. 2 = Incubation 2.

Normal lighting: Open columns.

Reversed lighting: Striped columns.

Amount CRF — Incubation 2, 13·30 hr = 1·0
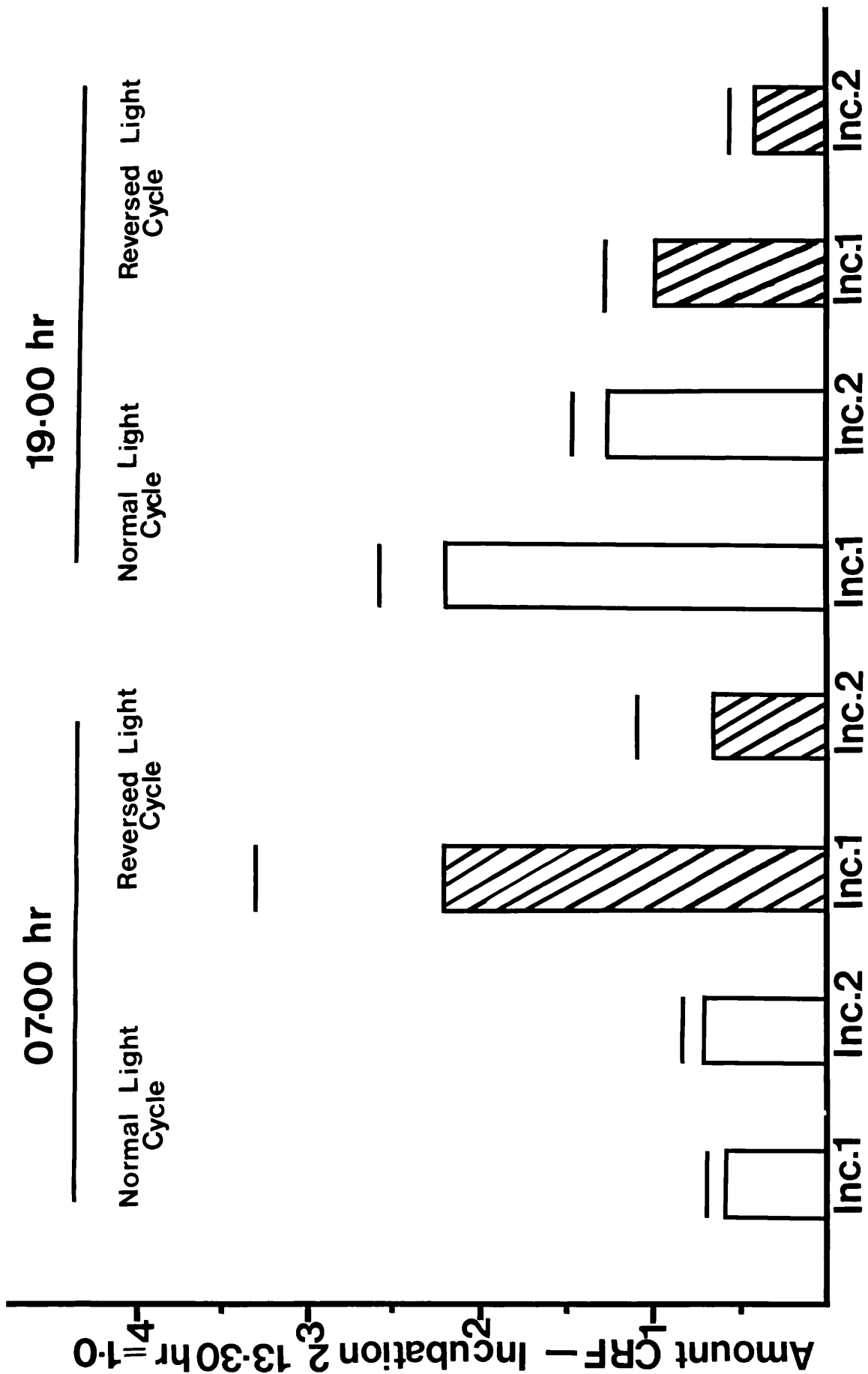
Table 5   Numerical and Statistical Data Pertaining to Figure 19

Time hr -Reversed Light Cycle	Period of Incubation	Number of Observations in Regression Analysis n	Number of Hypothalami Used in Assays Combined for Regression Analysis	Equivalent Dose of Standard Incubation 2 13.30 hr. MEO	Equivalent Dose of Unknown Incubation Medium. MEO	Log Potency ± Standard Deviation	Amount CRF (Potency) and 95% fiducial limits
07.00	Inc. 1	8	3	0.034	0.016	0.345 ± 0.073	2.216, 3.304, 1.486
	Inc. 2	6	3	"	0.052	-0.183 ± 0.082	0.657, 1.107, 0.389
19.00	Inc. 1	11	6	"	0.034	0.003 ± 0.05	1.006, 1.295, 0.781
	Inc. 2	13	3	"	0.081	-0.375 ± 0.058	0.422, 0.565, 0.316

Inc. 1 = Incubation 1.   Inc. 2 = Incubation 2.   MEO = Dose of CRF, Median Eminence Output.

lighting conditions at 7 to 10 days is not fully altered in phase by 12 hours; therefore, it is possible that 07.00 hr and 19.00 hr after 7 to 10 days of reversed lighting do not correspond to 19.00 hr and 07.00 hr under normal lighting conditions, but correspond to approximately 16.30 hr and 04.30 hr under normal lighting conditions; that is, the circadian rhythm under reversed lighting conditions may not have been examined at its peak and trough. It may be seen from Fig. 18 that between 16.30 hr and 04.30 hr there is a definite difference in CRF secretion in Incubation 1, but no, or very little, difference in CRF secretion in Incubation 2. Thus, the apparent lack of a circadian variation in CRF secretion in Incubation 2 under reversed lighting conditions is probably due to the limited amount of data gained and a rhythm could be present, though undetected.

These results (i.e. reversal of the plasma corticosterone rhythm and reversal of Incubation 1, but equivocal results from Incubation 2) imply that the circadian variation in output of CRF from the hypothalamus isolated and incubated in vitro is entrained by photo-periodic cues or associated phenomena in the light/dark cycle, and that a circadian variation in CRF secretion in Incubation 1 is involved in pituitary-adrenal circadian rhythmicity. Further, it may be concluded that the changes seen in the pattern of CRF secretion during the non-reversed Incubation 1 and Incubation 2 are physiological phenomena and are not due to incubation artefacts, since the effect is dependent on phenomena occurring in the whole experimental animal and not in the assay system, its derived tissues, nor its incubation media.

Although this is only preliminary data, the results reinforce the original reasons for verifying Incubations 1 and 2 in situations which affect the experimental animal only, in a manner which is within physiological limits and which does not affect the assay system nor its animals.

DISCUSSION

DISCUSSION

a)   CRF and its Bioassay

Some of the problems associated with the measurement of cortico-
trophin releasing factor (CRF) have been outlined in sections of the
Introduction, Methods, and Results.   The most striking aspect of work
involving the bioassay of CRF is the fact that CRF has, as yet, only
been identified in the following terms:- Firstly, CRF, as its name
implies, is a substance (or substances) which has (or have) the property
of being able, specifically and in small amounts, to cause the release
of corticotrophin (and possibly β-endorphin; Guillemin et al, 1977;
Vale et al, 1978) from the pituitary gland, a discovery which by now
is reasonably well established (Green and Harris, 1947; Harris, 1955;
Guillemin and Rosenberg, 1955; Saffran et al, 1955; Porter and Jones,
1956; Guillemin et al, 1957; Guillemin et al, 1959b; Schally et al,
1960; Guillemin et al, 1962; Schally et al, 1962a; Schally et al,
1962b; Guillemin and Schally, 1963; Schally and Bowers, 1964; Jones
et al, 1977).   Secondly, it is still in debate whether CRF does indeed
exist as a single entity or unique molecule, probably a peptide(s);
thus, although it is reasonable to assume that a specific corticotrophin
releasing hormone (CRH) exists, it is also true that corticotrophin
releasing activity has not, to date, been identified consistently with
a single specific peptide on gel chromatography, ion-exchange
chromatography, or electrophoresis (Schally et al, 1960; Guillemin et
al, 1962; Schally et al, 1962a; Schally et al, 1962b; Guillemin and
Schally, 1963; Schally and Bowers, 1964; Chan et al, 1969b; Pearl-
mutter et al, 1975; Cooper et al, 1976; Jones et al, 1977; Seelig
and Sayers, 1977; Schally et al, 1978a).   Further, in the light of
advances in the field of peptide hormone chemistry in the past 10 years
it would seem more reasonable to reserve any judgement about the

homogeneity of CRF, even if "heterogeneity" in the case of CRF proves

to be an artefact produced either by chemical extraction and purification

procedures (Cooper et al, 1976; Schally et al, 1978b) or by the

susceptibility of the pure CRF molecule to breakdown and cleavage.

On the other hand, it may be said that hypothalamic releasing hormones

are small peptides which do not, as yet, appear to exist as heterogeneous

peptides (see e.g. Schally et al, 1978a). Thirdly, the concept of CRF

has come to mean in practical terms corticotrophin releasing activity

which may be attributed to a substance(s) other than vasopressin,

vasotocin, or enkephalin and other β-endorphin peptide fragments.

Bioassays are therefore "screened" with a number of substances such as

those mentioned above (Portanova and Sayers, 1973a; Buckingham and

Hodges, 1977a; Gillies and Lowry, 1978; Buckingham and Hodges, 1979;

Hashimoto et al, 1979). The concept of a specific and single substance

capable of releasing corticotrophin is therefore still at issue (see

Introduction), and the concept of corticotrophin releasing bioactivity

may represent a more accurate description of most work in the field.

However, it should also be noted that the apparent lack of specificity

of the corticotroph in releasing ACTH to a number of substances may be

attributable to a number of non-physiological effects produced by the

suppression of the hypothalamo-pituitary-adrenal axis in in vivo bio-

assays and by the preparation of pituitary tissue in in vitro bioassays

(see Introduction). To date therefore, since there is as yet no

purified and identifiable molecule(s) of CRH, the effects of its (or

their) presence must be measured. The work set out in this thesis

aims to measure physiological effects of corticotrophin releasing bio-

activity and not the direct measurement of the substance itself. The

main problem encountered thereby in an assay designed to measure

corticotrophin releasing bioactivity was the lack of a purified standard

molecule of CRH. An internal assay standard of releasing activity

has been devised for these investigations. However, there are

limitations. Without a pure standard this approach to the study is

the best available given the absence of molecular and biochemical data

for CRH.

As the results in Fig. 6 and Fig. 7 demonstrate, the assay

provides a linear dose-response relation with which measurements of

CRF bioactivity may be made; this linear, ascending log dose-response

relation shows increasing responses to approximately a fifteen-fold

variation in the dose of CRF. The slope of the linear ascending part

of the log dose-response relation is not steep, a threefold increase

in dose producing only an 11% increase in response; on the other hand,

under the controlled conditions of the experiments the variance of the

responses is small, a characteristic which is established by handling

assay animals for two weeks prior to use (see Fig. 9), and this produces

an assay with a reasonable index of precision (λ = 0.13 $\pm$ 0.062; mean

$\pm$ standard deviation; n = 46). The assay is also sensitive in that

doses of $6.67.10^{-3}$ MEO Incubation 2 at 13.30 hr (Thomas and Sadow,

1975a) or $0.5.10^{-3}$ HE (or 10.5 μg) of the porcine CRF extract (D5

extract) are detected by the assay. However, sensitivity at this level

was not a function specifically sought after in these studies. Other

assay methods claim greater sensitivity but may not have the small index

of precision that was aimed for in this assay. Further, in the absence

of an international standard CRF preparation, assay sensitivity is hard

to define other than in terms of the potency of the CRF preparation.

The limited working range of the assay and relatively low slope of the

dose-response curve do not, on the face of it, provide a good assay -

however, in practical terms the assay is satisfactory because: Firstly,

in the event the fluctuations in the amounts of CRF measured were not

large in relation to the standard production of CRF in Incubation 2
at 13.30 hr; this is because the animals were not pre-treated and
were studied under non-stressed conditions in all the studies presented
here. This obviated the necessity of performing a large number of
assays in order to find the optimal doses of a CRF preparation for the
limited working range of the assay, and invariably this could be done
in less than 4 assays. Secondly, since a large number of responses
could be collected from a number of assays (a possibility permitted by
the ability to perform 3 to 5 assays per week under reproducible
conditions) the variance of the responses is low, thereby compensating
for the low slope of the log dose-response relation. Further, since
data were collected from a number of assays, the responses and estimates
of potency are less affected by variations in the sensitivity of the
assay. However, it is also true that it was rare for estimates of CRF
bioactivity to be arrived at in a single assay, both because of the
considerations above and because of the practical limitations in the
number of tissue samples that could be prepared in a single assay (see
Assay of CRF in Methods).

The results in Fig. 7 demonstrate that the assay measures total
"specific" CRF bioactivity. The assay responded to a purified CRF
extract (Extract D5 - Cooper et al, 1976) with dose-response character-
istics similar to those obtained with the CRF activity contained in
hypothalamic incubation media (Incubation 2 at 13.30 hr); this result
suggests the assay is measuring CRF bioactivity specifically, despite
the possibility that the composition of the corticotrophin releasing
activity in rat hypothalamic incubation media and in porcine hypothalamic
extracts may be dissimilar.

Whether the corticotrophin releasing activity in the two prepar-
ations mentioned above is similar or not cannot be decided simply on the

basis of assay data alone. However, when the whole dose-response curve for the hypothalamic incubation media is considered, it can be seen to be "bell-shaped"; that is, the hypothalamic incubation media do have a distinct corticotrophin release-inhibiting effect when given in supramaximal doses (Fig. 7 - Thomas, 1977), an effect which from the present preliminary results does not appear to be produced by the more pure porcine CRF extract D5. This effect may be due to a corticotrophin release-inhibiting factor (CIF) which may be released from the rat hypothalamus incubated in vitro under certain conditions (Thomas, 1977), or to some, as yet unknown, "ceiling effect" or autoregulatory phenomenon in the putative membrane receptor for CRF or in the synthesis and release of ACTH from the pituitary gland. Another possible explanation of the difference in the dose-response curves of the two preparations (D5 extract and incubate) may be that the extract, although extremely potent in corticotrophin-releasing activity, had been stored for one year in its dry form at $4^{O}C$; no information is available concerning the relative stability of any Inhibiting Factor despite the data accumulated concerning the stability of crude CRF material (see also below). Nonetheless, similar results demonstrating an "inhibitory effect" have been reported both with a purified bovine hypothalamic CRF extract (Guillemin et al, 1957) and with a crude rat median eminence CRF extract (Chan et al, 1969a), suggesting the effect is not peculiar to the method of obtaining CRF from the rat hypothalamus incubated in vitro. The dose required of the D5 porcine CRF extract to produce a response of 7.0 μg corticosterone/100 mg adrenal tissue/hr in the assay was $1.28.10^{-3}$ HE, and that of Incubation 2 at 13.30 hr using the larger 30 mg rat hypothalamic tissue block (see Removal of Hypothalamic Tissue) was 0.034 MEO (using the regression functions in Appendix D); the weight of an individual pig hypothalamus used in the preparation of

the porcine CRF extract was approximately 1.0 g using the figures given by Cooper et al (1976). It may be calculated, therefore, that on a weight basis the CRF bioactivity released from a single rat hypothalamus incubated in vitro at 13.30 hr is 1.25 times as potent as the CRF bioactivity extracted from a single pig hypothalamus. Taken together with the preliminary results obtained with the crude rat CRF extracts (CRF content data in Fig. 18 and Table 3), this result indicates that there is a difference of less than an order of magnitude in the CRF bioactivity which may be obtained from rat hypothalami (either by total extraction or by incubation) and from pig hypothalami (extract obtained from another laboratory) on a weight basis (in the following section the physiological release of CRF activity is discussed vis-a-vis the amount of CRF available for release; in the light of all the results available from the incubation studies it is concluded in this discussion that the release of CRF under these conditions is an active physiological process and not passive leakage). The above estimates also suggest the assay is measuring CRF bioactivity specifically and accurately. It should also be noted, however, that the estimates must be qualified by the fact that the losses of CRF bio-activity in the porcine extract during storage are uncertain (see below).

The use of an internal laboratory-standard CRF preparation (Incubation 3 at 13.30 hr) for the estimation of the amounts of CRF has its limitations, but, as discussed in the section Expression of Data and Statistical Methods, other methods of presentation of data are more direct but more prone to misinterpretation. Thus, presenting data in the form of the responses or percentage increases in response obtained with a fixed dose of each CRF preparation does not give any realistic indication of the relative amounts of CRF present (for the reasons enumerated in Expression of Data and Statistical Methods); further,

given the non-linearity of the log dose-response relation with maximal

doses of CRF and the working range of the assay, this method of

presentation could lead to serious errors of interpretation. Present-

ing the data in terms of the minimal effective dose (MED) of each

preparation, a procedure recommended for purified CRF extracts by

Schally et al (1968), would also be undesirable since this method

would be the most susceptible of all to day-to-day variations in the

sensitivity of the assay. Comparison of the equivalent doses of

different CRF preparations in the linear, working range of the assay,

that is, calculation of relative potencies, although providing an

indirect index of assay data, does provide a direct measure of the

relative amounts of CRF bioactivity in any preparation, and is a measure

which is less affected by variations in the assay sensitivity when, as

in this case, assay data are combined. The difference in the standard

used for the calculation of the results in earlier and later work (both

Incubation 2 at 13.30 hr), as pointed out in the section Assay of CRF

in the Results, is in the main attributable to the difference in the

tissue block removed from the brain; in later work most of the medio-

basal hypothalamus was used, a block of tissue approximately twice the

weight of the median eminence region used in earlier work (see Removal

of Hypothalamic Tissue). This result also implies that the extent of

the region producing CRF is not confined to the median eminence region

but is distributed throughout other areas of the mediobasal hypothalamus,

a finding which is in agreement with the findings of other workers that

CRF is distributed throughout the mediobasal nuclei of the hypothalamus

(Brodish, 1963; Martini et al, 1968; Krieger et al, 1977a; Yasuda et

al, 1977). This fact also justifies the use of the new standard CRF

Incubation 2 at 13.30 hr log dose-response relation for the calculation

of the results here, since in all the work presented here the larger

mediobasal hypothalamic tissue block was used. Further, as a consequence

certain differences exist between the results presented at an earlier stage (Thomas, 1977; Kamstra et al, 1978) and some of the results presented here, a finding which is discussed in the section Circadian Rhythmicity in the Hypothalamo-Pituitary-Adrenal Axis, and in the Results.

The results in Fig. 8 are evidence that the CRF bioactivity in hypothalamic incubation media (specifically Incubation 2 at 13.30 hr) is stable for up to 7 days if kept at -20°C. This finding is in agreement with the results obtained with CRF extracts by other workers who found that crude CRF extracts may be kept for prolonged periods at ~ 4°C (Guillemin et al, 1957; Schally et al, 1968; Chan et al, 1969b) or at -20°C (Yasuda and Greer, 1976c; Gillies et al, 1978a) without loss of CRF bioactivity. Purified CRF extracts, however, are reported to be highly unstable (Guillemin et al, 1957; Chan et al, 1969b). The crude rat CRF extracts prepared in the CRF-content experiments (Fig. 18 and Table 4) were used within 7 days of storage at -20°C, and it is therefore unlikely that any activity was lost in these extracts. The porcine CRF extract (extract D5 - Cooper et al, 1976) was a partially purified preparation and was stored in its air-tight container at 4°C for approximately one year after arrival at the laboratory - although this is a long period, CRF extracts have been reported to be stable for such lengths of time at ~4°C (Schally et al, 1968; Chan et al, 1969b). Once prepared in a stock solution (see Methods) the porcine extract was kept at -20°C and used within 6 weeks. The overall loss of activity in the porcine CRF extract is therefore uncertain.   Nonetheless, the extract retained considerable activity following its storage and preparation, and this suggests that the partially purified extract was stable.   Another aspect of the question of the stability of CRF bioactivity is the possibility raised by the results of the time-course-of-CRF-release experiments (Fig. 13) that CRF, when in contact with tissues, is destroyed or inactivated, possibly

by a proteolytic enzyme(s). This possibility was suggested in the
Results simply as an explanation of the low quantities of CRF present
in certain hypothalamic incubations, and the results by no means
provide conclusive evidence for this; it may also be noted that proteo-
lytic degradation of CRF has been shown to occur during extraction of
CRF from porcine hypothalami (Bristow et al, 1980). Nevertheless,
the existence of a proteolytic enzyme is a reasonable hypothesis and
suggests two courses of action for future experimentation; firstly, the
addition of a proteolytic enzyme inhibitor to the hypothalamic incubation
media; and secondly, the use of a superfusion technique for the
collection of CRF secreted from the isolated hypothalamus in vitro
(e.g. Vermes et al, 1977). It may also be noted that degradation of
LHRH (luteinizing hormone-releasing hormone) has been found to occur
both with hypothalamic fragments (Gallardo and Ramirez, 1977; Negro-
Vilar et al, 1979) and with hypothalamic synaptosomes (Marcano de Cotte
et al, 1980) in vitro; further, degradation of LHRH by hypothalamic
synaptosomes is stimulated by dopamine and is calcium-dependent
(Marcano de Cotte et al, 1980). Whether, in fact, degradation of CRF
is an important effect in the present studies is uncertain. However,
it may also be noted: Firstly, that the pituitary glands were rinsed
in KRBG prior to use in the assay in order to remove blood (see Assay
of CRF in Methods), a possible source of peptide-degrading enzymes; and
secondly, that it is likely that most of the enzymes which destroy
releasing hormones are located intracellularly (Gallardo and Ramirez,
1977). Further, as argued in the Results, all the experiments were
performed under strictly controlled conditions such that any effect
produced by losses of CRF bioactivity in vitro is consistent and does
not seriously affect the validity of the results.

The specificity of the assay has been demonstrated by a number
of criteria. Firstly, previous work in the laboratory (Sadow and
Penn, 1972) has shown that the assay does not respond to vasopressin.

The reason for this is that the pituitary tissue in this assay is not preincubated (see Assay of CRF in Methods), the responsiveness of pituitary tissue isolated and incubated in vitro to vasopressin increasing with increasing preincubation periods (Fleischer and Vale, 1968; Sadow and Penn, 1972; Lutz-Bucher et al, 1977); this effect may be due to the extent to which putative vasopressin receptor sites are occupied by endogenous hypophyseal vasopressin (Lutz-Bucher et al, 1977; Mialhe et al, 1979). The lack of responsiveness to vasopressin of the assay makes it unlikely that any neurohypophyseal vasopressin or any vasopressin in hypothalamic extracts (but not in hypothalamic incubation media; Bridges et al, 1975, see Introduction) interferes in the assay. Secondly, the results in Fig. 11 demonstrate that the hypothalamic incubation media do not have any significant ACTH-like activity (either by ACTH-activity, ACTH-preserving activity, or ACTH-potentiating activity), although the cerebral cortex incubation media did have some, insignificant, ACTH-like activity. The reason for the latter is unclear, especially as the quantities of immunoreactive ACTH are far greater in the hypothalamus than in the cerebral cortex (Krieger et al, 1977b). Nevertheless, the hypothalamic incubation media (specifically Incubation 1 at 13.30 hr) do not contain any detectable ACTH bioactivity, and to this extent the results do not represent measurements of ACTH bioactivity. Significant amounts of ACTH bioactivity have, on the other hand, been found in hypothalamic extracts (Arimura et al, 1967; Dhariwal et al, 1969; Cooper et al, 1976); further, the ACTH bioactivity contained in the crude rat hypothalamic CRF extracts (CRF content data Fig. 18 and Table 4) and in the porcine CRF extract (Cooper et al, 1976) was not assessed in this assay. However, it may be estimated that the quantities of ACTH contained in the extracts (less than 2 mU in the doses of extracts used; from data

by Cooper et al, 1976, and Krieger et al, 1977b) were too small to be detected by the assay (see Fig. 6, ACTH dose-response curve). It is therefore unlikely that the activity of these extracts was due to ACTH bioactivity. Thirdly, the results in Fig. 10 demonstrate that, used in the same doses as hypothalamic incubation media (Incubation 1 at 13.30 hr), cerebral cortex incubation media have less corticotrophin-releasing activity than hypothalamic incubation media. This result is in agreement with that obtained by Bradbury et al (1974) with cerebral cortex incubation media. However, if the weight of cerebral cortex tissue used is taken into consideration, the activity in cerebral cortex incubation media may be as potent as that contained in hypo-thalamic incubation media (see Results); further, although no dose- response effect was elicited by the two doses of cerebral cortex incubation media tested, this does not mean that no dose-response effect exists for other doses of cerebral cortex incubation media. It is therefore difficult, with the present data, to make any conclusions about the specificity of the assay to extra-hypothalamic ACTH-releasing activity except by virtue of the probable small amounts likely to be present. It is also true, however, that corticotrophin releasing bioactivity has been found in extra-hypothalamic brain extracts (cerebral cortex incubation media were used here) using both in vivo (De Wied, 1961b; Witorsch and Brodish, 1972; Dhariwal et al, 1969) and in vitro (Guillemin et al, 1957; Portanova and Sayers, 1973b; Yasuda and Greer, 1976c; Briaud et al, 1978) CRF bioassays. The results in Fig. 10 therefore suggest that this ACTH-releasing activity may be released from brain tissue isolated and incubated in vitro. Although, on purification, the nature of this ACTH-releasing activity may (Guillemin et al, 1957), or may not (Dhariwal et al, 1969) be similar to that of hypothalamic or neurohypophyseal CRF, it is evidently present in far

smaller quantities than CRF in the mediobasal hypothalamus or posterior pituitary (Yasuda and Greer, 1976c; Krieger et al, 1977a) and displays dose-response characteristics different from those of "specific" hypothalamic CRF (Yasuda et al, 1977). Fourthly, the quantities of cyclic-AMP in the hypothalamic incubation media, Incubation 1 and Incubation 2 at 13.30 hr, although sufficient (approximately 1 p mole/ml - see Results and Appendix C) to cause ACTH release in a pituitary segment perifusion system (e.g. Koch et al, 1979), in the present assay system approximately ten times (10 p mole/ml) the quantities of cyclic-AMP in the hypothalamic incubation media had no effect on the release of ACTH (see Table 1 in Results). In the experimental conditions used here, therefore, cyclic-AMP is not a non-specific ACTH-releasing agent. Fifthly, previous work in the laboratory (Thomas, 1977) has shown that TRH (thyrotrophin releasing hormone) does not cause the release of ACTH from hemipituitaries in the assay.

In addition to the experiments discussed above which demonstrate that the CRF bioassay is specific, there are a number of theoretical considerations which make this in vitro assay suitable for the assay of CRF. It has been suggested that the corticotrophin-releasing bioactivity of a putative CRF preparation should be demonstrated both in an in vivo assay and in an in vitro assay for CRF (Guillemin et al, 1957; Guillemin et al, 1959b; Schally and Bowers, 1964); further, in vitro bioassays may be criticized in that the response of the pituitary gland isolated and incubated in vitro may be unlike that of the pituitary gland in situ by virtue of its blood supply, position, and other factors. By these criteria the present studies are not entirely satisfactory. However, for a number of reasons the present in vitro bioassay is preferable to an in vivo bioassay. Firstly, it is likely that the procedures involved in suppressing endogenous CRF release in the in vivo assay (i.e. lesions

of the hypothalamus and treatment with glucocorticoids and tranquilizers)

result in an abnormal response of the pituitary gland to exogenous CRF.

Secondly, although there is little evidence to suggest that CRF causes

the release of ACTH from an extra-pituitary site (Guillemin et al, 1959b;

Witorsch and Brodish, 1972; Lymangrover and Brodish, 1973), in the in

vitro CRF bioassay the site of action of CRF in releasing ACTH is

restricted to the anterior and intermediate lobes of the pituitary

gland (Saffran et al, 1955; Guillemin et al, 1957; Kraicer and Morris,

1976a; Briaud et al, 1978) thereby providing "anatomical specificity".

An in vitro bioassay therefore also provides a means of determining the

site of action of substances which elicit an increase in hypothalamo-

pituitary-adrenal activity in vivo. For example, nerve growth factor

(NGF) has recently been found to elicit an increase in plasma ACTH

levels; whether its site of action is at the hypothalamus (eliciting

CRF release) or at the pituitary gland (stimulating ACTH release) is

unknown (Otten et al, 1979). Thirdly, in the in vivo bioassay CRF-

like artefacts are hard to exclude since ACTH release may be under the

physiological regulation of (i) a "tissue-CRF" (Lymangrover and Brodish,

1973), (ii) neurohypophyseal vasopressin (De Wied, 1961a; Greer et al,

1975), (iii) neurotransmitter agents released from the external layer

of the median eminence and the pars intermedia of the pituitary gland

(Bridges et al, 1973; Björklund et al, 1973; Hiroshige and Abe, 1973;

Palkovits, 1977; Hökfelt et al, 1978), and (iv) nerve growth factor

(NGF; Otten et al, 1979). Further, it is possible that the "sensitivity"

of in vivo assays may be confused with one of the above non-CRF-mediated

mechanisms of ACTH release. Fourthly, the losses of CRF bioactivity

due to degradation (see also discussion above) would be expected to be

greater in an in vivo bioassay than in an in vitro bioassay. However,

the sensitivity of in vivo bioassays implies that losses of CRF bio-

activity are minimal and investigation of this point may be important. Another advantage of the present bioassay is that the release of ACTH by the hemipituitaries _in vitro_ is measured in turn by a bioassay technique, the production of corticosterone by adrenal quarters incubated _in vitro_ (Saffran and Schally, 1955 – see also _Assay of Corticotrophin (ACTH)_ in the _Introduction_). This has the advantage that the total variety of ACTH bioactivity released in response to CRF is measured, rather than a particular species of immunoreactive ACTH peptide material using a radioimmunoassay. As a consequence, the index of CRF bioactivity (μg corticosterone/100 mg adrenal tissue/hr) provided by this assay is physiologically meaningful, and provides a sound reason for assuming that the fluctuations in the amounts of CRF assayed are physiologically significant.

b)   The Release of CRF from the Hypothalamus Isolated and Incubated
        In Vitro

The results in Fig. 12 demonstrate that the non-stimulated

release of CRF from the hypothalamus isolated and incubated in vitro

during Incubation 1 and during Incubation 2 at 13.30 hr is dependent

upon the presence of calcium ions in the incubation medium. This would

also indicate that the secretory mechanism responsible for the release

of CRF both in Incubation 1 and Incubation 2 is dependent upon extra-

cellular calcium, and that the release of CRF during both these

incubations does not have different secretory mechanisms. However,

it is also possible that during prolonged incubations (for example, in

experiments of the type shown in Fig. 13) that the release of CRF may

not be dependent upon calcium ions and that the secretion of CRF from

the hypothalamus isolated and incubated in vitro may not be entirely

dependent on a single, calcium ion-dependent, process because prolonged

incubations in calcium ion-free media were not performed. However

this may be,the results here are in agreement with those of Vermes

et al (1977) who found that the non-stimulated release of CRF from the

hypothalamus in a superfusion system is calcium ion-dependent. On the

other hand, Jones and Hillhouse (1976) found that the basal, non-

stimulated release of CRF from the hypothalamus isolated and incubated

in vitro is not inhibited by removal of calcium ions; removal of

calcium ions from the incubation medium only inhibited acetylcholine-

induced CRF release (Jones and Hillhouse, 1976) and electrically-

stimulated CRF release (Bradbury et al, 1974). These differences may

be due to differences in the depletion of calcium ions in the different

incubation protocols, or to the existence of a non-specific, calcium-

independent, release of CRF from the non-stimulated hypothalamus

isolated and incubated in vitro.   The basal, non-stimulated release

of CRF during Incubation 1 and Incubation 2 in the experiments described

here, therefore, is not a non-specific "leakage" or "diffusion"

phenomenon caused by the removal of the hypothalamus from the rest

of the brain. It has been calculated that the surfaces that are

cut by the preparation of a 100 mg slice of cerebral cortex tissue for

incubation in vitro represents 0.06% of the estimated total outer cell

surface area of the sample (McIlwain and Bachelard, 1971) - here,

where the shape of the hypothalamic tissue block used for incubation

is ellipsoidal or spherical in shape (see Methods), the surfaces that

are disturbed by cutting must represent an even smaller proportion of

the total cell surface area. Further, cut cells "seal" rapidly on

contact with physiological salines (e.g. the rapid sealing of brain

synaptosomes), and this process has been found to be calcium ion-

dependent in cardiac Purkinje fibres (Deleze, 1970). This latter

further emphasises the fact that leakage of CRF from cut surfaces is

insignificant, since in Fig. 12 removal of calcium ions caused a

reduction in CRF release, contrary to what would be expected if leakage

of CRF were important and calcium ions were required for sealing of cut

or damaged cells. It is therefore highly unlikely that cut cells

contribute to any significant leakage of CRF.

The reason for the dependence of the CRF release process on

calcium ions may be as follows:- an influx of calcium ions, following

sodium-dependent depolarization, is associated with the stimulus-

secretion process (Douglas, 1974) for vasopressin in the neurohypophysis

(Nordmann and Dyball, 1978), and a similar mechanism in the release of

CRF is suggested by (i) the fact that calcium ions may be replaced by

strontium ions in stimulating the release of CRF from the hypothalamus

isolated and incubated in vitro, whilst manganese inhibits CRF release

stimulated by 6.0 mM calcium (Jones and Hillhouse, 1976), implying the

existence of "late" calcium channels (similar to those described in

the squid giant axon; Baker et al, 1973) in the membrane of the

putative CRF neuron, and (ii) by the fact that veratridine (which

increases membrane sodium conductance) stimulates the release of

CRF in a hypothalamic superfusion system in a calcium ion-dependent

manner (Vermes et al, 1977). That membrane depolarization is the

"Primary stimulus" for the entry of calcium ions and secretion of

CRF from its neuron is suggested by the facts that high potassium

ion concentrations (Jones and Hillhouse, 1976), veratridine (Vermes

et al, 1977), and direct electrical stimulation (Bradbury et al, 1973;

Bradbury et al, 1974) all stimulate the secretion of CRF from the hypo-

thalamus isolated and incubated in vitro. Further, the fact that the

release of CRF from the hypothalamus incubated in vitro is stimulated

by acetylcholine and serotonin (Jones et al, 1976; Buckingham and

Hodges, 1977b) implies that postsynaptic depolarization of the "CRF

neuron" is produced by these neurotransmitter agents with the consequent

entry of calcium ions (Jones and Hillhouse, 1976). When incubated

for periods of up to 3 hours the hypothalamus loses its potassium

content, reaching a level of 50% of the normal tissue content between

2 and 3 hours of incubation (Bradbury et al, 1973; Bradbury et al,

1974) - during this time, however, the oxygen consumption of the tissue

remains constant (Bradbury et al, 1974) indicating that the tissue is

not subject to necrosis (see also below). The progressive loss of

potassium ions and gain of sodium ions by neural tissue isolated and

incubated in vitro for prolonged periods (Keesey et al, 1965; Bradbury

et al, 1974) may also contribute to a gradual depolarization of the

cells and, in this instance, consequent secretion of CRF. This may,

possibly, account for the continued gradual secretion of CRF during

incubations lasting up to 120 minutes in Fig. 13. However, it is

also true that membrane potentials of -60 mV have been encountered in

cells in cerebral cortex slices incubated in vitro (Keesey et al, 1965),

indicating that maintenance of membrane potentials remains relatively unaffected by placing neural tissues in physiological media. Whilst the above discussion is centred upon the effects possibly produced directly upon the electrochemical gradients and membrane potentials of a "CRF-producing neuron", it is also impossible to say whether any effect is a direct one upon the "CRF-producing neuron" rather than on other elements in a heterogeneous tissue such as the hypothalamus isolated and incubated in vitro. This makes the above discussion and the conclusions derived from Fig. 12 highly speculative in nature, and such discussion will remain, at best, speculative until the "CRF-producing neuron" has been identified and the appropriate micro-electrophysiological techniques (e.g. intracellular recording and ion-tophoresis) applied. It may also be added, however, that the demon-stration of CRF bioactivity in neurosecretory granules and synaptosomal preparations (Mulder et al, 1970; Fink et al, 1972; Mialhe et al, 1979) and the ability of hypothalamic synaptosomal preparations to release CRF in response to a number of agents (Edwardson and Bennett, 1974) provide good evidence for the existence of a "CRF-producing neuron".

Analysis of the time-course-of-CRF-release experiments presented in Fig. 13 and Fig. 14 suggests a number of conclusions, some of which were indicated in the Results. The pattern of CRF secretion both at 13.30 hr and at 16.00 hr is phasic. The first phase of CRF secretion both at 13.30 hr and at 16.00 hr lasts up to 2 minutes (Fig. 13) and is followed by a quiescent phase lasting up to 5 minutes of incubation. The amounts of CRF released during this first phase of secretion are not inconsiderable in relation to the amounts of CRF present at 15 minutes of incubation, but appear to be lost by 5 minutes of incubation, possibly because of degradation of CRF whilst no further release of CRF

occurs between 2 and 5 minutes of incubation (see previous section and below). The largest amounts of CRF secreted during the first 15 minutes of incubation (i.e. Incubation 1), both at 13.30 hr and 16.00 hr, appear during the period between 5 and 15 minutes of incubation, that is, during the second phase of CRF secretion. Thus, most of the CRF accumulated during Incubation 1 at 13.30 hr is secreted between 5 and 15 minutes of incubation (Fig. 13); similarly, Fig. 13 and Fig. 14 both show that most of the CRF secretion at 16.00 hr during the first 30 minutes of incubation occurs between 5 and 20 minutes of incubation. Therefore, both at 13.30 hr and at 16.00 hr there is a rapid phase of CRF secretion lasting up to 2 minutes of incubation, which is followed by a second phase of CRF secretion lasting between 5 minutes and 20 minutes of incubation accounting for most of the CRF accumulated in Incubation 1 - the latter phase of CRF secretion must be calcium ion-dependent as the results in Fig. 12 demonstrate (see also above). A third phase of CRF secretion occurs between 25 minutes and at least 30 minutes of incubation at 16.00 hr (Fig. 13), but not until at least 60 minutes of incubation at 13.30 hr - however, some phases of CRF secretion at 13.30 hr may have been missed between 30 and 45 minutes and between 45 and 60 minutes of incubation if degradation of CRF is taken into account (see previous section and below). That is, it appears that CRF may be released in bursts of activity; when secretion stops, then the amounts of CRF present in the incubation media may be seen to drop. The latter occurs from 2 to 5 minutes at both 13.30 and 16.00 hr; during 15 to 20 minutes at 13.30 hr and at 20-25 minutes at 16.00 hr; and finally again between 30 and 40 minutes at 13.30 hr (see also below). The underlying causes of the patterns of CRF secretion in Fig. 13 are uncertain; however, a number of hypotheses may be considered.

Firstly, the "phases" of CRF secretion may be the result of some aspect of the metabolism of the hypothalamus isolated and incubated in vitro. The respiration and glucose utilization of the hypothalamus incubated in vitro does not provide an adequate explanation of the results since: (i) the oxygen uptake of neural tissues in vitro is linear during two hour incubations (McIlwain and Bachelard, 1971; Bradbury et al, 1974), and (ii) the hypothalami were incubated in approximately 100 times their own volume of KRBG (3 ml), containing 11 mM glucose, and were gassed continuously with 95%/5% O_2/CO_2 - this provides the tissue with metabolic substrates for incubations of at least 3 hours duration (McIlwain and Bachelard, 1971). However, two aspects of the pattern of CRF secretion may have their cause in the metabolism of the hypothalamus isolated and incubated in vitro: (a) In the minute during which the donor animal is exsanguinated and brain tissues are removed, the levels of adenosine triphosphate (ATP) and phosphocreatinine in neural tissues fall considerably (McIlwain and Bachelard, 1971); however, once the brain tissue is placed in oxygen-ated glucose-saline, approximately 70% of the normal levels of ATP and phosphocreatine are restored within 10 minutes. This initial rapid restoration of energy-providing phosphate derivatives may account in some way for the initial phase of CRF secretion during the first 2 minutes of incubation, perhaps by providing ATP for the exocytosis of CRF. It is unclear, however, why CRF secretion should be reduced between 2 and 5 minutes of incubation whilst ATP and phosphocreatinine levels presumably continue to increase to a steady-state level, and to this extent the hypothesis is not entirely satisfactory - however, proper evaluation of any hypothesis cannot be made in the absence of relevant data; (b) Replacing the incubation medium after a 5 minute incubation has little effect on the release of CRF during the subsequent

15 minutes of incubation at 16.00 hr (Fig. 14 - 5 to 20 minute incubation at 16.00 hr) suggesting the accumulation of metabolites during 5 minutes of incubation has little effect on CRF secretion. The accumulation of metabolites or local inhibition of CRF on its own secretion during 15 minutes of incubation may, however, be important since replacing the incubation medium after 15 minutes of incubation (i.e. Incubation 1) has a significant effect on the release of CRF during the subsequent 15 minutes of incubation (30 minute incubations compared with Incubation 2 at 13.30 hr and 16.00 hr in Fig. 14). Whether the effect produced by changing the incubation medium after 15 minutes of incubation (i.e. Incubation 1 and Incubation 2 incubation procedure) is produced by the removal of metabolites or some local inhibition, it may be seen from Fig. 13 and Fig. 14 that the effect of any accumulated metabolites or local inhibition would be to cause a reduction in CRF secretion at 13.30 hr, whereas at 16.00 hr the effect is overridden and can perhaps only be slight. The manner in which metabolites may affect the release of CRF from the hypothalamus incubated _in vitro_ may be through an effect on the membrane electrochemical gradient (since a number of possible metabolites are known to affect the ion content of cortical tissue _in vitro_; Keesey _et al_, 1965) or through an effect on the synthesis or rate of formation of CRF in a "readily-released" pool of CRF (see below). Thus, the above hypothesis may provide some explanation of the patterns of CRF secretion in Fig. 13, to the extent that the effect of accumulated metabolites is to curtail CRF production (13.30 hr), or to override the effect (16.00 hr) allowing the second phase of CRF secretion to occur. Further, the hypothesis provides an explanation for the differences in the patterns of CRF secretion during continuous 30 minute incubations at 13.30 hr and 16.00 hr with the secretion of CRF in Incubation 1 and Incubation 2 at 13.30 hr and 16.00 hr (i.e. Fig.

14). Although a hypothesis is presented above in terms of the accumulation of metabolites, it is equally possible that accumulation of some other substance or factor may account for the results. One reservation may be made concerning the hypothesis of accumulation of metabolites or some other factor(s); that is, since the hypothalami were incubated in approximately 100 times their own volume of KRBG, it is unlikely that the concentrations of any metabolite or factor reached after 15 minutes of incubation are large. However, the hypothalamus incubated in vitro may be sensitive to even extremely small amounts of substances (e.g. pg quantities of neurotransmitters; Jones et al, 1976). Overall consideration of the metabolic consequences of incubating the isolated hypothalamus in vitro therefore provides some explanation of the results in Fig. 13 and Fig. 14, and vindicates the physiological validity of the Incubation 1 and Incubation 2 incubation procedure using changes of the incubation medium at 15 minute intervals.

Secondly, it is possible that the phasic pattern of CRF secretion may be due to the release of CRF from distinct storage pools in the hypothalamus; this hypothesis has been previously suggested in connection with some of the results presented here (Thomas, 1977; Kamstra et al, 1978). CRF appears to be stored in neurosecretory granules (Ishii et al, 1969; Mulder et al, 1970; Fink et al, 1972; Mialhe et al, 1979), and may be associated with a neurophysin (Vandesande et al, 1974; Bock, 1977), although 50 to 60% of the total CRF activity stored in nerve endings may be in a free cytoplasmic form (Mulder et al, 1970). The release of CRF from the hypothalamus incubated in vitro may occur preferentially from a newly synthesised or newly formed store, as does the release of radiolabelled vasopressin from the neurohypophysis in vitro (Sachs and Haller, 1968; Burford et

al, 1973). Although no similar data have been published on the specific activity of released CRF peptide material and CRF peptide material stored in the hypothalamus following incubation of hypothalami with ^{3}H-valine (Insall et al, 1977a), there is no reason to suppose the release of CRF from the hypothalamus in vitro is any different in this respect from the release of vasopressin, or from other instances where hormones (Howell and Taylor, 1967; Grodsky et al, 1969) or neurotransmitters (Collier, 1969; Potter, 1970; Stjärne and Wennmalm, 1970) are released preferentially from a newly formed store in vitro. Although the results of the experiments described here do not provide proof of the two-compartmental storage of CRF, such a hypothesis may provide an explanation of some of the results. Thus, in Fig. 13 and Fig. 14 it is possible that the secretion of CRF during the early stages of incubation (possibly up to 30 minutes of incubation) occurs mainly from a readily-released pool of newly synthesised CRF, whereas the secretion of CRF during the later stages (up to 120 minutes) of incubation occurs mainly from a less-readily released pool of "depot" CRF. Further, if the "depot" pool of less-readily released CRF forms part of the total measurable CRF bioactivity contained in the hypothalamus, then variations in CRF content may not necessarily be manifested as variations in CRF secretion since the "depot" pool of CRF is not readily released - this would explain the results in Fig. 18 showing an increase in CRF content at 16.00 hr with no increase in CRF secretion in Incubation 1 or Incubation 2 (although an increase in CRF secretion during a 30 minute incubation at 16.00 hr is evident in Fig. 13; this difference with Incubation 2 has been considered above), and the results of other workers who have found that CRF secretion and CRF content may vary independently when the hypothalamus is incubated in vitro with

noradrenaline, glycine (Buckingham and Hodges, 1977b), serotonin, or acetylcholine (Jones et al, 1976). Variations in the rate of formation of CRF in the newly-synthesised, readily-released pool of CRF may cause a phasic pattern of CRF secretion (Fig. 13) and also explain the circadian variations in the secretion of CRF in Incubation 1 and Incubation 2 over the 24 hour cycle (Figures 16, 17, 18 and 19) – further aspects of the synthesis of CRF are considered below. It may also be postulated that the release of CRF from the readily-released pool of CRF is subject to (i) regulation by neural afferents, (ii) glucocorticoid and ether stress treatment to the whole animal (Thomas and Sadow, 1975b; Thomas and Sadow, 1976), (iii) the calcium ion concentration of the external medium in vitro (Fig. 12), and (iv) accumulation of metabolites (or other factors) by the isolated hypothalamus incubated in vitro (see above, and Fig. 13 and Fig. 14). A two-compartmental-storage-of-CRF hypothesis, therefore, may explain a number of aspects of the results presented here and it is also compatible with a number of the above-mentioned hypotheses; i.e. the release of CRF from the newly-synthesised, readily-released pool of CRF being calcium ion-dependent and being subject to the accumulation of metabolites by the isolated hypothalamus incubated in vitro. The release of CRF from the less-readily released pool of CRF may be stimulated by prolonged incubations in vitro (Fig. 13) or by pharmacological or electrical stimulation of the hypothalamus. Thus, as already suggested above, the gradual loss of potassium ions and accumulation of sodium ions by the hypothalamus during a two hour incubation (Bradbury et al, 1973; Bradbury et al, 1974) may promote the release of CRF from the less-readily-released pool of CRF. Evidence for this scheme from another source is the finding that calcium ion-dependent basal CRF release from the superfused hypothalamus

is blocked in in vitro conditions by dexamethasone, and by prior in vivo treatment with corticosterone given to the whole animal, whereas veratridine-stimulated (see above) CRF release is not affected by glucocorticoid treatment. These results were interpreted as evidence that veratridine may release CRF from a pool that is distinct from the pool that is responsible for basal CRF release (Vermes et al, 1977).

Therefore, the two hypotheses considered above, that is, the accumulation of metabolites (or other factors) by the hypothalamus isolated and incubated in vitro and the two-compartmental storage of CRF, together provide an explanation of the results presented in Fig. 13 and Fig. 14 in addition to some aspects of the results presented in Fig. 18 and Fig. 19 (see also below). Although the "phasic" pattern of CRF secretion in Fig. 13 may be explained in the light of the above discussion, it is necessary to consider another hypothesis at this point. The secretion of CRF from the hypothalamus may occur episodically. This hypothesis is difficult to establish with the data in Fig. 13, since the secretory episodes are most probably of very short duration, as the data in the first 5 minutes of incubation may suggest, and the sampling in Fig. 13 may have missed some secretory episodes. Pulsatile, episodic, secretion has been evidenced in plasma GH (growth hormone - Martin et al, 1974) and plasma ACTH (Krieger, 1975a) levels. That the pulsatile patterns of adenohypophyseal secretion are regulated by the hypothalamus is suggested by the finding that, in the rat, the pulsatile pattern of GH secretion is attenuated by lesions of the ventromedial nuclei (Martin et al, 1974). It is possible therefore, that an episodic pattern of CRF secretion may in turn regulate an episodic pattern in adenohypophyseal ACTH secretion. Alternatively, pulsatile secretion of CRF may have some other function at the anterior pituitary gland; for example, "pulses" of CRF may be

a requirement for optimal stimulation of ACTH release, as has shown

to be the case for the stimulation of LH release by LHRH (luteinizing

hormone-releasing hormone) in the monkey (Belchetz et al, 1978).

Although the above hypothesis relates primarily to the question of the

overall pattern of CRF secretion in vivo, it may be irrelevant to the

results presented here, obtained in vitro with the isolated hypothalamus

devoid of its neural connections with the rest of the brain; further,

extrapolation of the pattern of CRF secretion seen during incubation

studies to the possible pattern of CRF secretion in vivo may not be

justified where, as in this context, complex neural organization may

be involved.

Another aspect of Fig. 13 is that, since Fig. 13 is a cumulative

representation of the secretion of CRF, decreases in the amounts of CRF

must represent, at the least, periods of reduced or absent CRF secretory

activity; further, since the amounts of CRF are significantly

decreased at certain times, this must either represent inconsistencies

in the secretion of CRF from hypothalami in different experiments, or

losses in CRF bioactivity during periods of quiescence. As suggested

in the Results, the decreases in the amounts of CRF in both curves in

Fig. 13 (13.30 hr and 16.00 hr) are in themselves consistent enough to

warrant consideration of the possibility that CRF bioactivity may be

degraded during the course of hypothalamic incubations. The existence

of a CRF-degrading enzyme(s) in tissues has been discussed in the

previous section, and will not therefore be commented upon further here.

However, if this hypothesis is correct it follows that the amount of CRF

bioactivity present in any incubation is the net balance between CRF

secretion and degradation of CRF; it also follows that the net amount

of CRF present in any incubation may not be the actual total amount of

CRF secreted (as cautioned in the Results) and that the patterns of CRF

secretion in Fig. 13 may represent an attenuation of the actual total amounts of CRF released in each phase of CRF secretion; further, the phase of apparent quiescence in CRF secretion at 13.30 hr, that is, between 15 and 60 minutes of incubation, may actually be a phase during which CRF is released at a slow, steady rate (perhaps inhibited by accumulated metabolites - see above). The overall effect of the activity of a CRF-degrading enzyme may therefore be to mask phases of slow CRF secretion, accentuate phases of arrested CRF secretion, and attenuate phases of rapid CRF secretion. However, it is likely that most CRF-degrading activity takes place intracellularly rather than extracellularly, and that the amounts of CRF degraded are small when the decay of the curve at various points in Fig. 13 is considered. This is because, assuming the secretion of CRF has stopped completely during the periods in which significant losses of CRF occur at a maximal rate (i.e. between 1 and 5 minutes of incubation at 13.30 hr and between 2 and 5 minutes of incubation at 16.00 hr), the maximal rate of degradation of CRF is 20-30% of the maximal rate of secretion of CRF (i.e. between 0 to 1 and 10 to 15 minutes of incubation at 13.30 hr, and between 15 to 20 and 25 to 30 minutes of incubation at 16.00 hr) taking the rate of degradation of CRF into account. However, this figure may be inaccurate since it is difficult to make any assumptions about the rate of CRF secretion in the periods when losses of CRF occur or about the activity of an enzyme (e.g. whether the enzyme is itself inactivated) in the absence of data using added enzyme inhibitors. It may also be noted that the amounts of LHRH lost when incubated or super-fused with hypothalami in vitro are reported to be up to 20-25% of the amounts of LHRH added to the media (Gallardo and Ramirez, 1977; Negro-Vilar et al, 1979).

The time course of CRF release at 13.30 hr (Fig. 13) also shows

that the hypothalamus isolated and incubated in vitro continues to secrete CRF for up to 120 minutes of incubation. This would indicate that the tissue is viable in vitro for this length of time. Other workers have shown a linear uptake of oxygen by neural tissues incubated in vitro for prolonged periods (McIlwain and Bachelard, 1971; Bradbury et al, 1974; see also above). Further, the fact that the amount of CRF secreted at the end of 120 minutes of incubation at 13.30 hr is greater than the estimated initial hypothalamic content of CRF at 13.30 hr (compare Fig. 13 and Fig. 18) implies that CRF is synthesised de novo by the hypothalamus isolated and incubated in vitro. Little is known of the biosynthesis of the releasing factors, although a ribosomal site of synthesis for luteinizing hormone-releasing hormone (LHRH) and somatostatin has been suggested (McKelvy and Epelbaum, 1978), whilst for the smaller tripeptide thyrotrophin releasing hormone (TRH) a non-ribosomal site of synthesis may exist (Mitnick and Reichlin, 1971; McKelvy and Epelbaum, 1978). Cycloheximide in vitro inhibits the incorporation of ^{3}H-valine into a peptide that behaves like CRF on gel filtration and electrophoresis (Insall et al, 1977a), and blocks serotonin-induced release of CRF (Insall et al, 1977b). In vivo, cycloheximide abolishes a peak of CRF content occurring 30 minutes following ether-laparotomy stress, but not the peak occurring at 2 minutes, that is, immediately following the stress (Fujieda and Hiroshige, 1978). These results suggest that CRF synthesis may be ribosomal, and that serotonin-induced release of CRF and stress-induced increases of CRF content are dependent on peptide or protein synthesis. The immediate release of CRF and increase of CRF content following a stimulus may not be dependent on ribosomal synthesis, but, as suggested by Jones and Hillhouse (1977), on cleavage of CRF from a prohormone; further evidence for this scheme of rapid activation of cleavage of CRF

from a prohormone, and slower ribosomal synthesis of the prohormone (and possibly a cleaving enzyme) is, however, lacking. Nevertheless (as implied above), the amounts of CRF released by the isolated hypothalamus incubated in vitro even during short unstimulated incubations, are large in relation to the hypothalamic content of CRF, possibly as much as 50% of the CRF content in the present studies (Fig. 18; 100% or more in the prolonged incubations in Fig. 13), and slightly less than (Buckingham and Hodges, 1977b; Buckingham, 1979), or even more than (Jones and Hillhouse, 1977; Jones et al, 1979b) the total CRF content in other studies, differences probably being attributable to differences in the preparation of hypothalamic tissue used or in the incubation protocols. In contrast, the amounts of luteinizing hormone-releasing hormone (LHRH) released from hypothalami incubated in vitro are 3% (Gallardo and Ramirez, 1977) or less (Negro-Vilar et al, 1979) of the tissue content of LHRH, and the amounts of vasopressin released from the neurohypophysis incubated in vitro are approximately 10% of the tissue content (Sachs and Haller, 1968). This suggests a number of conclusions: Firstly, CRF stores may be small and CRF secretion in vitro may depend not on the release of a large pre-formed store of CRF (except during prolonged incubations), but on the rapid synthesis and release of CRF from a readily-released pool of CRF (see above). Secondly, it is also possible that CRF stores may in fact be larger than the present data suggest since stored CRF may exist largely in an inactive precursor form. To this extent, the hypothesis of Jones and Hillhouse (1977) that CRF is rapidly cleaved from an inactive precursor molecule may be correct. Thirdly, CRF may be released in unusually large quantities in in vitro conditions and although no comparison can be made at present with the secretion of CRF in vivo this could be an effect produced by the removal of the hypothalamus from the rest of the brain and incubation in vitro; further, pre-formed stores of CRF

may be depleted by the decapitation and exsanguination of the donor animal prior to the removal of the hypothalamus. The latter conclusion does not deny the validity of the previous two conclusions since it may be seen that all three conclusions are mutually compatible. The above discussion would therefore suggest that the rate of formation of CRF in the readily-released pool of CRF (considered above) is extremely rapid. Further, since the accumulation of metabolites, or other factors, in the incubation system influences the secretion of CRF (Fig. 13 and Fig. 14 - see above) it is possible that the rate of formation of CRF in the readily-released pool of CRF is influenced by metabolites or its own secretion, as mentioned above. Since the secretion of CRF is also subject to a circadian rhythm (Figures 13, 14, 16, 17, 18 and 19), it is also possible that the rate of formation of CRF in the readily-released pool of CRF is subject to a circadian rhythm.

Although hypothalamic CRF content and CRF release are not necessarily related since CRF may be stored in a less-readily released pool of CRF (see above; and Fig. 18), measurements of CRF content can provide to some extent a measure of CRF synthesis. Following administration of sham-adrenalectomy, laparotomy, leg-break or ether stress in vivo, hypothalamic CRF content has been shown to follow a biphasic pattern of increases, with a rapid (within 2 minutes) increase in CRF content followed by a slow (lasting up to 80 minutes) increase in CRF content (Hiroshige et al, 1971; Hiroshige et al, 1974; Sato et al, 1975; Sakakura et al, 1976). This pattern of fluctuations in CRF content following stress presumably follows a similar pattern of CRF synthesis, and it may be seen from Fig. 13 that a biphasic pattern is also evident in the secretion of CRF from the hypothalamus in vitro during the first 15 to 20 minutes of incubation (both at 13.30 hr and 16.00 hr) following decapitation and exsanguination of the donor animal

and removal of the hypothalamus. This may reinforce the suggestion made above that the phasic pattern of CRF secretion in Fig. 13 is the result of a phasic pattern of CRF synthesis in the readily-released pool of CRF (since variations in the formation of CRF in the readily-released pool of CRF would contribute to variations in the total hypothalamic content of CRF). Thus, the phasic pattern of CRF secretion evident in Fig. 13 may be the result of the stimulation of CRF synthesis (or formation of CRF from a prohormone) caused by decapitation and exsanguination of the donor animal. However, this effect of decapitation and exsanguination and removal of the hypothalamus may apply only to the overall pattern of CRF secretion and synthesis evident in Fig. 13, and not to the circadian variations in the amounts of CRF secreted. This is because the response in hypothalamic CRF content to ether-laparotomy stress is greater in the morning than in the evening (Hiroshige et al, 1969; Takebe and Sakakura, 1972), contrary to the results obtained here with CRF secretion (Figures 16, 17 and 18) implying greater synthesis of CRF in the evening than in the morning. Therefore it is possible that the effect of decapitation and exsanguination of the donor animal may only be to determine the pattern of CRF secretion (and synthesis) from the hypothalamus incubated in vitro, whilst the amounts of CRF secreted are not determined by this stimulus but by physiological variations in the experimental animal, in this instance a circadian rhythm. This does not mean, however, that there is a division between the effects produced by a "stress" and the effects produced by other physiological variations or manipulations in vivo, since ether stress and injection stress treatment in vivo cause an increase in the amount of CRF secreted by the hypothalamus incubated in vitro (Thomas, 1977), but rather the effect of a particular procedure, the decapitation and exsanguination of the donor animal and removal of the hypothalamus, may be to produce a characteristic pattern of CRF

secretion, whilst, as already stated, the effects of physiological variations or manipulations _in vivo_ are to affect the amounts of CRF secreted. The above hypothesis also implies that patterns in the synthesis of CRF seen in the intact animal (i.e. the content data of Hiroshige _et al_, 1971; Hiroshige _et al_, 1974; Sato _et al_, 1975; Sakakura _et al_, 1976) following a surgical procedure are also seen in the hypothalamus isolated and incubated _in vitro_ (i.e. CRF secretion data in Fig. 13), and further experiments using protein synthesis inhibitors given both to the intact animal and applied in _in vitro_ conditions would provide a test of the hypotheses presented above.

The results shown in Figures 13, 14, 16, 17, 18 and 19 demonstrate that hypothalami removed and incubated _in vitro_ at different times of the day show a circadian rhythm in the amounts of CRF secreted. The explanation for this circadian variation in the secretion of CRF from the isolated hypothalamus incubated _in vitro_ may be discussed in terms of the hypotheses outlined above. It is possible that the circadian variation in the secretion of CRF is the result of a circadian rhythm in the synthesis of CRF, that is, in the rate of formation of CRF in a newly-synthesised readily-released pool of CRF. That the circadian variations in the secretion of CRF both in Incubation 1 and Incubation 2 (Figures 16, 17 and 18) are the result of the same underlying circadian rhythm in the rate of formation of CRF in a readily-released pool of CRF is suggested by the fact that there is a significant correlation between variations in CRF secretion in Incubation 1 and variations in CRF secretion in Incubation 2 over the 24 hour cycle (Fig. 18; see Results), and by the fact that, at 13.30 hr, Incubation 1 and Incubation 2 show a similar dependence on extracellular calcium ions (Fig. 12; see above). Further, the "circadian variation" in CRF

secretion between 13.30 hr and 16.00 hr during a 30 minute incubation but not in Incubation 1 or Incubation 2 (Fig. 14; see above) may be due not to the circadian rhythm in the rate of formation of CRF in a readily-released pool of CRF, but to an effect produced by the accumulation of metabolites (or some other factor) during the first 15 minutes of incubation (see above); that is, the secretion of CRF in Incubation 2, following a change of the incubation medium, is not affected by the production of metabolites by the hypothalamus in vitro (Fig. 14; see above), and the circadian rhythm in CRF secretion in Incubation 2 is not therefore an artefact produced by the metabolism of the hypothalamus in vitro but, like Incubation 1, the result of a circadian rhythm in the rate of formation of CRF in a readily-released pool of CRF. It may also be seen from the already-discussed results in Fig. 13 and Fig. 14, and from the results in Fig. 18 (and possibly in Fig. 19) that the secretion of CRF in Incubation 2 is "more resistant" or "less affected" by the changes in CRF secretion evident in Incubation 1 (Fig. 18; and possibly Fig. 19) and evident during 30 minute incubations at 13.30 hr and 16.00 hr (Fig. 13 and Fig. 14). As discussed above, this may be due to the fact that in Incubation 2 fresh incubation medium is provided, thereby removing any metabolites (or other factors such as CRF) accumulated during the first 15 minutes of incubation and providing an incubation protocol which, although not as ideal as a superfusion technique, resembles to some extent the continuous flow of blood through the hypothalamus in vitro. The secretion of CRF in Incubation 2 may therefore represent a more stabilized phase of CRF secretion (and synthesis) than the secretion (and synthesis) during Incubation 1 or during prolonged incubations (see also above concerning recovery of ATP levels in vitro, and patterns of CRF secretion and synthesis, following preparation of the hypothalamus for incubation). Although hypothalamic content of CRF is not directly related to CRF

secretion (for the reasons pointed out above) the circadian variations

in hypothalamic CRF content in the results presented here (Fig. 18)

and in the results of other workers (Ungar, 1967; Hiroshige et al,

1969; David-Nelson and Brodish, 1969; Takebe and Sakakura, 1972;

Ixart et al, 1977) also provide further evidence for assuming that

there is a circadian rhythm in the rate of synthesis of CRF. The

circadian variations in the synthesis of CRF in the intact animal (as

evidenced by the determination of CRF content) may therefore also

occur in the hypothalamus isolated and incubated in vitro at different

times of the day. It should also be re-emphasised, however, that

there is an important difference in the two methods of making inferences

about CRF synthesis; that is, CRF secretion may be more closely related

to newly-formed and readily-released CRF than CRF content which

represents an accumulation of CRF which may not be readily-released.

This does not imply that the two systems are not interchangeable and

in dynamic equilibrium with each other. CRF secretion also represents

a more physiologically relevant parameter than CRF content since it is

the secretion of CRF that is more closely allied to and probably

regulates adenohypophyseal ACTH secretion, not CRF content. Nonetheless,

it is likely that there is a circadian rhythm in CRF synthesis and

release in vivo and that this rhythm in the intact animal is also

manifested in the hypothalamus isolated and incubated in vitro at

different times of the day. The neural pathways and neurotransmitter

agents that may regulate the circadian rhythm of CRF synthesis and

secretion in vivo have been outlined in the Introduction, and the short-

term effects of the disruption of these pathways (in contrast to the

long term effects examined in vivo by: Halasz et al, 1967a; Halasz

et al, 1967b; Palka et al, 1969; Allen et al, 1972; Krey et al, 1975;

Spies et al, 1979) on the secretion of CRF by the isolated hypothalamus

incubated in vitro have been discussed above from a number of aspects.

It may be seen from this discussion that the rapid isolation and
incubation of the hypothalamus in vitro, whilst undoubtedly disrupting
moment-to-moment changes in the signals from extra-hypothalamic areas
and destroying the integrity of the neural networks regulating CRF
secretion, does not disrupt the dynamic state of CRF secretion at the
time at which the animal is sacrificed but, rather, provides a measure
of it. This allows physiological changes in the neural regulation of
CRF secretion produced in the whole animal to be measured in isolated
conditions in vitro. Therefore whilst incubation of the hypothalamus
in vitro destroys physiological neural regulation, it also provides a
measure of the neural regulation of CRF secretion in vivo prior to
sacrifice. Further aspects of the neural regulation of circadian CRF
secretion are considered in the next section of the Discussion.
Although by incubating the isolated hypothalamus in vitro one would
expect that neural function in terms of impulse activity is grossly
disturbed, in this instance where the (putative) neuron concerned
has an endocrine function and its secretion is tonic in nature (by
comparison to neurotransmitter-producing neurons), changes in the
secretion of CRF may occur on a different time scale to those occurring
in neurotransmitter-producing neurons; in this sense the neurons
producing CRF in the hypothalamus may "store" the information in
afferent impulse activity in the biosynthetic processes producing CRF.
That is, if the CRF-producing neuron is like the vasopressin- and
prolactin-producing neurons (Cross et al, 1974), impulse activity may
be more closely related to afferent stimulation per se rather than to
CRF secretion, although impulse activity would obviously still be
involved in secretion. This speculation also provides a further
reason for supposing that CRF secretion within the whole animal prior
to sacrifice is reflected in the secretion of CRF by the hypothalamus
isolated and incubated in vitro immediately following removal from

the animal.

A number of hypotheses concerning the secretion of CRF by the hypothalamus isolated and incubated in vitro have therefore been considered above. Much of this discussion is necessarily speculative since it is difficult to account for any phenomenon in the absence of relevant data by which to assess these hypotheses. It should also be emphasised that full investigation of the processes occurring in the isolated hypothalamus incubated in vitro would require a study dedicated to this problem exclusively, and that this was not the intention of the present studies but rather to investigate the neuroendocrine regulation of the H-P-A (hypothalamo-pituitary-adrenal) axis during the 24-hour, circadian, rhythm. It was felt that whilst investigation and validation of a particular experimental approach is invaluable (viz. calcium-free incubation media, time-course-of-CRF-release, and CRF content experiments), it is also desirable to make a study of physio-logical phenomena (viz. circadian rhythmicity in the H-P-A axis) rather than of possible technical limitations. Nevertheless, a number of important factors likely to govern the secretion of CRF from the isolated hypothalamus incubated in vitro have been considered and some conclusions have been derived from these considerations. A summary of some of the above suggestions which, at present, appear relevant is therefore presented in Fig. 20 and the accompanying legend. It may be seen that the present studies were directed towards the regulation of CRF secretion from the hypothalamus in vivo rather than towards "regulation" that may be produced in vitro. This is inherently more logical because neural regulation cannot be investigated in vitro in the light of the fact that the neural organisation of the hypothalamus in vitro is disrupted. Therefore, the studies were concerned with physiological neural regulation in vivo as it may be examined by the technique of incubating the isolated hypothalamus in vitro. As the

FIGURE 20

Possible scheme for the two-compartmental storage of CRF in the hypothalamus; suggestions made in the text concerning the secretion of CRF from the hypothalamus isolated and incubated in vitro are related to the diagram and summarized below.

Pool A: Newly-synthesised (or biologically active) CRF which is readily released at a rate K_1. CRF secretion in Incubation 1 and Incubation 2 occurs primarily from this pool. The release of CRF from Pool A is calcium-dependent. Metabolites or other factors locally present may affect the rate of synthesis (or formation) or CRF in Pool A and/or the secretion of CRF from Pool A. The rate of synthesis (or activation) of CRF in Pool A and the rate of secretion of CRF from Pool A is subject in vivo to regulation by neural afferents involved in circadian rhythmicity and stress responses in the H-P-A axis; physiological variations in this activity in vivo are evident in the amounts of CRF released in Incubation 1 and Incubation 2 in vitro. The pattern of CRF synthesis (or formation) and secretion in vitro following the removal of the hypothalamus from the animal may be biphasic.

Pool B: Store of CRF which may be filled from newly-synthesised (or formed) CRF in Pool A at a rate K_2. CRF is released from Pool B less readily than from Pool A at a rate of K_3 ($K_3 < K_1$). CRF secretion during prolonged incubations or during acute stimulation may occur at an increased rate from Pool B. The release of CRF from Pool B may be cation dependent. Since Pool B is filled from Pool A at a rate K_2, changes in the synthesis (or formation) of CRF in Pool A may affect Pool B; physiological changes in the rate of synthesis (or formation) and release of CRF in Pool A may also, therefore, occur in Pool B.

Incubation Medium: Ionic composition influences the secretion of CRF both from Pool A and from Pool B. Metabolites accumulate in the medium, in addition to CRF and other substances. CRF may also be degraded in the extracellular medium although degradation may occur primarily intracellularly (viz. Pool B).

The conclusions and speculations given above relate to the results presented in Figures 12-19; a more complete explanation is given in the text preceding this summary.

Median Eminence

Pool A   Pool B

K_2

K_1 K_3

Incubation   Medium

Cell
Body
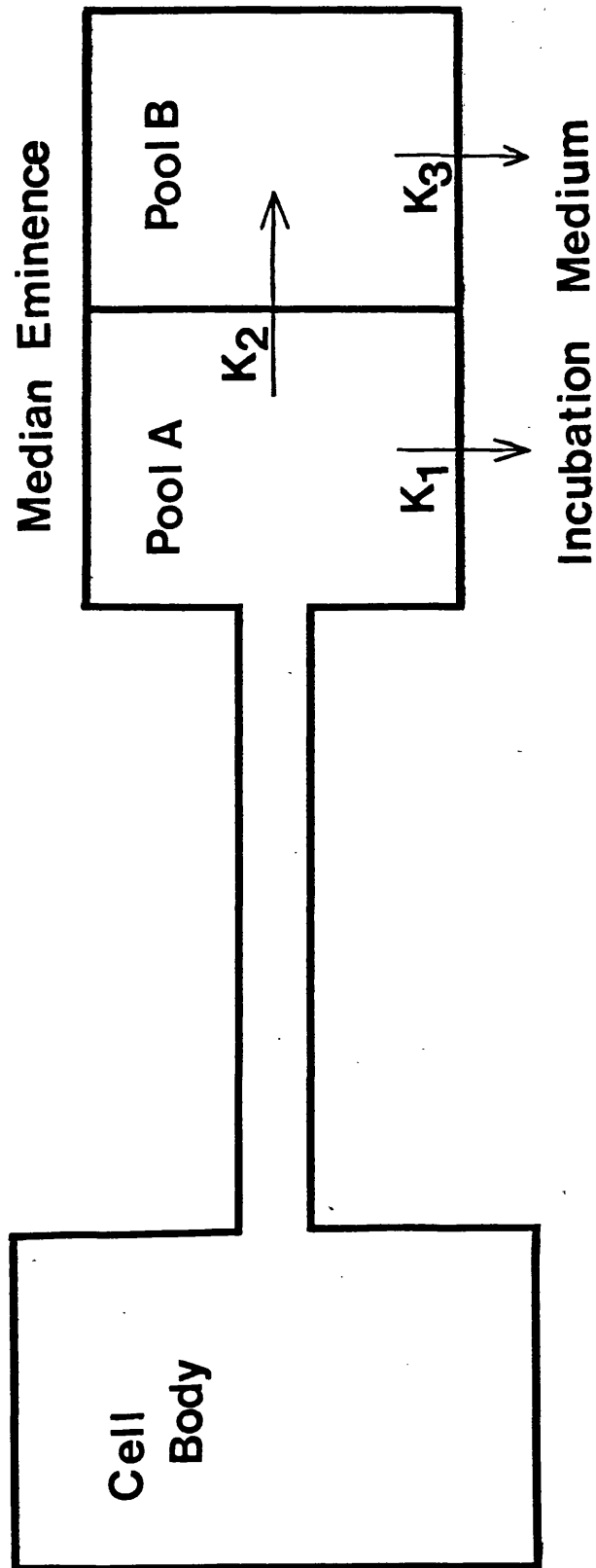
legend accompanying Fig. 20 suggests, the relation of CRF secretion

in vitro to CRF secretion in vivo is of primary importance and has

been considered in this discussion inasmuch as this is possible given

the lack of data available specifically on CRF secretion within the

whole animal at this present time. The latter, at present, is a

major technical obstacle and it may be hoped that, in future, methods

of obtaining CRF (in sufficient quantities) from unstressed intact

animals may be developed.

c)   Circadian Rhythmicity in the Hypothalamo-Pituitary-Adrenal Axis

The results presented in Fig. 15 demonstrate that a circadian

rhythm in plasma corticosterone levels existed in the animals used for

the investigation of CRF secretion at different times of the 24-hour

cycle.   The circadian rhythm obtained with animals kept under "normal"

lighting conditions (with lights on between 07.20 hr and 19.20 hr) shows

a 2.5-fold variation in plasma corticosterone levels with a significant

(p < 0.01) peak-to-trough difference of 17.6 µg corticosterone/100 ml

plasma.   Although precise comparison with circadian rhythms in plasma

corticosterone levels obtained in other laboratories is not warranted

given the differences in the animals and experimental conditions used

in different laboratories, the circadian rhythm in Fig. 15 (obtained

with male rats using an artificial 12:12 hr light:dark schedule, with

food and water available ad libitum; plasma corticosterone levels

were determined fluorimetrically) is like that obtained by other workers

using similar conditions (e.g. Simon and George, 1975) although the

trough levels may be slightly elevated (see e.g. Hiroshige et al, 1969).

The latter is unlikely to be due to any disturbances in the environmental

conditions in which the animals were kept since trough levels appeared

in the early morning, a quiet period in the building.   Further,

collections of samples were postponed if there had been any disturbance

or significant deviation in temperature in the 24 hours prior to any

collection (see Methods).   The secondary peak in the circadian rhythm

during the dark period between 24.00 hr and 01.00 hr (Fig. 15) is not

always evident in the studies of other workers who use independent

samplings of groups of animals, a "plateau" after the peak in the

circadian rhythm usually being found (e.g. Guillemin et al, 1959c;

Hodges and Mitchley, 1970b; Hiroshige et al, 1969; Simon and George,

1975); however, using serial samplings, secondary peaks in the circadian

rhythm in plasma corticosterone levels have been found both in individual

animals (e.g. Honma and Hiroshige, 1978) and in groups of animals (e.g.
Krieger, 1973b). This could indicate that the circadian rhythms in
the animals kept in the conditions used in this laboratory (see Methods)
are highly synchronized; possible reasons for this may be that the
animals were kept in unstressed conditions (see Methods) and that the
animals were handled daily at approximately 16.30 hr thereby possibly
providing an additional cue for the circadian rhythm. The reason for
the occurrence of secondary peaks in the circadian rhythm of plasma
corticosterone levels may be associated with the activities of the
animals during the dark period since the peak locomotor activity of
rats has been found to occur approximately 5 hours following the peak
in corticosterone levels at "lights off" (Honma and Hiroshige, 1978).
Further, the peak in plasma corticosterone levels at "lights off" has
been found to occur immediately prior to the peak in the circadian
rhythm in food intake (Takahashi et al, 1976; Morimoto et al, 1977;
Wilkinson et al, 1979), and the secondary peak in plasma corticosterone
levels may also, therefore, be associated with secondary "peaks" or
meals in the feeding pattern of the animals before the onset of light
(Petersen, personal communication). Whether the intake of food causes
an increase or decrease in plasma corticosterone levels is unclear,
since the actual peak intake of food is associated with decreasing
plasma corticosterone levels (even during reversal of the circadian
rhythms in plasma corticosterone and food intake under "reversed"
lighting conditions; Morimoto et al, 1977) whilst the period leading
up to feeding is associated with an increase in plasma corticosterone
levels (Takahashi et al, 1976; Morimoto et al, 1977). This effect
is especially evident in animals kept under restricted feeding
schedules (Krieger, 1974; Moberg et al, 1975; Morimoto et al, 1977;
Heybach and Vernikos-Danellis, 1979; Morimoto et al, 1979; Wilkinson
et al, 1979). A similar relation has been found to occur between

plasma corticosterone levels and intake of water (Johnson and Levine, 1973; Gray et al, 1978; Levine et al, 1979), and in more general terms it has been suggested that consummatory behaviour results in suppression of pituitary-adrenal activity (Levine et al, 1979); the association between feeding and the circadian rhythm in plasma corticosterone levels is discussed further below. The time of the peak in the circadian rhythm in animals kept under normal lighting conditions is at 19.00 hr, that is, the time of "lights off" in the lighting schedule. The fitted cosine function (Appendix E) gives a peak at approximately 21.00 hr and this emphasizes the fact that the circadian rhythm is not a smooth cosine function but shows a sharp increase in the late afternoon between 18.00 hr and 19.00 hr followed by a sharp decrease between 19.00 hr and 22.00 hr. It should also be emphasized that the present studies were performed with animals kept in an artificial 12:12 hr light cycle (see Methods) and not in natural lighting conditions. The reason for doing this is that the light cycle is controlled both with respect to the light intensity during the day and with respect to the day-length, such that climatic and seasonal variations do not influence the results obtained (plasma corticosterone levels from animals kept in natural daylight conditions have previously been obtained in a similar laboratory - Sadow and Thomas, personal communication). The circadian rhythm obtained with animals kept under "reversed" lighting conditions is more like a cosine function (Fig. 15 and Appendix E) and the fitted curve gives a peak at 06.22 hr, close to the observed peak at 07.00 hr. The differences between the circadian rhythms obtained with animals kept under "normal" lighting conditions and animals kept for 7 to 10 days under "reversed" lighting conditions are considered further below.

Since the plasma corticosterone levels in Fig. 15 represent

the "total" corticosterone concentrations in contrast to the free

or bound corticosterone concentrations it is also important to

consider the circadian rhythm in corticosteroid binding activity

(transcortin and albumin). Circadian rhythms in corticosteroid

binding activity (CBA) have been reported recently for the rat;

Ottenweller et al (1979) found a bimodal rhythm in CBA with a peak

occurring in the morning and a second major peak occurring in the

evening in phase with the peak in plasma corticosterone levels,

whereas Calvano et al (1979) found the peak in CBA to occur 6 hours

following the peak in plasma corticosterone levels. It is unclear

how the rhythm in CBA may be regulated: Although long-term injections

of ACTH cause a decrease in transcortin (Acs et al, 1967) whilst

adrenalectomy causes an increase in transcortin (Gala and Westphal,

1966), both these effects are contrary to what would be expected if

the rhythms in plasma ACTH or corticosterone regulated the circadian

rhythm in CBA. Further, in the short-term, increases in CBA occur

in the morning in the absence of an increase in plasma corticosterone

(Ottenweller et al, 1979) or with a phase-delay of 6 hours following

the peak in plasma corticosterone levels (Calvano et al, 1979). It

has been suggested, rather, that the circadian rhythm in CBA may be

due to recompartmentalization of proteins caused by the vascular

changes associated with the activity cycle (Ottenweller et al, 1979);

that is, the circadian rhythm in CBA may be produced by the circadian

rhythm in locomotor activity. The circadian rhythm in CBA may have a

number of functions: Firstly, it may regulate the duration of the

evening surge in plasma corticosterone levels by reducing metabolic

clearance of corticosterone, which is at its lowest at this time (see

Introduction); and secondly, as suggested by Ottenweller et al (1979),

changes in the ratio of bound-to-free corticosterone during the day may

change the site of action of corticosterone from vascular beds

impermeable to proteins (e.g. brain), to vascular beds permeable to
proteins (e.g. liver, heart). The latter may also regulate cortico-
steroid negative feedback in the brain during the circadian rhythm
(see Introduction) releasing the hypothalamus from negative feedback
during the peak of the circadian rhythm. It may be seen from the
above discussion, therefore, that activity cycles and the circadian
rhythm in the H-P-A axis are inter-related in a number of ways. This
is logical since as Scharrer (1964) pointed out in his description
of photoneuroendocrine systems, light acts as a synchronizer by which
an organism may schedule metabolic adjustments so that incompatible
activities do not conflict.

The results in Figures 16, 17 and 18 demonstrate that there is
a circadian variation in the secretion of CRF from the hypothalamus
isolated and incubated in vitro. Although the results differ in some
respects from the data published earlier (Thomas, 1977; Kamstra et al,
1978), they confirm the original findings that there is a circadian rhythm
in CRF secretion, both in Incubation 1 and Incubation 2. The possible
reasons for the differences have been enumerated in the relevant
sections of the Methods and Results, and as detailed in the Results,
the main differences occur in the secretion of CRF during Incubation 1.
As pointed out in the previous section CRF secretion in Incubation 1
is also subject to larger changes over the 24-hour cycle than CRF
secretion in Incubation 2. The lack of difference between the earlier
results and the present results in CRF secretion during Incubation 2
(taking the Incubation 2 at 13.30 hr standard into account) confirms
the conclusion made in the previous section that CRF secretion in
Incubation 2 is more "stable" and resistant to changes than CRF
secretion in Incubation 1, except perhaps at 16.00 hr when the hypo-
thalamo-pituitary-adrenal axis is in a phase of dynamic change as

evidenced by the increasing plasma corticosterone levels and increasing CRF content (Fig. 18). CRF secretion in Incubation 1 goes through a 3.8-fold circadian variation, increases in CRF secretion occurring during the daylight period between 08.00 hr and 10.00 hr and between 16.00 hr and 19.00 hr. CRF secretion in Incubation 2 goes through a 2-fold variation over the 24-hour cycle, and shows a similar pattern of bimodal increases during the daylight hours between 08.00 hr and 10.00 hr and between 16.00 hr and 17.30 hr. The phase-advance of the increase in CRF secretion in Incubation 2 on the increase in CRF secretion in Incubation 1 in the afternoon confirms the earlier results (Thomas, 1977; Kamstra et al, 1978) although the timing of the increases are not the same (see Results). Nevertheless, this repeated finding would suggest that although CRF secretion in Incubation 1 and Incubation 2 may occur from the same newly-synthesized, readily-released pool of CRF (see previous section) there may be some difference in the underlying processes in the two incubations, though not in the calcium ion-dependence of the two incubations (Fig. 12; see previous section). This difference could be due to the biphasic pattern evident in CRF secretion from the hypothalamus isolated and incubated in vitro (Fig. 13; see previous section), CRF secretion in Incubation 1 probably encompassing the first and only part of the second phase of secretion, whereas CRF secretion in Incubation 2 measures the second, more prolonged, phase of secretion. The relation between CRF secretion in Incubation 1 and Incubation 2 to CRF content has been discussed in the previous section and summarized in Fig. 20. Thus, from Fig. 18 it is evident that CRF content increases in phase-advance of an increase in CRF secretion both in Incubation 1 and Incubation 2; this could be because an increase in CRF synthesis may occur during the early afternoon, newly-synthesized CRF being stored as CRF content, possibly in a less-readily-released pool of CRF

(Pool B, Fig. 20), before an increase in secretion of newly-synthesized CRF from a readily-released pool of CRF is evident (Pool A, Fig. 20).

As explained above, the changes in CRF secretion do not necessarily parallel changes in CRF content (viz. Fig. 18). However, to the extent that CRF content may measure variations in CRF synthesis (viz. Fig. 20) it is interesting to note that whilst some investigators have found the peak in CRF content to occur in the afternoon or early evening (Ungar, 1967; Hiroshige et al, 1969; David-Nelson and Brodish, 1969; Takebe and Sakakura, 1972; Ixart et al, 1977), others have found no significant circadian fluctuations in CRF content (Retiene and Schulz, 1970; Yasuda and Greer, 1976b) or peak amounts occurring in the morning (Chiappa and Fink, 1977) whilst in each case a "normal" afternoon or evening peak in plasma corticosterone levels was found. If variations in CRF secretion are responsible for the circadian rhythm in plasma corticosterone levels, this would reinforce the suggestion made above that fluctuations in CRF secretion do not necessarily parallel fluctuations in CRF content. However, as implied at the beginning of this section, it is difficult to make comparisons between the circadian rhythms obtained in different laboratories, in conditions that are not identical, and even differences in the amount of tissue used for extraction of CRF may cause discrepancies between laboratories (Chiappa and Fink, 1977). A difference of another kind obtained with measurements of CRF is the finding that when hypothalami are preincubated and stimulated with serotonin in vitro, CRF secretion is greater in the morning than in the evening (Dallman et al, 1977b; Jones et al, 1979b), in contrast to the present, and opposite, findings obtained with non-stimulated hypothalami isolated and incubated in vitro. This difference may be due to the fact that Jones et al investigated CRF secretion at only two time points in the 24-hour cycle,

thereby possibly missing a preceding peak in CRF secretion (see, for example, Fig. 19 and the discussion in the Results accompanying Fig. 19); alternatively, the secretion of CRF in the conditions used by Jones et al may represent a quite different phenomenon to the secretion of CRF examined in the studies presented here using non-stimulated hypothalami incubated in vitro immediately following removal of the hypothalamus from the animal. The latter may be because, as suggested in the previous section, stimulation may cause the release of CRF from a less-readily released pool of CRF (Pool B Fig. 20) in addition to release from a readily-released pool of CRF (Pool A, Fig. 20). Another reason for the difference may be that the secretion of CRF, as examined in the laboratory of Jones et al, may be more closely related to the circadian rhythm in the stress response since the plasma corticosterone response to the stresses of injection of saline (Haus, 1964), handling (Ader and Friedman, 1968), ether anaesthesia (Dunn and Carillo, 1978), and laparotomy (Engeland et al, 1977), the plasma ACTH response to the stresses of ether anaesthesia (Yasuda et al, 1976), histamine injection, and laparotomy (Engeland et al, 1977), and the increase in hypothalamic CRF content following ether-laparotomy stress (Hiroshige et al, 1969; Takebe and Sakakura, 1972), are all greater in the morning than in the evening; this phenomenon may be due to the greater response of the central nervous system to stress in the morning than in the evening, in contrast to the greater sensitivity of the pituitary and adrenal glands to CRF and ACTH respectively, in the evening (see Introduction). The results presented here are unlikely to be related to a stress response, therefore, since greater secretion of CRF and CRF content were found in the late afternoon and early evening (Fig. 18); further, at 13.30 hr, handling of the animals for two weeks results in a diminished output of ACTH by hemipituitaries isolated and incubated in vitro (Fig. 9), indicating that the animals

were not stressed at the time of death (see also previous section).
Thus it may be seen that circadian rhythms both in CRF content and
CRF secretion are highly sensitive to methods and conditions in each
laboratory.

The circadian rhythms evident in CRF secretion in Incubation
1 and Incubation 2 and the circadian rhythm in plasma corticosterone
levels may be seen from Fig. 18 to parallel each other to some extent
over the 24-hour cycle, CRF secretion in Incubation 1 showing a larger
variation more like that in plasma corticosterone levels than CRF
secretion in Incubation 2; the latter does not mean, however, that
the pattern of CRF secretion in Incubation 1 is more closely related
to the regulation of circadian rhythmicity in plasma corticosterone
levels than the pattern in Incubation 2, since parallel co-variations
may not necessarily infer a causal relation in events as separated as
the secretion of CRF and the fluctuations in plasma corticosterone
levels. The most suitable method of assessing the phase relations
between the circadian rhythms in CRF secretion in Incubation 1 and
Incubation 2 and the circadian rhythm in plasma corticosterone levels
would be to perform a cross-correlation analysis; however, the data
are not suitable for this form of analysis since the samplings were
not made at similar regular intervals and interpolation would be
necessary, and in any event it is more usual to perform this kind of
analysis on simultaneous, serially-dependent samplings. However, a
significant correlation existed between the fluctuations in CRF
secretion in Incubation 1 and Incubation 2 (r_s = 0.786; see Results)
inferring there is little or no phase difference in the two circadian
rhythms over most of the 24-hour cycle, although as noted above the
increase in secretion of CRF in Incubation 2 in the afternoon occurs
in phase-advance of the increase in Incubation 1. Further, fitting a

cosine function to the rhythm in plasma corticosterone levels (Appendix E) gives a peak in plasma corticosterone levels at approximately 21.00 hr; this would indicate that the peak in the circadian rhythm in CRF secretion occurs 2 to 3½ hours before the "peak" in plasma corticosterone levels. However, it is obvious that the circadian rhythm under normal lighting conditions does not conform to a cosine function (although the fit is significant; Appendix E) since, as mentioned above, there is a sharp increase in plasma corticosterone levels between 18.00 hr and 19.00 hr; this deviation from a smooth cosine function occurs during the sharp increase in CRF secretion in Incubation 1 between 17.30 hr and 19.00 hr and 2 hours after the increase in CRF secretion in Incubation 2 between 16.00 hr and 17.30 hr. It may be seen, therefore, that sharp increases in CRF secretion from the hypothalamus isolated and incubated in vitro precede the acute rise in plasma corticosterone levels just before "lights off". These findings have a logical explanation since as discussed in the Introduction (see e.g. Fig. 3) the circadian rhythm in plasma corticosterone levels is determined not by one factor, the stimulation of the H-P-A axis provided in the secretion of CRF, but a number of endogenous circadian rhythms; that is, in the sensitivity of the adrenal glands to ACTH, cholesterol-side-chain-cleaving activity in the adrenal gland, corticosteroid binding activity, metabolic clearance of corticosterone, sensitivity of the pituitary gland to CRF, and pituitary ACTH content. It was concluded in the Introduction that whilst circadian rhythmicity in ACTH secretion may not be necessary for the appearance of these circadian rhythms, circadian rhythmicity in ACTH secretion is necessary for the full expression and synchronization of these circadian rhythms. It may be seen, therefore, that all that is physiologically required of the hypothalamo-pituitary unit is not a "driving" circadian rhythm, but

a sharp pulse in the evening in order to synchronize circadian rhythms in the H-P-A axis and to maintain the full expression of the circadian rhythms; this suggestion would be in agreement with what the results in Fig. 18 also suggest. The results in Fig. 18 are therefore in logical agreement with what has been determined about circadian rhythmicity in the H-P-A axis in vivo, and this also reinforces the suggestion made in the previous section that CRF secretion from the hypothalamus isolated and incubated in vitro reflects accurately hypothalamic secretion of CRF in vivo immediately prior to sacrifice. The reason for the elevated plasma corticosterone levels at 24.00 hr and 01.00 hr (discussed above) whilst CRF secretion is decreased in both Incubation 1 and Incubation 2 is most probably that a peak in CRF secretion may have occurred between 19.00 hr and 01.00 hr; further, as implied above, increased CRF secretion at 01.00 hr is not a physiological requirement since the circadian rhythm in plasma corticosterone levels is in a descending phase at this time (see also Appendix E). The reason for the increase in CRF secretion in the morning between 08.00 hr and 10.00 hr is unclear especially as this is not accompanied by an increase in plasma corticosterone levels; the reason for the latter may be that, as the sensitivity of the pituitary gland to CRF and of the adrenal glands to ACTH are at their lowest in the morning (see Introduction), an increase in CRF secretion may not be manifested as an increase in plasma corticosterone levels. The reason for the increase in CRF secretion at this time of the day may be for a number of reasons: Firstly, as noted earlier, an increase in corticosteroid binding activity has been found to occur in rats at this time of the circadian rhythm (Ottenweller et al, 1979) and this was attributed to the possibility that the animals were disturbed by daily activity in their surroundings. Therefore, it is possible that the rise

in CRF secretion may be associated with disturbances in the animal

house during the day; however, this is unlikely since precautions

were taken to prevent disturbances by noise or other environmental

factors (see Methods). Alternatively, it may be tentatively

speculated that the increase in corticosteroid binding activity and

CRF secretion at this time of the day may not be the result of

activity in the animals or disturbances (this is unlikely since the

animals were asleep at 10.00 hr - personal observation) but the

consequence of a metabolic adjustment during the sleep period; further,

the decrease in "free" corticosterone in the morning may result in a

decreased negative feedback of corticosteroids on the brain and a

consequent increase in CRF secretion, and this may in turn initiate

the gradual increase in corticosterone levels in the afternoon. Secondly,

the increase in CRF secretion in the morning, whether neural or

metabolic in origin, may have a function not only in initiating the

gradual increase in plasma corticosterone levels in the afternoon but

also in "priming" the pituitary gland for the evening surge in ACTH

secretion. It is known that vasopressin may "prime" the pituitary

gland to release ACTH in response to CRF (see Introduction) and CRF

may have similar activity although recent work would suggest that this

does not occur in vitro (Pickering and Fink, 1979). Whatever the

reason for the increase in CRF secretion in the morning may be, this

pattern was also evident in the earlier data (Thomas, 1977; Kamstra

et al, 1978) and its origin or possible function remain to be clarified.

The neural pathways involved in the regulation of circadian

rhythmicity in the H-P-A axis have been outlined in the Introduction;

however, further consideration of the nature of hypothalamic regulation

of circadian rhythmicity is important. The role of the suprachiasmatic

nuclei as an entrainer or "coupler" of circadian rhythms is prepotent in

current literature on the subject (see for example reviews by Menaker et al, 1978, and Rusak and Zucker, 1979). Implicit in this concept are the neuroanatomical findings that the suprachiasmatic nuclei receive afferents from the retinohypothalamic tract (e.g. Moore, 1979), that neurons in the suprachiasmatic nuclei respond to photic stimulation (Nishino et al, 1976), that efferent fibres from the suprachiasmatic nuclei project to the caudal border of the ventromedial nuclei, arcuate nuclei and median eminence (Swanson and Cowan, 1975), and that neurons in the ventromedial nuclei and lateral hypothalamic area show circadian patterns of activity (Koizumi and Nishino, 1976), providing evidence that the hypothalamus recieves information of an external "Zeitgeber", the light/dark cycle. Further, section or ablation of the anterior afferent pathways to the mediobasal hypothalamus abolishes circadian rhythmicity in plasma corticosterone levels (Halasz et al, 1967a; Lengvari and Liposits, 1977) and in pituitary ACTH content (Halasz et al, 1967b) without abolishing the response to neurogenic stress (Slusher, 1964; Palka et al, 1969) or to electrical stimulation of the median eminence region (Makara et al, 1978), suggesting CRF-producing neurons and pathways mediating the neurogenic stress response are left intact, whilst pathways mediating circadian rhythmicity are removed. Destruction of the suprachiasmatic nuclei disrupts circadian rhythms of feeding (Van den Pol and Powley, 1979), drinking (Rusak, 1977; Van den Pol and Powley, 1979), locomotor activity (Krieger et al, 1977c; Rusak, 1977; Szafarczyk et al, 1979), plasma ACTH levels (Szafarczyk et al, 1979), and plasma corticosterone levels (Krieger et al, 1977c; Raisman and Brown-Grant, 1977; Abe et al, 1979). Further, when food is freely available, light appears to be the pre-dominant synchronizer of circadian rhythms, and this conclusion is further underlined by the "free-running" rhythms in plasma cortico-

sterone levels (Honma and Hiroshige, 1978; Morimoto et al, 1979) and

in locomotor activity (Honma and Hiroshige, 1978) in animals kept in

constant light. However, when availability of food is restricted

(Krieger, 1974; Moberg et al, 1975; Morimoto et al, 1977; Heybach

and Vernikos-Danellis, 1979; Morimoto et al, 1979; Wilkinson et al,

1979) food intake takes on the central role as the synchronizer of

the circadian rhythm in plasma corticosterone levels, even when food

is presented in the morning within a "normal" lighting schedule. This

inter-relation between circadian rhythms in feeding and plasma cortico-

sterone levels is also demonstrated at a neural level when both

circadian rhythms are disrupted by lesions of the ventromedial nuclei

(Bellinger et al, 1976). However, like light, food intake only acts

as a synchronizer when it is an external synchronizer in the form of a

restricted feeding schedule. Thus, when the circadian rhythm in plasma

corticosterone levels is abolished by suprachiasmatic nuclear lesions

(Krieger et al, 1974; Abe et al, 1979) or by constant lighting

conditions (Morimoto et al, 1979) the rhythm in plasma corticosterone

levels is not entrained by the rhythmicity of food intake. Thus it

may be seen that the circadian rhythm in plasma corticosterone levels

corresponds to the stress response (or "general adaptation syndrome" -

Selye, 1976) in that the circadian rhythm is synchronized by external

stimuli of biological significance such as the transition from light to

dark and restricted availability of food, whilst it "free-runs" under

conditions of constant light or darkness or freely available food in

the absence of light synchronization. Implicit in the circadian

rhythm in plasma corticosterone levels is the concept of "anticipation"

or "deprivation"; this is because the rise in plasma corticosterone

levels occurs before the transition from light to dark and before the

presentation of food (see above). This also has biological value

since events that are repeated every 24 hours (unlike the unpredictability in stressors) may be prepared for in advance so that the animal may exact the best use of the availability of food or darkness. This aspect of the circadian rhythm must involve complex neural organization and the hippocampal formation may provide exactly such a neural mechanism since the hippocampal formation is classically involved in memory functions. Further, in the context of availability of food, the hippocampal formation is also purported to mediate the anticipation of "rewards" (Gray, 1971). The involvement of afferents from the hippocampal formation in the rhythmicity of plasma corticosterone levels is uncertain since, whilst circadian rhythmicity of plasma cortico-sterone was abolished following lesions of the hippocampus or the fornix, the effect is not consistent or permanent (Moberg et al, 1971; Seggie and Brown, 1971; Lengvari and Halasz, 1973; Wilson and Critchlow, 1973/1974; Wilson and Critchlow, 1974; Lanier et al, 1975). This inconsistency may be due to neural regeneration following the lesions (Stenevi et al, 1973). However, when lack of an effect on the circadian rhythm in plasma corticosterone levels was obtained, only two time points in the circadian rhythm were examined (e.g. Lengvari and Halasz, 1973; Wilson and Critchlow, 1974) which does not rule out the possibility that the rise in plasma corticosterone levels prior to "lights off" was affected; further, in one study there was an obvious increase in the evening rise in plasma corticosterone levels following dorsal and ventral hippocampectomy (Lanier et al, 1975). This latter suggests that the hippocampus may mediate its effect on the circadian rhythm in plasma corticosterone levels by an inhibitory mechanism; this is also suggested by the finding that stimulation of the dorsal and ventral hippocampus in the afternoon produces an initial increase in plasma corticosterone levels, followed by a delayed inhibition of the circadian rise in plasma corticosterone levels (Casady and Taylor,

1976). Thus, whilst the suprachiasmatic nuclei may be essential for photoperiodic entrainment, the hippocampal-fornix system may have an important role, as yet to be determined, in the timing of the rise in hypothalamo-pituitary-adrenal activity prior to the light/dark transition or the availability of food.

As stated in the Introduction acetylcholine and serotonin are implicated in the regulation of circadian rhythmicity in the hypothalamo-pituitary-adrenal axis (Krieger et al, 1968; Krieger and Rizzo, 1969; Scapagnini et al, 1971; Moroji et al, 1973; Vermes et al, 1974; Simon and George, 1975; Heybach et al, 1979). It would, therefore, be of interest to investigate the effects of pharmacological agents known to affect cholinergic and serotonergic metabolism, given previously to the whole animal, on the circadian rhythm in CRF release in vitro.

Having established the circadian rhythm in plasma corticosterone levels and CRF secretion as shown in Incubation 1 and Incubation 2 from isolated hypothalami derived from animals kept under "normal" lighting conditions (Fig. 18), these circadian rhythms were re-examined in animals kept for 7 to 10 days in a "reversed" lighting cycle. This was done both in order to test the photoperiodic entrainment of the circadian rhythms, and in order to verify that the circadian rhythms were not artefacts produced by the experimental methods or conditions other than the light cycle. The results in Fig. 15 demonstrate that the circadian rhythm in plasma corticosterone levels can be reversed in animals kept for 7 to 10 days in "reversed" lighting conditions. The peak and the trough of the circadian rhythm obtained in such animals kept in "reversed" lighting may be seen to have been subject to a "phase-shift" of 12 hours. Although the results of the cosine function-fitting procedure imply that the circadian rhythm under "reversed"

lighting conditions has been subject to a phase-shift of 9½ hours

following 7 to 10 days of the "reversed" lighting schedule (see

Appendix E), it may be seen that this is only because the cosine

function for the circadian rhythm under "normal" lighting conditions

does not give a peak that concurs with the observed peak at 19.00 hr,

but at approximately 21.00 hr, whereas the fitted cosine function for

the "reversed" circadian rhythm gives a peak at approximately 06.30 hr

which concurs with the observed peak at 07.00 hr.   This result for

the "normal" circadian rhythm has been discussed above and is

attributable to the fact that at the peak of the circadian rhythm the

fit to a cosine function is poor.   Thus, the peak of the circadian

rhythm in blood corticosterone levels is reversed after 7 to 10 days

of "reversed" lighting.   However, it may readily be seen from Fig. 15

that the circadian rhythm obtained in animals kept in "reversed"

lighting is not like the circadian rhythm in "normal" lighting

conditions, in that there is not the sharp rise in plasma corticosterone

levels before the peak which is evident in the circadian rhythm obtained

in animals kept in normal lighting conditions, and the plasma cortico-

sterone concentrations at the 90° phase angle (or approximately the

mean level) is lower in the circadian rhythm in "reversed" lighting

conditions (see Results and Appendix E).   This result may indicate

that although the peaks and troughs of the plasma corticosterone

circadian rhythm have reversed after exposure of the animals to the

"reversed" lighting schedule for 7 to 10 days, the circadian rhythm

may not have fully adapted to the reversed lighting conditions at the

time at which the animals were studied.   Similar results with circadian

rhythms in plasma corticosterone levels which are reversed are evident

in the studies of Haus (1964), who found that, in mice, the circadian

rhythm in plasma corticosterone levels was reduced both in amplitude

and mean 24-hour levels 14 days after exposure of the animals to

reversed lighting. In the studies of Morimoto et al (1977), the amplitude of the circadian rhythm in plasma corticosterone levels in rats was reduced during the 3 days in which the circadian rhythm reversed. In humans, reduction of the amplitude of the circadian rhythm in cortisol levels during reversal of the rhythm is a common finding (see e.g. reviews by Aschoff, 1978 and 1979). Further, Perkoff et al (1959) found that the reversed circadian rhythm in plasma 17-OH corticosteroids was reduced in amplitude in human subjects kept for 5 to 10 days in a "reversed" light/dark, sleep/work cycle. Again, in man a relation exists between the amplitude of the circadian rhythm in urinary 17-OH corticosteroids and ability to move the peak of the rhythm (Reinberg et al, 1978). It has been suggested that ability to "phase-shift" circadian rhythms may be associated with a small amplitude in the rhythm (Aschoff, 1978). Thus, attenuation of the amplitude and mean level of the circadian rhythms in plasma corticosteroids is a feature of adaptation to "reversed" lighting schedules. It may also be noted that in humans the circadian rhythm in plasma cortisol levels is particularly slow to adapt to altered lighting schedules (see e.g. review by Aschoff, 1979). The results in Fig. 15 would therefore indicate that the circadian rhythm in plasma corticosterone levels has reversed in phase following exposure of the rats to "reversed" lighting for 7 to 10 days, but that the circadian rhythm may not have fully adapted to the "reversed" lighting schedule at this time.

The preliminary results obtained with the secretion of CRF in Incubation 1 and Incubation 2 from hypothalami obtained from animals kept in reversed lighting conditions are shown in Fig. 19; it may tentatively be concluded that these results demonstrate that the morning/evening difference in the secretion of CRF in Incubation 1 is reversed but that there is no significant morning/evening difference

in the secretion of CRF in Incubation 2 from hypothalami obtained from animals kept for 7 to 10 days in reversed lighting conditions. The lack of a significant morning/evening difference in CRF secretion in Incubation 2, as pointed out in the Results, does not mean however that there is no circadian rhythm in Incubation 2 following reversal of the light cycle, since only two times in the 24-hour cycle were sampled in these preliminary experiments. The circadian rhythm may not, therefore, have been sampled at its peak and trough. It is also possible, as the peripheral measurements of plasma corticosterone levels would indicate, that the circadian rhythm in the hypothalamo-pituitary-adrenal axis may not have fully adapted to the reversed light cycle. Nonetheless, these preliminary results do indicate that reversal of photoperiodic cues causes a reversal of the morning/evening difference in CRF secretion in Incubation 1 concomitantly with reversal of the circadian rhythm in plasma corticosterone levels. Further, the results, together with the results obtained with animals kept in "normal" lighting conditions, imply that a circadian variation in CRF secretion evident in Incubation 1, and probably in Incubation 2, is involved in pituitary-adrenal circadian rhythmicity. The results also demonstrate that the changes seen in CRF secretion during Incubation 1 and Incubation 2 at different times in the day are physiological phenomena and are not due to incubation artefacts, since the effect observed over the 24-hour cycle is dependent on phenomena occurring in the whole experimental animal and not in the assay system, its derived tissues, nor its incubation media.

APPENDIX

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A. **Transfer of the Laboratory Animal Housing to the New Animal House**

In December 1977 the new Animal House in the Medical Sciences Building, University of Leicester, was completed. The animals kept in the first Animal House, in the Adrian Building, University of Leicester, were all used in experiments by the end of January 1978, and a new colony of animals of exactly the same origin as that used in the first animal laboratory (Wistar strain, Bantin and Kingman Ltd., Grimston, North Humberside) was established in the new animal laboratory in the Medical Sciences Building. The new laboratory was equipped with sound insulation and time switches for the lighting in the same way as had been established for the first laboratory. The cages, soil-trays, cage-racks, water and food hoppers, water bottles, incubation baths, centrifuges, torsion balances, gassing equipment, dissection instruments, bags of food, and all other items which could be transported, were transferred from the old laboratory to the new laboratory on completion of the sound insulation of the new laboratory in January 1978. Thus, the new animal colony was re-established in exactly the same conditions as those provided in the first laboratory, and the regime of animal care and experimentation was identical with that in the first laboratory.

In order to verify that the reactivity of the tissues obtained from animals kept in the second location was similar to that in the first, the standard output of non-stimulated ACTH release from pituitaries isolated and incubated in vitro was measured by the indirect measurement of corticosterone output from incubated quartered adrenal glands (Incubation 3 - Controls, Fig. 5; see Assay of CRF in Methods) and a comparison was thereby made of the animals kept in the two locations. The data for animals kept in the first animal laboratory (Adrian Building) was derived from CRF assays conducted in the month prior to removal of the animal laboratory; the data for animals kept in the second animal laboratory (Medical Sciences Building) was obtained in an experiment conducted in March 1978, one month after the establishment of the new animal laboratory. The results are presented below in tabular form:-

Table 6   Comparison of the non-stimulated pituitary-adrenal release of hormones in vitro from tissues obtained from animals kept in two locations

Location	Non-stimulated Pituitary-Adrenal Release of Hormones. μg Corticosterone/100 mg adrenal tissue/hr Mean $\pm$ SEM	n
First Animal Laboratory	5.404 $\pm$ 0.247*	12
Second Animal Laboratory	5.432 $\pm$ 0.137**	12

* not significantly different from **, p $>$ 0.1 (t test).

It may be seen from Table 6, therefore, that the output of

pituitary-adrenal hormones from tissues removed from animals kept in both locations was not significantly different.

B   Preparation and composition of Krebs-Ringer bicarbonate glucose
     (KRBG) solution

Krebs-Ringer bicarbonate glucose solution (KRBG) (2, 3)

STOCK SOLUTIONS:

5.75% KCl	4 ml
6.1% $CaCl_2$ (.OH_2O)	3 ml
10.55% KH_2PO_4	1 ml
19.1% $MgSO_4$.7H_2O	1 ml

AND   Just before use; 4.5% NaCl   ... ...   100 ml

109 ml

ADD   Deionized water up to final volume of 272.5 ml -   163.5

272.5

To 216 ml of this double ringer add just before use:-

        (216 ml Double Ringer)
        116 ml Deionized Water
        84 ml of 1.3% $NaHCO_3$ which has been gassed for ½ to ¾ hr with

        95% O_2-5% CO_2 at room temperature.

        104 ml 1% glucose

Total   520 ml - Gas this final solution for 10 mins with 95% O_2 - 5% CO_2
Vol.

Concentrations of Salts (mM):-

        KCl   - 4.695
        $CaCl_2$ - 2.513
        KH_2PO_4 - 1.183
        $MgSO_4$ - 1.181
        NaCl   - 117.385
        $NaHCO_2$ - 24.997
        D-Glucose - 11.101

Concentrations of Ions (approx.) mM:-

        K^+ - 5.9
        Ca^{2+} - 2.5
        Mg^{2+} - 1.2
        Na^+ - 142.4

In Rat Plasma concentrations are:- (1)

        K^+ - 3.5 mEq/l
        Na^+ - 137.0 mEq/l
        Cl^- - 98.3 mEq/l
        Glucose   - 91.1 mg/100 ml

Concentrations in KRBG:-

K^+ -   5.9   mEq/l

Na^+ -   142.4 mEq/l

Cl^- -   127.1 mEq/l

Glucose - .199.8 mg/100 ml

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(1)   Information supplied by animal breeders, Bantin and Kingman Ltd.

(2)   'Manometric Techniques' - Umbreit, W.W., 4th Edition. Burgess, Minneapolis, 1964.

(3)   Birmingham, M.K., Elliot, F.H. and Valere, P.H.L. Endocrinology 53, p. 687, 1953.

Calcium-free Krebs-Ringer bicarbonate glucose solution

As in normal KRBG except:

STOCK SOLUTIONS:

5.75% KCl	4 ml
6.1% $CaCl_2(.OH_2O)$	Omitted
10.55% KH_2PO_4	1 ml
19.1% $MgSO_4.7H_2O$	3.13 ml

AND   Just before use; 4.5% NaCl ... ...   100 ml

ADD   Deionized water up to final volume of 272.5 ml   ...   164.37

Calcium Free Double Ringer   272.5

Final Concentrations of ions (approx.) mM:-

K^+ -   5.9

Ca^{2+} -   0

Mg^{2+} -   3.69

Na^+ -   142.4

Calcium-enriched Krebs-Ringer bicarbonate glucose solution

As in normal KRBG except:

STOCK SOLUTIONS:

5.75% KCl	4 ml
6.1% $CaCl_2(0.H_2O)$	4.41 ml
10.5% KH_2PO_4	1 ml
19.1% $MgSO_4.7H_2O$	Omitted

AND   Just before use; 4.5% NaCl ... ...   100 ml

                                                      109.41 ml

<u>ADD</u>   Deionized water up to final volume of 272.5 ml   ...   <u>163.09</u>

<u>Calcium-enriched Double Ringer</u>   <u>272.5</u>

Final concentrations of ions (approx.) in mM:-

K^+ -   5.9

Ca^{2+} -   3.69

Mg^{2+} -   0

Na^+ -   142.4

2.128 ml of calcium-enriched solution added to 1 ml of calcium-free medium brings the ionic composition of the final medium back to that of normal KRBG, i.e.:

K^+ - 5.9, Ca^{2+} 2.51,

Mg^{2+} 1.18, Na^+ 142.4 mM.

C   Assay of Cyclic-AMP
         (Brown et al, 1972)
         The principles of protein binding assays are described by
         Chard (1978).

Reagents

1.   Preparation of Stock Binding Protein

         Bovine adrenal glands were obtained from a local (Leicester)
         abattoir and transported on ice to a cold room ($\sim 4^{o}C$).
         They were then dissected clean of fat, and the medullary
         tissue was scraped from the cortex. The cortical tissue was
         then placed in 2 volumes of Littlefields medium (Medium A,
         Brown et al, 1972 - see below) and homogenized in a blender-
         mixer.

         Littlefields Medium

              0.25 M   sucrose
              0.025 M   KCl
              0.005 M $MgCl_2.6H_2O$
              0.05 M   Tris-HCl buffer pH 7.4
              0.006 M   mercaptoethanol

         The homogenate was then centrifuged at 2000 x g for 5 mins at
         $4^{o}C$. The supernatant was removed and re-spun at 5000 x g for
         15 mins at $4^{o}C$. This final supernatant was divided into 0.5
         ml fractions and stored at $-22^{o}C$.

2.   Assay Buffer

              50 mM   Tris-HCl buffer pH 7.4

              8 mM   theophylline hydrate
              6 mM   mercaptoethanol
              4 mM   EDTA

         The pH value was checked on a pH meter.

3.   Tracer Radioactive Cyclic AMP H^3)

         Tritium labelled cyclic-AMP was obtained from the Radiochemical
         Centre, Amersham. The stock solution was diluted to 0.5 p
         mole/10 μ l before use.

4.   Chemical Standard Cyclic AMP

         Cyclic AMP was obtained from Boehringer, and a 1 mM (34 mg/100
         ml) stock solution stored at $-22^{o}C$.

5.   Charcoal Suspension for Separation of Bound and Free Ligand

              1 g   Norit   GSX
              1 ml   10%   BSA
            25 ml   Deionized water

         This was prepared immediately before use.

Optimisation of Assay

The dilution of stock binding protein at which 30% of the radioactive ligand is bound was found by adding a series of dilutions (i.e. 1 : 10 to 1 : 160) of stock binding protein to 0.5 p mole tritiated cyclic AMP and performing the assay procedure (see below). A curve of bound ratioactivity against protein dilution was then drawn. The protein dilution at which approximately 30% of the radioactivity was bound was then selected.

Assay Procedure

1.   The stock binding protein was diluted by the appropriate amount in assay buffer.

2.   Stock radioactive tracer was diluted to 0.5 p mole/10 μl.

3.   A series of dilutions of standard cyclic-AMP were made up in the range 0.25 to 8 p moles/100 μl.

4.   The hypothalamic incubation media, Incubation 1 and Incubation 2 kept at -20°C from an assay performed the previous day, were used undiluted.

5.   The following were added to a series of tubes, each tube being prepared in duplicate, in the following order:-

      (i)   100 μl standard, blank, or hypothalamic incubation medium

      (ii)   10 μl tritiated cyclic AMP

      (iii)   200 μl diluted binding protein

according to the scheme:-

	Standard/ blank	Tracer	Incubation Media	Binding Protein
Standards	100 μl	10 μl	–	200 μl
Incubation Media	–	10 μl	100 μl	200 μl
Blank (H$_2$O)	100 μl	10 μl	–	200 μl
Non-specific binding (1mM stock cyclic AMP)	100 μl	10 μl	–	200 μl
Total counts	100 μl	10 μl	–	200 μl

The tubes were then placed in a shaker for approximately 5 mins, and then left to stand at 4°C for at least 1½ hours.

During this time, the charcoal suspension was prepared and kept at 4°C until use.   500 μl of the charcoal suspension was added to all the tubes except those marked 'Total';   500 μl assay buffer was added to 'Total' tubes.   The tubes were then shaken

on a 'Rotamixer' and centrifuged at 1200 x g for 15 mins at 4°C. 500 μl supernatant was transferred from each tube to counting vials, and scintillation fluid added to the counting vials. The samples were then counted in a scintillation counter, using a pre-selected counting time for each sample (approximately 2 mins).

A standard curve was then constructed by plotting the ratio

$$\frac{C_o}{C_s} \quad \text{against} \quad \left[\text{Cyclic AMP} \right]$$

where C_o is the number of counts obtained with the blank samples, C_s the number of counts obtained with each of the standard samples, and $\left[\text{cyclic AMP} \right]$ is the relevant concentration of standard cyclic AMP. The concentration of cyclic AMP in the unknown samples (i.e. the hypothalamic incubation media) was then calculated from this standard curve using the ratio $\frac{C_o}{C_u}$, where in this instance C_u is the number of counts for the unknown samples. The results indicated that the concentrations of cyclic AMP in Incubation 1 and Incubation 2 were near the lower detection limits of the assay, and values of 1.12 p moles/ ml and 1.05 p moles/ml were calculated for Incubation 1 and Incubation 2 respectively.

The description of the assay procedure above is modified after instructions for a class practical written by Dr S Nahorski; his expert direction and that of Mr A Wilcocks is gratefully acknowledged. Permission to use laboratory equipment belonging to Professor R Whittam for parts of the assay is also especially appreciated.

D.   Regression Analysis of CRF Assay Data Obtained With Incubation
      1 and Incubation 2 at 13.30 hr and with the D5 CRF Extract

        The raw data obtained from a number of assays (indicated below)
was subjected to linear regression analysis using the procedures
described in Expression of Data and Statistical Methods in Methods. The
parameters calculated from the linear regression analysis of the data
obtained with Incubation 1 and Incubation 2 at 13.30 hr and with the D5
CRF extract are presented below.

Incubation 1 at 13.30 hr

Responses to doses
$\leq$ 0.05 MEO were included in the analysis (see Assay of CRF in Results).

Number of assays included in regression analysis = 16

Number of observations included in regression analysis, n = 208

Corrected sum of squares for responses, Syy = 173.883

Corrected sum of squares for log doses, Sxx = 41.775

Corrected sum of products, Sxy = 58.951

Slope, b = 1.411

Residual variance, s^2 = 0.441

Variance of slope = 0.0106

95% Interval estimate of b = 1.411 $\pm$ 0.201

95% Interval estimate of intercept, α = 9.184 $\pm$ 0.09

Calculated Index of Precision for Incubation 1 (n = 208), λ = 0.0326

λ may appear small mainly due to the size of n. The assay performance,
as measured by the residual variance, is consistent and within expected
limits (see also below).

Log equivalent dose Incubation 1 at 13.30 hr (i.e. dose producing a
response of 7.0 μg corticosterone/100 mg adrenal tissue/hr) = - 1.5474

Equivalent dose   =   0.0284

Variance of log doses   =   0.2018

Variance of log equivalent dose   =   0.000367

Relative potency:-

Log equivalent dose Incubation 2 at 13.30 hr, potency 1.0, = - 1.464
(see below)

Variance of log equivalent dose Incubation 2 at 13.30 hr = 0.000315 (see
                                                                  below)

Log potency Incubation 1 at 13.30 hr $= -1.464 - (-1.5474)$
$$= 0.0832$$

Variance of log potency $= 0.000367 + 0.000315$
$$= 0.00068$$

95% Interval estimate of potency Incubation 1 at 13.30 hr
$$= 1.211, 1.363, 1.077$$

Incubation 2 at 13.30 hr

Responses to doses ≤ 0.1 MEO were included in the analysis (see Assay of CRF in Results).

Number of assays included in regression analysis $= 17$

Number of observations included in regression analysis, n $= 76$

Corrected sum of squares for responses, $S_{yy} = 43.926$

Corrected sum of squares for log doses, $S_{xx} = 1.493$

Corrected sum of products, $S_{xy} = 2.207$

Slope, $b = 1.478$

Residual variance, $s^2 = 0.549$

Variance of slope $= 0.368$

95% Interval estimate of $b = 1.478 \pm 1.189$

95% Interval estimate of intercept, $\alpha = 9.164 \pm 0.167$

Calculated Index of Precision for Incubation 2 (n = 76), $\lambda = 0.0575$

λ may appear small mainly due to the size of n. The assay performance, as measured by the residual variance, is consistent and within expected limits.

Log equivalent dose Incubation 2 at 13.30 hr (i.e. dose producing a response of 7.0 μg corticosterone/100 mg adrenal tissue/hr) $= -1.464$

Equivalent dose $= 0.03436$

Variance of log doses $= 0.0199$

Variance of log equivalent dose $= 0.000315$

Potency:-

Log potency $= -1.464 - (-1.464)$
$$= 0$$
that is, Incubation 2 at 13.30 hr is allocated a potency of 1.0, and is the standard CRF preparation from which the relative potency of other preparations is calculated.

Variance of log potency   =   0.000315

95% Interval estimate of potency   =   1.0, 1.083, 0.923

D5 CRF Extract

a)   Responses to doses between $0.25.10^{-3}$ HE and 2.10^{-3} HE

Number of assays included in regression analysis   =   8

Number of observations included in regression analysis, n = 58

Corrected sum of squares for responses,   Syy = 40.591

Corrected sum of squares for log doses,   Sxx =   4.562

Corrected sum of products,   Sxy = 6.15

Slope,   b   = 1.348

Residual variance, s^2 = 0.577

Variance of slope   =   0.126

95% Interval estimate of   b   = 1.348 ± 0.697

95% Interval estimate of intercept, $\propto$ = 10.90 ± 0.195

Calculated Index of Precision for D5 Extract (n = 58), λ = 0.074

λ may appear small mainly due to the size of n.   The assay performance, as measured by the residual variance, is consistent and within expected limits.

The relative potency of the D5 CRF extract (i.e. HE D5 Extract/ MEO Incubation 2 at 13.30 hr) was calculated, but is not presented since this procedure may not be valid given the lack of evidence for assuming that the CRF activity of the two preparations is similar.   However, a comparison of the CRF activity of the two preparations on the basis of the weight of the tissues from which the two preparations were derived (i.e.   pig hypothalamus and rat hypothalamus) is made in the Discussion .

b)   Responses to doses between 2.10^{-3} HE and 10.10^{-3} HE

Doses of D5 extract in this range produced no further increments in responses - the data obtained with these doses were analysed separately (see Assay of CRF in Results).

Number of assays included in regression analysis   =   5

Number of observations included in regression analysis,   n   =   29

Corrected sum of squares for responses,   Syy   =   10.075

Corrected sum of squares for log doses,   Sxx   =   2.522

Corrected sum of products,   Sxy = 0.5698

Slope, b =   0.226

Residual variance, s^2 = 0.3684

Variance of slope   = 0.146

95% Interval estimate of b =   0.226 $\pm$ 0.784 (not significantly different from 0)

95% Interval estimate of intercept, α =   7.754 $\pm$ 0.231

E.   Partial Linear Regression Analysis of the Circadian Rhythm in
       Plasma Corticosterone Levels Under "Normal" and "Reversed"
       Lighting Conditions

The mean plasma corticosterone levels at each of the times

sampled were fitted by partial linear regression analysis to a cosine

function, with the cycle period, Υ, fixed at 24.0 hr (i.e. $\frac{360}{24} = 15°/hr$).

The assumption that the cycle period is equal to 24.0 hr was made;

firstly, because the sampling method used does not provide data suitable

for analysis of rhythms of other than 24 hr periodicity (i.e. there is

an a priori assumption of 24 hr periodicity, since different groups of

6 animals were sacrificed at each time on different days, and not

continuously over a period of, say, 48 hrs, such that artefacts caused

by any disturbance at the sampling times would be illuminated – see

Methods), and secondly, because all that was required was a rudimentary

analysis (given the calculating facilities used) of the times of the

peaks and troughs, mean levels, and amplitude of the circadian rhythms.

The function used for this analysis was:-

$$y = \alpha + \beta \cos 15 (x - z),$$ where y = plasma corticosterone
concentration

α = intercept, or $90°$ and $270°$ phase-angle value of the plasma

corticosterone concentration (approximately the mean

concentration over 24 hours)

β = ½ amplitude of the circadian rhythm (approximately the

difference between peak or trough values and the mean)

x = time at which the samples were taken

z = time of $0°$ or $180°$ phase, or time of peak or trough of the

circadian rhythm

This function may be restated as:-

$$y = \alpha + \beta \cos (15x - 15z)$$

$$= \alpha + \beta \cos 15x \cos 15z + \beta \sin 15x \sin 15z$$

then, if $\beta \cos 15z = \beta_1$

$$\beta \sin 15z = \beta_2$$

$$\cos 15x = x_1$$

$$\sin 15x = x_2$$

$$y = \alpha + \beta_1 x_1 + \beta_2 x_2 \quad (1)$$

$$\text{and} \quad \frac{\beta_2}{\beta_1} = \tan 15z \quad (2)$$

Using this function (1) the data may then be subjected to partial

linear regression analysis (Bailey, 1959). From this, the values

of α, β, and z (2) were calculated for each set of plasma cortico-

sterone concentrations, under normal lighting conditions and under

reversed lighting conditions.

"Normal" Lighting (Lights on 07.20 hr, Lights Off 19.20 hr)

$\alpha = 17.144 \pm 0.876$ ($\pm$ Standard deviation)

$\beta = 6.831$

$z = -3.039 = 20.58$ hr (peak)

$\bar{y} = 17.646$

χ^2 "Goodness-of-fit" test:-

Time hr	Calculated Corticosterone Concn. μg/100 ml	Observed Corticosterone Concn. μg/100 ml
07.00	11.194	12.340
08.00	10.528	13.450
10.00	10.564	11.783
12.00	12.363	11.808
14.00	15.443	12.725
16.00	18.979	16.867
18.00	22.023	20.8
19.00	23.094	29.425
20.00	23.759	26.048
22.00	23.723	18.162
24.00	21.924	21.7
01.00	20.499	21.875
04.00	15.309	12.420

χ^2 = 5.775, p > 0.8, ν = 11

Thus, the peak and the trough of the circadian rhythm of plasma corticosterone levels under normal lighting conditions are at 20.58 hr and 08.58 hr respectively.

"Reversed" Lighting (Lights on 19.20 hr, Lights off 07.20 hr)

α = 8.726 $\pm$ 0.779

β = -4.515

z = -5.640 = 18.22 hr (trough)

$\bar{y}$ = 9.406

χ^2 "Goodness-of-fit" test:-

Time hr	Calculated Corticosterone Concn. μg /100 ml	Observed Corticosterone Concn. μg /100 ml
07.00	13.177	17.175
08.00	12.830	13.608
10.00	11.340	8.020
12.00	9.15	9.817
14.00	6.846	6.5
16.00	5.045	10.063
18.00	4.231	5.683
20.00	4.621	4.608
22.00	6.111	4.675
24.00	8.301	9.113
04.00	12.406	14.208

$\chi^2 = 8.466, \quad p > 0.3, \quad \sqrt{} = 9$

Thus, the peak and the trough of the circadian rhythm of plasma cortico-sterone levels under reversed lighting conditions are at 06.22 hr and 18.22 hr respectively.

α for "normal" lighting and "reversed" lighting differ significantly $(p < 0.001)$.

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ABSTRACT

Studies of Circadian Patterns in CRF Secretion

from the Rat Hypothalamus Isolated and Incubated In Vitro

by Gerald S. Kamstra

An in vitro bioassay which discriminates between vasopressin and CRF was used in an investigation of the secretion of CRF from the isolated rat hypothalamus incubated in vitro for varying periods of time at different times in the 24-hour cycle. A porcine hypothalamic CRF extract was also tested in the assay and elicited responses similar to those elicited by rat hypothalamic incubation media.

During continuous incubations sampled at a series of times (up to 120 minutes) the pattern of CRF secretion is seen to be phasic. The secretion of CRF, when the hypothalamus is incubated for two consecutive 15 minute incubation periods, is shown to be calcium ion-dependent and to display a circadian rhythm with peaks occurring in the afternoon when studied at different times in the 24-hour cycle. From this, the secretion of CRF by the hypothalamus isolated and incubated in vitro is considered not to represent non-specific diffusion or "leakage" of CRF, but a phenomenon of physiological significance which reflects a circadian rhythm in CRF secretion in vivo.

The secretion of CRF during two consecutive 15 minute incubations measured systematically at different times of the day shows a circadian rhythm which parallels the peaks and troughs in the circadian rhythm in plasma corticosterone levels, but which shows a phase delay with the increase in hypothalamic CRF content in the afternoon. Reversal of the light/dark cycle results in a reversal of the morning/evening difference in the pattern of CRF secretion during the first 15 minutes of incubation and a concomitant phase-reversal of the circadian rhythm in plasma corticosterone levels. These findings imply that CRF secretion is involved in the regulation of circadian rhythmicity in plasma corticosterone levels, and that circadian rhythmicity in CRF secretion may be entrained by photoperiodic cues.