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Polypeptides of porcine posterior pituitary

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POLYPEPTIDES OF PORCINE POSTERIOR PITUITARY

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HISTORICAL

The hypophysis cerebri (pituitary gland) is attached to the tuber cinereum region of the hypothalamus, a part of the vertebrate forebrain forming the floor of the third ventricle, by the infundibular stalk (64). The hypophysis is a composite structure consisting of an anterior lobe (adenohypophysis or pars distalis), an intermediate lobe (pars intermedia) and a posterior lobe (neurohypophysis or pars nervosa).

In his review of the first evidence suggesting a function for the pituitary gland, Sir Henry H. Dale (16) recalls the discovery in 1895 of a pressor (blood pressure increase) effect due to an intravenous injection of pituitary gland extract. In 1898, other investigations showed this pressor action was obtained only from the posterior lobe. Eight years later, after discovering the oxytocic (uterine contracting) properties of a posterior lobe extract, Dale verified the reported antidiuretic properties of these extracts. Observations of the destruction of these activities by hot acids and alkalis and tryptic digestion led Dale to assume a single substance having the character of a relatively simple polypeptide.

Abel and Pincoffs (1), in 1917), reported that secondary albumoses (proteoses) and possibly peptones (polypeptides) were found to be present in all of the therapeutically used extracts of the posterior lobe of the hypophysis cerebri which

were examined.

Smith (122), in 1927, observed that hypophysectomized animals, that is, animals from which the entire pituitary gland was removed, decreased in body weight, had inhibited skeletal growth, and pronounced atrophy of the adrenal cortex, thyroids and reproductive organs. Also noted were decreases in size of the liver, spleen and kidney, a cessation of follicular growth and a filling of the ovaries with interstitial tissue in the female or a loss of libido sexualis and spermatogenesis in the male. Daily transplantations of anterior lobe tissue in these animals caused a resumption of body growth and a return of the reproductive organs to their normal size. Li and Evans (67) proposed the name adrenocorticotrophic hormone (ACTH) for the substance in these implants which affects the adrenal cortex in preference over the names adrenotrophic or corticotrophic hormone. In 1951, the Journal of the American Medical Association (127) suggested the corticotropin nomenclature for this substance.

In 1928, Kamm and his co-workers (57) were able to separate the oxytocic principle from the pressor principle of acetone-desiccated posterior lobe tissue by a hot dilute acetic acid extraction followed by salt and solvent precipitation techniques. They concluded that these substances were of a basic nature and probably amines. By 1933 the peptide nature of these biologically active materials had been

revealed (25) and work continued on the purification and chemical properties (25, 54, 55, 87, 88) of these hormones using electrophoretic, countercurrent distribution and paper chromatographic methods.

In June of 1953, Tuppy (130) reported the amino acid sequence of oxytocin which was conclusively proved by the synthesis reported in July by du Vigneaud et al. (24). du Vigneaud and his co-workers (24) also postulated the structure of lysine vasopressin (LVP), the porcine hormone, and reported the synthesis of this octapeptide. The structure of bovine vasopressin (AVP) was published in September by R. Acher and Jacqueline Chauvet (3) and verified by the synthesis of this hormone by du Vigneaud et al. (23) the following year. The bovine hormone differed from the porcine molecule in that arginine in the former replaced the lysine residue in the latter. An excellent review of the isolation, proof of structure and synthesis of the vasopressins has been written by du Vigneaud (22).

It was obvious in the purification of oxytocin and vasopressin that the posterior lobe of the pituitary gland contained a rich pool of peptides of varying size and complexity. Winnick et al. (134) and Ramachandran and Winnick (92) in their study of the peptides of beef and pork posterior pituitary lobe (pars nervosa plus pars intermedia) found that peptides constituted 3-4% of the weight of desiccated tissue.

In addition to oxytocin, vasopressin, and melanocyte-stimulating hormone (MSH or intermedin), 30 different peptides, comprising approximately one-third of the total peptide material, were isolated and studied. In size the peptides ranged from simple dipeptides to one with perhaps as many as 84 residues. Hypothalamic extracts (134) showed no significant peptide pool and resembled brain in having very high levels of glutamine and glutamic acid.

In 1951, Bargmann and Scharrer (9) proposed that in higher vertebrates the synthesis of the posterior lobe peptide hormones occurred in neurosecretory cells of the nuclei supraopticus and paraventricularis of the hypothalamus from whence they pass to the pars nervosa by way of the hypothalamohypophyseal tracts. A review of the data for this conclusion was given by Scharrer and Scharrer (116) at the 1953 Laurentian Hormone Conference. McCann and Brobeck (72) showed that hypothalamic lesions which destroyed a significant fraction of the supraopticohypophyseal tract blocked ACTH secretion as measured by ascorbic acid depletion, adrenal weight maintenance or blood ACTH concentration. They also reported that commercial Pitressin, of which presumably vasopressin was the active factor, produced ACTH secretion in the lesioned animals when injected intravenously. The conclusion reached was that the supraopticohypophyseal tract may play a role in the regulation of ACTH secretion by release of antidiuretic hormone

into the hypophyseal portal vessels.

Investigations into the factors underlying the normal secretion of ACTH concerned two aspects of the problem. First, the method of maintaining a steady secretion of ACTH in the normal animal; and, secondly, the mechanism involved in increasing ACTH secretion under physiological or psychological stress. As previously cited (67), ACTH maintains the structure and function of the adrenal cortex. In his review of the features of the adrenal cortex, Sayers (106) discusses the pituitary adrenocortical system and its role in homeostasis, maintenance of a steady state in an organism through all types of stress; the reader is referred to that article for a detailed presentation of the experimental evidence. Indices for the assessment of adrenocortical activity are four:

I. Alterations in the adrenal cortex.

1. Size of the gland - enlargement of the gland occurs within 6 to 24 hours after severe stress. Adrenal weight increases proportionately to the doses of administered ACTH.

2. Histochemical changes - A close parallelism exists between intensity of sudanophilic substance and the concentration of cholesterol in the gland which fluctuates according to the ACTH level. The glomerular, sudanophobic, fascicular and reticular zones of the

adrenal cortex may show different degrees of depletion and accumulation of lipid in response to stress.

3. Chemical changes.

   a. Cholesterol - approximately 5% of the net weight of the tissue is cholesterol, which is depleted by ACTH; much evidence suggests that cholesterol is a precursor to the cortical steroids.

   b. Ascorbic acid - administration of ACTH causes the concentration of adrenal ascorbic acid to fall appreciably within 20 minutes after injection into rats. This phenomenon constitutes the basis of the well-known Sayers ACTH assay (107).

   c. Phosphate - administration of ACTH in the rat increases the rate of turnover of radioactive phosphorus in the adrenal cortex.

II. Alterations in the organism.

1. Lymphocytes - Lymphocytosis in the adrenolectomized animal or of a patient with Addison's disease reflects low adrenocortical hormones while the lymphocytopenia of Cushing's syndrome is a result of an elevated cortical hormone titer. ACTH initiates lymphocytopenia and this can be employed as a measure of adrenocortical activity.

2. Eosinophils - The drop in the number of eosinophils after the administration of ACTH is expressed in per-

centage of the pre-injection count and is a less variable response than the lymphocyte response. Intravenous injections of epinephrine also produces a fall in the circulating eosinophils.

3. Metabolic changes - Other changes due to deficiencies in cortical hormones include insulin hypersensitivity, lack of diuresis after administration of water, and failure to retain sodium under circumstances of sodium deprivation.

III. Urinary steroids - The cortical hormones, including cortisone and 17-hydroxycorticosterone, extracted from human urine promote liver-glycogen deposition (LGD), maintain life, offer cold-protection (CP), improve work performance and prevent water intoxication when injected into adrenolectomized animals. The LGD and CP tests are relatively good indices of adrenocortical activity but are tedious and time-consuming. Chemical measurement of cortical steroids extracted from urine is done by phosphomolybdic acid reduction, copper reduction, or formaldehyde production by periodic acid. Sayers (106) does not believe that a measurement of 17-ketosteroids in the urine is of value in assessing the rate of the cortical hormone secretion of man.

IV. Blood corticoids - Although appreciable quantities of cortical hormones are present in adrenal venous blood, unequivocal responses by bioassay methods are not obtained. However, Guillemin et al. (28, 30, 41) have perfected a method for assessing the concentration of plasma free corticosteroids using a fluorometric technique. Mote's review (82) discusses the physiological bases for the action of ACTH in human beings.

In his review of the neural control of the pituitary gland in 1951, Harris (44, p. 563) concluded that:

> The glandular tissue of the neurohypophysis, which exists in the infundibular lobe, infundibular stem and median eminence of the tuber cinereum, is richly innervated by the supraoptico-hypophysial tract of nerve fibers.

(See composite diagram of a sagittal section through the hypothalamus and pituitary gland, Fig. 1.)

Psychological or physiological stress causes the release of antidiuretic hormone through excitation of the gland mediated by the supraoptico-hypophysial tract in the normal animal. Electrical stimulation of this tract causes an inhibition of a water diuresis; increase in the relative urinary chloride; increase in the uterine activity in the oestrous or oestrogenized animal; increase in intestinal activity; appearance of an antidiuretic substance in the urine; and the ejection of milk from the cannulated lactiferous duct of

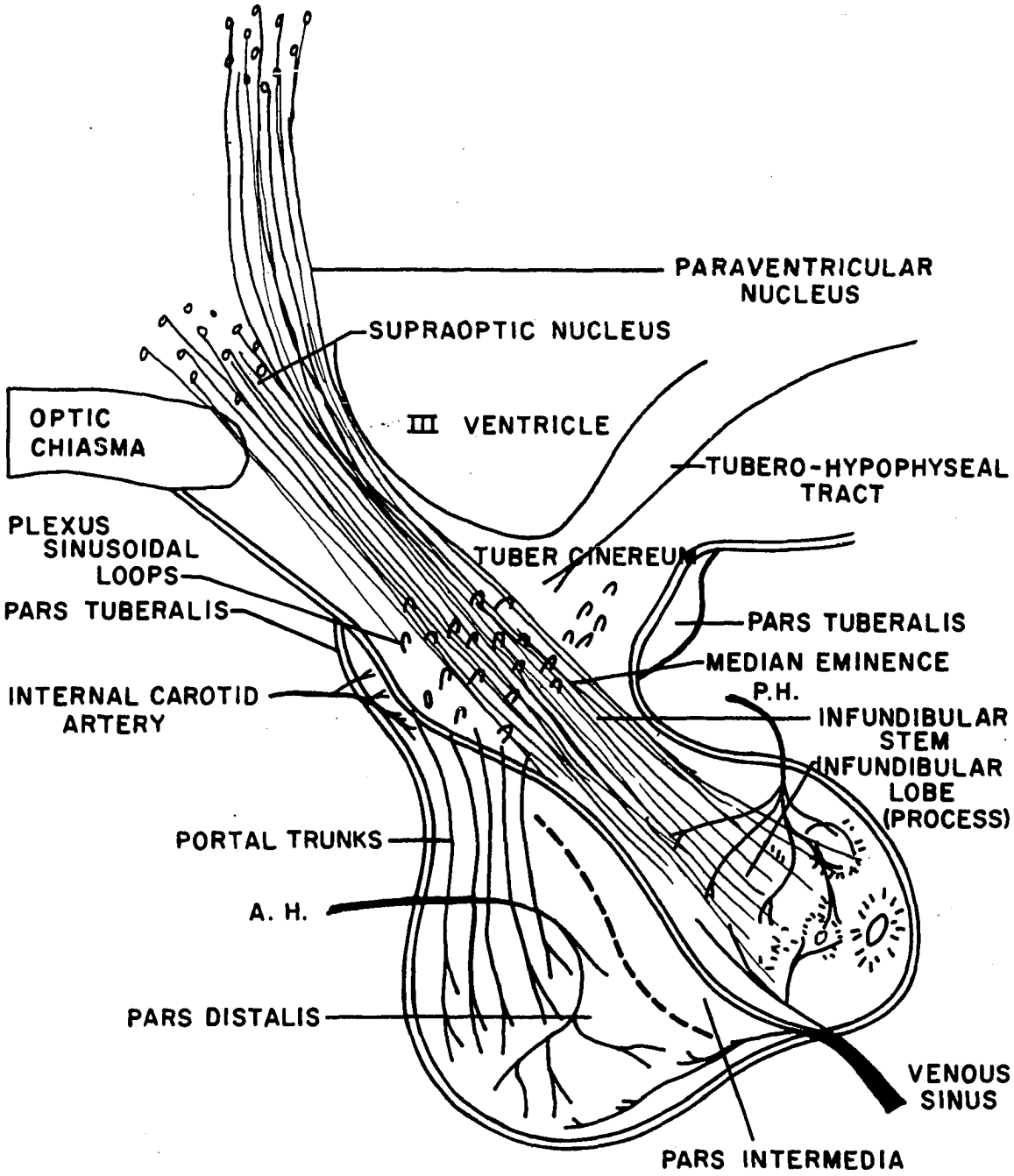


Fig. 1. Schematic drawing of sagittal section of the hypothalamus and hypophysis

a lactating mammary gland. These changes are caused by the liberation of the antidiuretic (pressor) and oxytocic substances into the blood.

Harris (45) in reviewing the regulation of ACTH release from the adrenohypophysis considered three views put forward at that time: 1) ACTH secretion is regulated by the systemic blood level of adrenalin (epinephrine); 2) ACTH secretion is regulated by the systemic blood level of the adrenal cortical hormones; 3) ACTH secretion is controlled by the hypothalamus acting through the hypophyseal portal vessels. For a detailed discussion of these views the reader is referred to this review. In brief the arguments martialed against adrenalin as the underlying mechanism of ACTH release are: 1) completely sympathectomized animals resist many forms of stress almost as well as intact animals and 2) audicgenic stimulation, known to cause discharge of ACTH in normal rats, produced a response in rats with removed adrenal medullas. Pretreatment of an animal with adrenal cortical extract or crystalline cortical steroids prevents adrenal ascorbic acid depletion which normally follows temperature or chemical stress. The level of the adrenal cortical hormones influences but is not the only factor which regulates the secretion of ACTH since adrenal atrophy occurs in rats with anterior pituitary gland transplants. Pituitary transplants and grafts show that normal ACTH secretion requires the hypophyseal portal

vessels connecting the hypothalamus and adrenohypophysis.

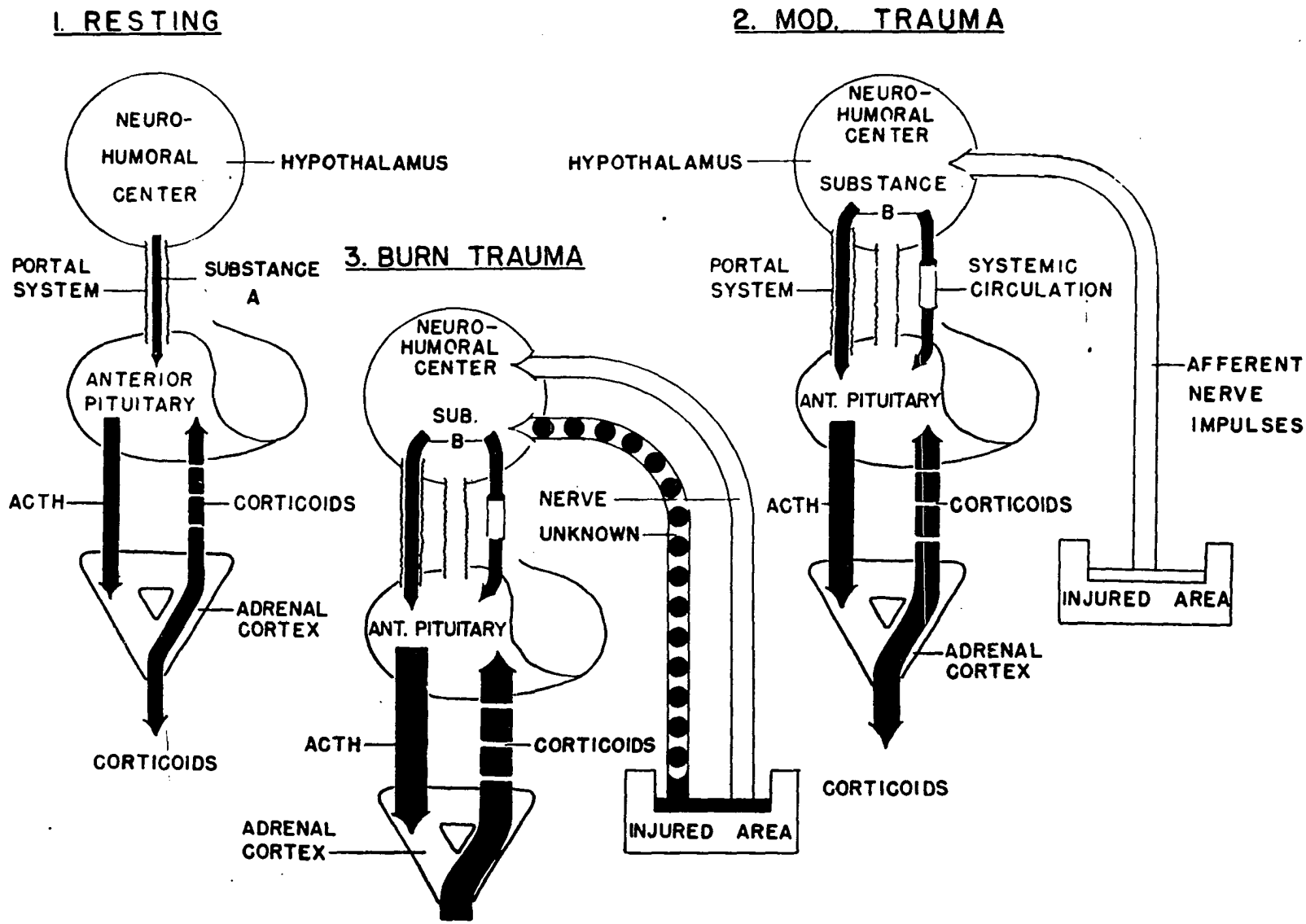
Harris (45, p. 633) concludes, with Fortier, that a dual mechanism of ACTH release operates

> . . . one purely humoral in response to systemic stimuli, and the other probably neuro-humoral mediated by the hypothalamo-hypophyseal neuro-vascular pathway and coming into play under the influence of nervous or emotional stimuli.

Hume (53) proposed a theory on the neuro-endocrine response to injury based on the experimental evidence obtained in his own laboratory and by other workers. In the normal state, a humoral substance is secreted by the hypothalamus, moves through the portal system to the adenohypophysis and maintains the normal production of ACTH. See Fig. 2-1.

In emergency mechanisms, the traumatized area causes nerve impulses to actuate the hypothalamus to release a humoral substance B (Fig. 2-2), which stimulates ACTH production. The hormone probably reaches the pituitary partly by the hypophyseal portal system because the speed of ACTH release after stress, in the form of histamine, would not permit systemic circulation to act upon the hypophysis and lesions in the median eminence region prevent increased ACTH release following trauma. Severe trauma, as shown in Fig. 2-3 probably occurs through a similar mechanism as moderate trauma but with an additional humoral substance acting directly on the hypothalamus. It is possible, Hume (53) suggests, that substances A and B can be identical. Arguments

Fig. 2. Diagrams illustrating the hypothalamic-pituitary-adrenal relationships (53)


1. RESTING
NEURO-
HUMORAL
CENTER
HYPOTHALAMUS
PORTAL
SYSTEM
SUBSTANCE
A
ANTERIOR
PITUITARY
ACTH
CORTICOIDS
ADRENAL
CORTEX
CORTICOIDS
3. BURN TRAUMA
NEURO-
HUMORAL
CENTER
SUB.
B
NERVE
UNKNOWN
ANT. PITUITARY
ACTH
CORTICOIDS
ADRENAL
CORTEX
INJURED AREA
2. MOD. TRAUMA
NEURO-
HUMORAL
CENTER
HYPOTHALAMUS
SUBSTANCE
B
PORTAL
SYSTEM
SYSTEMIC
CIRCULATION
AFFERENT
NERVE
IMPULSES
ANT. PITUITARY
ACTH
CORTICOIDS
ADRENAL
CORTEX
CORTICOIDS
INJURED AREA

are presented against Sayers' (106) theory of decreased circulating corticoids causing the stimulation of ACTH release although they may account for deceleration of ACTH secretion when the stressing stimulus is lessened. In elegant experiments employing a radio coil allowing remote control stimulation of the hypothalamus, Hume (53) demonstrated that stimulation of the median eminence or the posterior tuber cinereum of the dog produced ACTH release as measured by eosinopenia. Since this is also true in the sympathectomized dog, ACTH release does not depend on epinephrine release.

Guillemin and Fortier (31) showed that rats could be brought to a "state of resistance" by Phenergan, an antihistaminic drug, which caused a complete inhibition of the normal adrenal ascorbic acid discharge to histamine injection. However, neurotropic stimuli including auditory stimulation and immobilization; and systemic stress, cold exposure, resulted in a depletion of adrenal ascorbic acid. They conclude that histamine is not an important factor in the hypothalamohypophyseal interaction due to neurotropic stress and can also be disregarded as the only humoral agent responsible for pituitary stimulation by systemic stress.

Slusher and Roberts (121), in 1954, were able to extract an aqueous non-protein fraction from bovine brain containing active material which caused eosinopenia in rats and an increase in oxygen consumption by rat adenohypophyseal tissue

in vitro; however no adrenal ascorbic acid depletion in the rat was evident. Another material, lipoprotein in nature, found only in the posterior hypothalamus caused eosinopenia and adrenal ascorbic acid depletion. The lipid portion of this active complex, which was thought to represent the ACTH-releasing neurohumor, and could be removed after prolonged dialysis, was non-saponifiable and not cholesterol.

Guillemin (39) reported that SKF-501 (N-[9-fluorenyl]-N-ethyl-β-chloroethylamine, HCL), SKF-688A, Dibenzyline, (N-phenoxy-isopropyl-N-benzyl-β-chlorothylamine, HCL) and atropine sulfate were effective blocking agents against the stress-inducing, normally ACTH releasing compounds: adrenaline, non-adrenaline and acetylcholine respectively. However, these pharmacological blocking agents did not significantly alter the ACTH release measured by adrenal ascorbic acid depletion (AAAD) induced by other systemic or neurotropic stresses; thus neither adrenaline, non-adrenaline nor acetylcholine can be the sole humoral agent involved in pituitary stimulation. Guillemin (39) showed that explants of posterior hypothalamus or eminentia media caused the release of ACTH in vitro from anterior pituitary tissue cultures. Saffran and Schally (103), using an in vitro assay (104) to determine factors stimulating ACTH release, found hypothalamic tissue or brain cortex, in the presence of epinephrine or arterenal, increased the release of ACTH. The greatest stimulation of

ACTH release occurred with posterior pituitary lobe tissue plus arterenol; they concluded that the neurohypophysis is probably involved in the adenohypophyseal-adrenocortical response system. Contrary to the claims of Slusher and Roberts (121), Saffran and Schally (103) found that the anterior and posterior parts of the hypothalamus were equally effective in causing ACTH release. In a later publication in the same year, Saffran et al. (105) extracted a corticotropin-releasing factor (CRF) from beef and hog posterior pituitaries and showed it to be distinct from vasopressin and oxytocin by its behavior in several paper chromatography solvent systems. Crude vasopressin (200 U/mg.) prepared from beef posterior pituitaries contained CRF activity but further purification to 400 U/mg. caused a loss of most of the CRF activity. Since CRF contaminated vasopressin and appeared at ninhydrin-staining areas on paper chromatography, it was assumed to be probably peptide in nature. These investigators believed that McCann and Brobeck's (72) results with Pitressin could be explained by CRF contamination and further postulated that CRF is elaborated within neurosecretory cells in the hypothalamus (as stated previously for oxytocin and vasopressin (116)) and stored until needed in the neurohypophysis. Guillemin and Hearn (32) also found, in a similar assay, that Pitressin increased ACTH release whereas highly purified arginine-vasopressin had no effect. In work conducted at the

same time, Martini and Morpurgo (71) noted that Pitressin produces a significant fall of adrenal cholesterol after subcutaneous injections of 0.3 U/rat whereas Pitocin at the same concentration has no significant effect. They cited these results in support of the argument that posterior pituitary hormones may activate the anterior lobe to discharge ACTH and that antidiuretic hormone may be the neurohumoral transmitting agent.

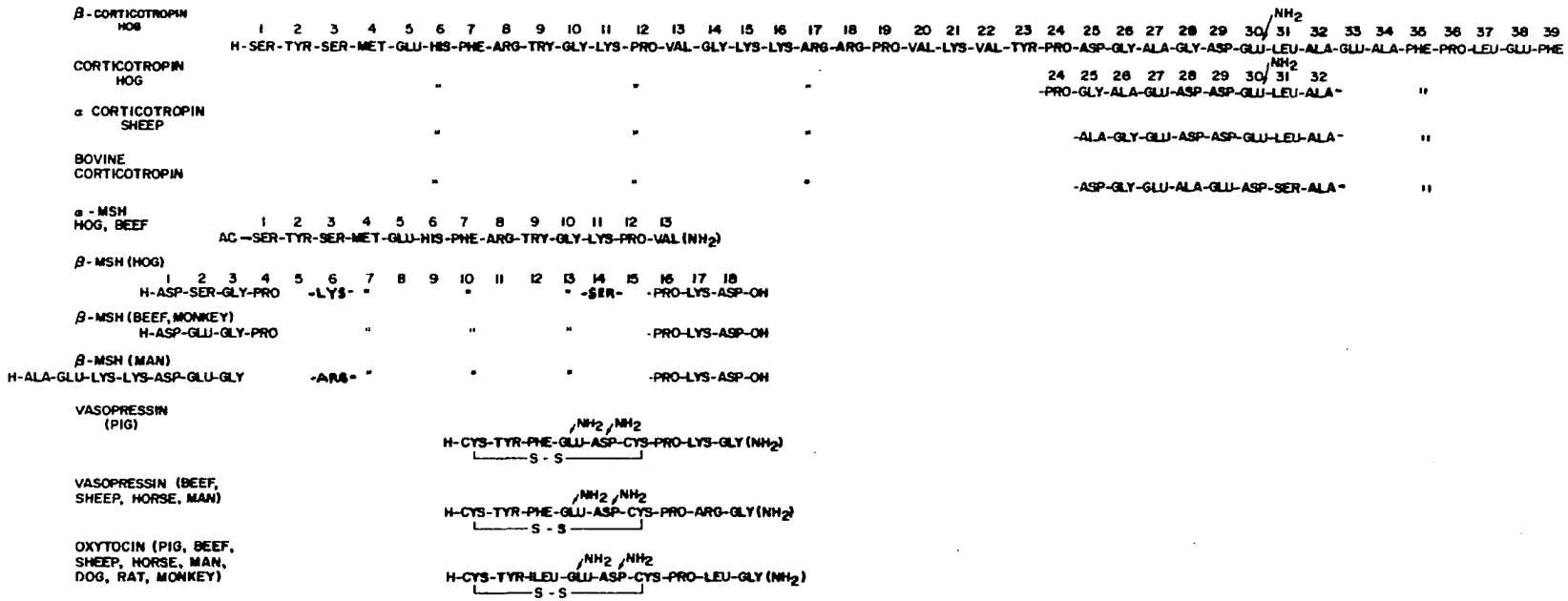
For a more detailed review of the literature concerning the pituitary-adrenal relationship to June of 1955, the publication of C. N. H. Long (69) may be consulted.

Meanwhile, work had continued on the purification, isolation and amino acid sequence of corticotropin and finally resulted in the complete elucidation of the structure in 1954. An excellent early review of this work is presented by Hays and White (47) while a more complete discussion is offered by C. H. Li (66). Scientists associated with the American Cyanamid Company (10, 66, 117, 118) published a series of papers on β-corticotropin which constitute an excellent review of the methodology involved in peptide hormone research. Seven active components, β-corticotropin representing the most abundant one (ca. 50%), were isolated from the porcine oxycellulose-treated starting material. β-Corticotropin gave three smaller active molecules P_2, P_3 and P_4 having 28, 30 and 31 amino acid residues respectively upon

pepsin digestion (10, 118). As in other peptide hormones, a species difference exists for the ACTH molecule in the pig, cow and sheep. The corticotropins have a definite melanocyte-stimulating activity which can be readily understood in their sequential similarity to the MSH molecules as indicated in Fig. 3.

In early 1956, Porter and Rumsfield (90) reported adrenal ascorbic acid depletion (AAAD) in the hydrocortisone-inhibited rat upon injection with lyophilized hypothalamico-hypophyseal portal vessel plasma. This plasma showed no ACTH activity in the hypophysectomized rat. Similarly tested lyophilized carotid artery plasma gave no ACTH secretion in the hydrocortisone-treated intact rat. The active substance in portal plasma was non-dialyzable and present as a single fraction of the alcohol fractionated plasma proteins. A significant AAAD occurred when Pitressin was injected into the hydrocortisone-inhibited, intact rats confirming the work previously cited. Rumsfield and Porter concluded that the active substance "is either a large protein molecule or is bound to a large protein molecule and is probably not identical with vasopressin."

Guillemin et al. (33) reported preliminary studies on the isolation of a CRF active (as assayed by the in vitro technique) complex or mixture of polypeptides, DΔ, from hog and beef hypothalamus. One active fraction, D, obtained by


β-CORTICOTROPIN
HOG
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39
H-SER-TYR-SER-MET-GLU-HIS-PHE-ARG-TRY-GLY-LYS-PRO-VAL-GLY-LYS-LYS-ARG-ARG-PRO-VAL-LYS-VAL-TYR-PRO-ASP-GLY-ALA-GLY-ASP-GLU-LEU-ALA-GLU-ALA-PHE-PRO-LEU-GLU-PHE
CORTICOTROPIN
HOG
24 25 26 27 28 29 30 31 32
-PRO-GLY-ALA-GLU-ASP-ASP-GLU-LEU-ALA-
α CORTICOTROPIN
SHEEP
-ALA-GLY-GLU-ASP-ASP-GLU-LEU-ALA-
BOVINE
CORTICOTROPIN
-ASP-GLY-GLU-ALA-GLU-ASP-SER-ALA-
α-MSH
HOG, BEEF
1 2 3 4 5 6 7 8 9 10 11 12 13
AC-SER-TYR-SER-MET-GLU-HIS-PHE-ARG-TRY-GLY-LYS-PRO-VAL
β-MSH (HOG)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
H-ASP-SER-GLY-PRO -LYS- -SER- -PRO-LYS-ASP-OH
β-MSH (BEEF, MONKEY)
H-ASP-GLU-GLY-PRO -PRO-LYS-ASP-OH
β-MSH (MAN)
H-ALA-GLU-LYS-LYS-ASP-GLU-GLY -ARG- -PRO-LYS-ASP-OH
VASOPRESSIN
(PIG)
H-CYS-TYR-PHE-GLU-ASP-CYS-PRO-LYS-GLY
S - S
VASOPRESSIN (BEEF,
SHEEP, HORSE, MAN)
H-CYS-TYR-PHE-GLU-ASP-CYS-PRO-ARG-GLY
S - S
OXYTOCIN (PIG, BEEF,
SHEEP, HORSE, MAN,
DOG, RAT, MONKEY)
H-CYS-TYR-ILEU-GLU-ASP-CYS-PRO-LEU-GLY
S - S

paper chromatography, was extracted from Protopituitrin, Pitressin, hypothalamus and posterior pituitary with the hypothalamic fraction approximately 100 times more active than posterior pituitary D. D was well separated from vasopressin, had no ACTH potentiation of the adrenals and showed MSH activity. This work was reported in greater detail in 1957 (34). Schally and Saffran (114) reported that histamine and purified lysine vasopressin did not accelerate the release of ACTH by isolated cultures of rat pituitaries. In contrast, purified CRF preparations in minute doses significantly increased the release of ACTH. Swingle et al. (128) showed that commercial Pitressin exhibited ACTH-releasing activity when incubated with pituitaries and the incubation fluid infused intravenously into hypophysectomized rats. The CRF could be separated from the pressor component by Craig countercurrent distribution or by destroying the latter by acid hydrolysis. These investigators concluded that the ACTH-releasing agent was not histamine or a histamine-like substance. McDonald et al. (76, 77) studied the effect of Pitressin on the adrenocortical activity in man and reported a stimulation of the pituitary-adrenal system. Commercial and synthetic lysine vasopressin were found equipotent in producing AAAD in rats with hypothalamic lesions; therefore, McCann and Fruit (73) concluded that LVP is the neurohumor responsible for ACTH release. McDonald et al. (75) conducted

investigations in normal human subjects by simultaneous determinations of urine osmolality and plasma hydrocortisone level under stresses of fluid deprivation, hypertonic saline, nicotine, hand immersion in ice water and insulin induced hypoglycemia. The data indicated that endogenous ADH release may occur without an increase of ACTH and an increase in ACTH release may occur without evidence of ADH. These observations failed to support the concept that endogenous ADH release stimulates the release of ACTH.

Guillemin et al. (29) reported that the variation in concentration of plasma free corticosteroids gave an excellent ACTH log dose response and further showed (35) that fraction D, previously described and characterized by its in vitro activity, stimulated the discharge of ACTH in vivo in rats with either hypothalamic lesions or pharmacological blockade. Royce and Sayers (95) published experiments showing that one unit of Pitressin was equivalent to 0.1 mu of ACTH measured by AAAD in the hypophysectomized rat and achieved similar results with highly purified arginine vasopressin (AVP). Pitressin at 0.2 U has no significant AAAD in the hypophysectomized rat but potentiates the activity of ACTH by a factor of 1.55 and Pitressin also depletes AAA in the decapitated-lesioned rat, showing it to have an extrapituitary action. Hilton et al. (48) showed a direct vasopressin stimulation of the isolated adrenal gland to produce 17-hydroxycorticoids in

the dog. In a later report in 1958, Royce and Sayers (96) reported an acid extraction of a CRF active, pepsin labile fraction from calf brain median eminence area tissue. The activity was not adsorbed by oxycellulose and could not be accounted for by the pressor activity present and its known ACTH-releasing action. Royce and Sayers (96) found no difference in the AAAD response to ACTH in the hypophysectomized or median eminence lesioned animals. Schally et al. (115) showed that hog hypothalamic and hog and beef posterior pituitary extracts gave a CRF activity as measured in the in vitro assay. Porcine brain extracts showed no activity. Purified CRF, obtained by paper chromatography, of neurohypophyseal origin significantly increased the release of ACTH at doses of 1 μmg., and upon hydrolysis gave cystine, aspartic acid, glutamic acid, glycine, proline, lysine, phenylalanine, alanine, serine and histidine as the amino acid composition. De Garihle et al. (18) were able to obtain peptide fractions containing CRF activity by using dialysis and Amberlite IRC-50 column chromatography on extracts of posterior pituitary of beef and hog. The active fractions showed CRF activity in both in vitro and in vivo assays and contained minute amounts (1-2 U/mg.) of vasopressin, indicating CRF and vasopressin to be two chemically distinct substances.

In a presentation at a meeting in April, 1959, of the Federation of American Societies for Experimental Biology,

Royce and Sayers (97) reported experiments showing a crude acetic acid extract of pituitary stalk-median eminence tissue derived from calf hypothalamus containing a pepsin-labile factor capable of inducing near-maximal AAAD in rats with hypothalamic lesions at a dose of 24-53 γ of protein. This same dose failed to elicit AAAD in intact rats pretreated with desoxycorticosterone acetate (DCA or DOCA); therefore, they concluded that one site of DCA blockade of ACTH release must occur at the adenohypophysis level. Schally and Guillemin (111) were able to concentrate CRF by chromatography on carboxymethyl cellulose (CMC) column employing a protopituitrin or pitressin intermediate starting material. The pressor and CRF activity curves were almost identical and the assays were conducted at "safe" doses corresponding to 10 or 30 mU of pressor activity. Guillemin et al. (30) reported later that doses of 30 mU of highly purified natural lysine vasopressin (LVP 287 U/mg.) are inactive in rats with hypothalamic lesions or pharmacological blockade. They concluded that the CRF activity of their fraction D must be attributed to a substance other than vasopressin. In opposition to Royce and Sayers (96), however, Guillemin et al. (30) found that animals with "effective" hypothalamic lesions have an inferior sensitivity to that of hypophysectomized rats toward ACTH. The smallest effective dose is 1 mU versus 0.1 mU for the hypophysectomized rat. Lerner and Lee (65) reported the

absence of corticotropic activity in α-MSH as measured by AAAD. Steelman and Guillemin (124) showed that highly purified α-MSH ($1x10^7$ U/mg.) when studied for ACTH activity *in vivo* gave a response of less than 0.18 ACTH USP U/mg. for the AAAD assay and 0.1 ACTH USP U/mg. for the plasma B assay. The *in vitro* assay gave 3.6 ACTH USP U/mg. This difference in activity, approximately 30 fold, was tentatively related to unequal rates of absorption by the adrenal tissue *in vitro* and unequal rates of inactivation and disappearance of the substance *in vivo*. β-MSH did not show adrenocorticotropic activity. In their re-evaluation of the *in vitro* pituitary incubation system used to demonstrate CRF activity, Guillemin and Schally (36) showed that the effect of CRF material, prepared as described earlier (111), could not be explained on the basis of the inhibition of ACTH inactivation. Furthermore CRF did not act by potentiating ACTH at the adrenal level or by an inherent adrenocorticotropic activity, and the most active fraction stimulated ACTH-release *in vivo* at a dose of 60 mr.

Nichols and Guillemin (84) in experiments designed to study the effects of endogenous and exogenous vasopressin on ACTH in unanesthetized trained dogs showed that no correlation was observed between the discharge of ACTH as measured by plasma free 17-hydroxycorticosteroid (17OHC) and the release or inhibition of release of antidiuretic hormone (ADH).

These investigators further commented that if this phenomena is due only to a large difference in sensitivity to their respective affectors, this must mean a factor of 3.5×10^3 to 7.1×10^3 between the amount of vasopressin necessary for maximal antidiuresis and the threshold dose for ACTH stimulation; or, a differential release of vasopressin from the posterior lobe - to the periphery for antidiuresis or to the anterior pituitary for ACTH secretion. McCann and Haberland (74) reported that rats with "acute" hypothalamic lesions were one-third as sensitive to ACTH than the hypophysectomized rats, a result agreeing qualitatively with Guillemin et al. (30) but disagreeing with Royce and Sayers (96). Using this observation to interpret their assay results, McCann and Haberland (74) were able to show the presence of a CRF in acid extracts of beef or rat stalk-median eminence area with 10 to 20% of the CRF activity apparently due to vasopressin. Although Royce and Sayers (96) could effect some purification by oxycellulose treatment, McCann and Haberland found that oxycellulose almost quantitatively adsorbed all active material. They also published data indicating that the hydrocortisone inhibited rat, as used in the Porter and Jones (89) assay, gives a response significantly less, 82 ± 10 to 133 ± 14 for 6 mU of ACTH, than that of the lesioned animal. DeWied and Mirsky (21) found that the minimal dosage of intraperitoneally injected Pitressin necessary to produce a sig-

nificant decrease in AAA was more than 10 times greater in the hydrocortisone-treated than in the normal rat. Twice the intravenous dosage was required for AAAD in the treated animal than for the untreated animal. Identical adrenal cortical responses were obtained in the hydrocortisone-treated rats with similar intravenous doses of Pitressin, arginine vasopressin and synthetic lysine vasopressin. Rumsfield and Porter (100) demonstrated the presence of ACTH-releasing activity in protein fraction III_0 of portal vessel plasma as measured by AAAD in the steroid-depressed rat. The activity attributable to III_0 was lost during dialysis against a buffer containing 15.2% ethanol and was not due to contamination with nonepinephrine, epinephrine, histamine or serotonin. Porter and Rumsfield (73) showed that fraction III_0 consisted of three major protein components with isoelectric points at pH 4.4, pH 5.1 and pH 6.6 and the active peak had a mobility in veronal buffer (pH 8.6; $\mu = 0.1$) of -1.91 ± 0.41 cm.^{2} volt^{-1} sec.$^{-1}$ $\times 10^{-5}$ by starch column electrophoresis. The CRF activity of fraction III_0 was trypsin and pepsin labile, destroyed by refluxing with 0.1 N NaOH but not by refluxing in 0.1 N HCl for two hours. Preliminary results indicated the lyophilized portal plasma dissolved in Cohn buffer at pH 6.9 lost the active substance upon dialysis against distilled water suggesting that the plasma protein serves a role in transport of the CRF in portal blood.

Rumsfield and Porter (99) made an acetone extraction of beef hypothalamus which was evaporated to dryness and taken up in ether. The ether solution was extracted with 0.9% saline solution which showed ACTH-releasing activity as measured by the Porter and Jones (89) assay. An aliquot of the saline phase equivalent to 15 "hypothalami" was lyophilized, dissolved in ammonium formate buffer and chromatographed on a Dowex 50x2 column. Ninhydrin analysis revealed 18 peaks with only one, containing two ninhydrin positive components as indicated by paper chromatography in five solvent systems, possessing ACTH-releasing activity. Countercurrent distribution of the active peak gave partition coefficients in sec-butanol-0.05% acetic acid of approximately 0.11 and 0.37. Descending paper chromatography gave R_F values much higher than those obtained for the vasopressins. Gros and De Garihle (42) obtained an ACTH-releasing preparation by submitting hog posterior pituitary powder to an acetic acid extraction which was lyophilized to a small volume and acetone precipitated. The fraction containing small amounts of vasopressin and ACTH-releasing activity was subjected to countercurrent distribution for ≥5 transfers. The CRF fraction, assayed by the <u>in vitro</u> method (18), was well-removed from the vasopressin area and showed three or four peptides when paper electrophoretographed. Column electrophoresis of the CCD CRF fraction gave an apparently homogeneous peptide which

gave an excellent CRF response at doses of 1γ.

In the spring of 1960, Guillemin et al. (38) reported that when CRF concentrates (111) are subjected to countercurrent distribution in three systems, LVP and CRF activities are widely separated. Since no CRF activity was demonstrable under the LVP peak, the distinct chemical entity of each of these substances was confirmed. However, since the CRF activity corresponded with the α-MSH peak in the three CCD's and were not separable by paper strip or zone electrophoresis, it was uncertain whether α-MSH and CRF were one and the same molecule. Schally and Guillemin (112) studied the behavior of oxytocin, lysine and arginine vasopressin, ACTH, α-MSH and CRF on CMC and IRC-50 cation exchange columns. CRF activity based on an in vivo assay followed oxytocin and preceded LVP and AVP, α-MSH and ACTH on CMC, but followed oxytocin and LVP on IRC-50. In their investigation of the ACTH-releasing factor present in Pitressin, Sideman and Sobel (120) studied its electrophoresis fractions and found the pressor and ACTH-releasing activities occurred in the same area. Amino acid analysis of the peptide gave only those eight contained in LVP and comparison of the two activities during mild acid and alkaline hydrolysis, iodination and incubation with placental extract indicated parallel alteration. These researchers concluded that LVP causes ACTH release in the guinea pig and may be a hypothalamic mediator in that species.

Royce and Sayers (98) subjected a glacial acetic acid extract of a portion of calf tissue including median eminence and pituitary stalk (SME) to chromatography on a carboxymethyl cellulose column using a discontinuous gradient elution of 0.05, 0.1, 0.2 and 0.4 M ammonium acetate buffer. Corticotropin releasing activity was found in the 0.01 M and 0.10 M fractions; the 0 05 M fraction had no activity whereas the bulk of the pressor activity emerged with the 0.2 M buffer with a small response elicited by the 0.40 M fraction which contained the ACTH activity exclusively. The 0.10 M fraction had no detectable pressor or ACTH activity at a dose of 0.3 SME (4.3 μg of protein) but gave significant CRF response. The data indicated the possibility of the existence of two chemically distinct CRFs in SME tissue if the fractionation techniques were adequate.

In June of 1960, Guillemin et al. (37) and in a more detailed report later (109) reported the chemical and physiological evidence for the existence of two corticotropin-releasing factors in hog posterior pituitary extracts. A single peak obtained by CMC chromatography contained LVP, α-MSH and all the CRF activity (112); this was subjected to CCD and LVP was well separated from α-MSH (38). The α-MSH area contained CRF activity which could be separated on analytical scale CMC columns. The CRF was a peptide or group of peptides containing the amino acids of α-MSH plus

threonine, alanine and leucine. This peptide, designated ∝-CRF, was active in releasing ACTH only at relatively high levels of 2-3 γ *in vivo* or 0.5 γ *in vitro* and had an inherent ACTH activity of 0.1 U/mg., pressor activity of 0.1 to 0.2 U/mg. and MSH activity of 0.1 to 0.3×10^7 U/mg. Earlier work (34, 36, 112, 115) indicated a vasopressin-related CRF with activities at lower dose levels and thus the CCD pressor area was carefully explored. Paper chromatography revealed a small peptide, related to vasopressin on the basis of amino acid composition, active at 0.05 γ *in vitro* or 0.1 γ *in vivo* and designated β-CRF. These investigators (37, 109) believe ∝-CRF may serve as a biosynthetic precursor of ACTH and further propose β-CRF as the true chemical mediator for the release of ACTH. De Garihle *et al*. (18) fractionated hog posterior pituitary by 1) extracting with dilute acetic acid; 2) fractional precipitation with acetone; 3) countercurrent distribution; and 4) zone electrophoresis on a cellulose column. Peaks 3 and 4 of the electrophoresis possessed CRF activity and represented approximately 10% of the starting material; peak 3 gave an amino acid analysis including those found in ∝-MSH plus threonine and leucine. Fraction 3 possessed an MSH activity amounting to 1-2.5% of that of pure ∝-MSH and gave CRF activity at doses of 0.5-1.0 γ ; fraction 4 showed the same degree of CRF activity. In addition De Garihle *et al*. (19) tested the *in vitro* CRF activity of sev-

eral peptides synthesized by Kappeler and Schwyzer (58). Two of these peptides, a heptapeptide having an amino acid sequence of α-MSH from position 4 through 10 with a glutamine at 5, and a decapeptide with the sequence of the first 10 amino acids of α-MSH with the exception of the acetyl group on serine 1 and a glutamine at 5, gave excellent ACTH-releasing activity at 0.1γ. Kappeler and Schwyzer (58) report that the heptapeptide has 2.8×10^5 U/g. MSH-activity, as compared with α-MSH (2×10^{10} U/g.), β_b-MSH (2×10^9 U/g.) and α_b-ACTH (0.5×10^8 U/g.), but no ACTH activity.

DISCUSSION

Statement of Problem

In the spring of 1956, at the time the work reported here was begun, reports in the literature strongly suggested the existence of a neurohumor which was released from the hypothalamus under conditions of psychological or physiological stress. This chemical mediator, transmitted by the portal vessels, brought about secretion of adrenocorticotropic hormone from the adenohypophysis. The goals of this investigation were: 1) to isolate the corticotropin-releasing factor (CRF); 2) to determine its chemical nature and molecular structure; and 3) to confirm the structure by synthesis if feasible. Because of evidence that CRF was probably a peptide or family of peptides, it was considered useful to make preliminary investigations of the nature of the peptide pool contained in hog hypothalamic and posterior pituitary lobe starting material.

Experimental Results

In 1954, McCann and Brobeck (72) reported that secretion of ACTH in rats was brought about by large doses of Pitressin, a commercial product obtained from porcine posterior pituitary lobe by a procedure first employed by Kamm and co-workers (57). Later, Saffran et al. (103, 104, 105) reported the

presence of a corticotropin-releasing factor in water extracts of rat neurohypophysis and Guillemin and Hearn (32) attributed the ACTH hypophysotrope activity of Pitressin to a contaminant of probable hypothalamic origin. Guillemin et al. (33) obtained a CRF active fraction from Pitressin Intermediate B, a fraction in the Kamm procedure (132). Because of the ease of procurement of porcine posterior pituitary, and its reported CRF activity, it was selected as the chief starting material in this investigation.

In the beginning years of this work, the only assay facilities available to us were located at the Baylor University College of Medicine, Houston, Texas under the supervision of Dr. Roger Guillemin. This assay limitation necessitated repetition of proven methods known to result in highly active fractions to produce starting materials for further purification procedures. It was thought that this path would yield the greatest information with the smallest possible number of assays. The procedure for the preparation of "D" material reported in abstract by Guillemin et al. (32) and later in greater detail (34) was the method chosen at the beginning.

Acetone-desiccated hog posterior pituitary lobe, generously donated by the Armour Laboratories or Princeton Laboratories Inc. was subjected to the Kamm extraction procedure (57). Seventy grams of this material gave an average of 4.7 g. of Pitressin Intermediate B (132), a light, tan powder

assaying 4-5 U/mg. of pressor activity, and referred to as Kamm product.

A methanol extract of the Kamm product prepared by extracting 3 g. with methanol:water (10:1 v/v) resulted in average yields of 0.8 g. of lyophilized product having a pressor activity of 2-3 U/mg. This product gave a cleaner pattern in the subsequent paper chromatography step and aided in obtaining a nearly pressor-free active fraction.

A modification of the solvent system used by Guillemin et al. (32) was employed for further fractionation of methanol extract by paper chromatography. Their solvent I consisting of acetone:0.05% aqueous urea:diethylene glycol (6:3:1) was modified by using deionized water instead of the urea solution. Twenty papers, each streaked with 50 mg. of methanol extract for a total of 1.0 g., gave an average yield of 0.05 g. of CRF active fraction D found at R_F 0.9-1.0. Solvent I revealed at least eight ninhydrin positive components, all presumably heterogeneous, present in methanol extract. A pooled sample of fraction D (FD 126) amounting to 0.588 g. had a pressor activity of 1-2 U/mg.; an MSH Melanophore Index of 3-4 for 2γ; 4, 5^+ for 4γ and 5-5^+ for 8γ compared to 2-3 for 1.25γ and 3-4 for 2.5γ of standard and CRF activity giving a 210% increase in compound B at a dose of 100γ. Of the CRF activity, approximately 180% of the 210% increase can now be explained by the presence of vasopressin.

Many experiments were performed on FD 126 or similarly obtained fractions with the object of selecting a desirable method for purifying the active CRF material.

Paper chromatography of FD 126 in BAW (4:1:1) gave eight ninhydrin positive spots at R_F's of 0.07^{+3}, 0.16^{+4}, 0.22^{+2}, 0.28^{+3}, 0.32^{+2}, 0.44^{+2}, 0.55^{+4} and 0.74^{+4}.

Carboxymethyl cellulose, prepared by the method of Sober and Peterson (85), was used for a column (CMW 2) employing 0.2 M ammonium acetate, pH 4.5 as the eluting buffer. Folin analysis revealed the presence of five peaks in 24.8 mg. of a D-like starting material, all showing heterogeneity on chromatography on paper in BAW (4:1:1). At least 13 different ninhydrin positive components were present in the fractions investigated.

A 210 mg. sample of FD 126 was subjected to 450 transfers at 25°C in the Automatic Countercurrent Distribution apparatus in a solvent system of sec-butanol-0.5% trichloroacetic acid (CCD 24). A ninhydrin analysis of the completed distribution gave eight peaks, none of which conformed to theoretical curves calculated at the corresponding partition coefficient value. Paper chromatography of the four fractions investigated at K values of 0.142, 0.223, 0.332 and 0.574 showed from three to five ninhydrin positive components present in each fraction. The speed and resolving power of this technique made it a valuable tool in later purification steps.

The technique of one dimensional paper chromatography followed by electrophoresis in a direction 90° to the solvent development (59, 86) was used for analysis of FD 126; the presence of 10 to 14 peptides was revealed; on elution and hydrolysis, these were found to range in size from 5 to 13 amino acids.

In summary, fraction D appears to consist of at least eight and possibly as many as 14 peptides ranging in size from 5 to 13 amino acids. Countercurrent distribution affords a rapid method for obtaining useful quantities of peptides which could be subjected to more refined techniques yielding homogeneous materials.

The tedium and limitations involved in the preparation of fraction D by paper chromatography led to a search for other methods of producing fraction D or D-like material of equal biological activity. Crude starting materials were used in experiments employing chromatopiles, hyflo-supercel columns, powdered cellulose columns, countercurrent distribution and ion exchange columns.

A sample of protopituitrin (Parke-Davis), subjected to chromatography by the chromatopile technique (Chromatopile 2), gave seven peaks when developed with Solvent I. All fractions obtained were heterogeneous as revealed by paper chromatography in Solvent I. Fraction 400-550 gave a potency of 2.0 for a dose of 100 γ and fraction 550-750 assayed at 2.1

while fraction 750-950 showed no activity at the same dose in the in vitro assay. The recovery of material amounted to only 44% of the starting material. This method, considering the low recovery, low resolving power, and work involved seemed to offer no advantage to the paper chromatographic preparation of D.

A column (Cell. 5) prepared by packing a heavy slurry of Whatman Cellulose powder in n-butanol under 60 cm. of air pressure was charged with a sample of Kamm product dissolved in a solution of n-butanol, acetic acid and water. A stepwise gradient elution scheme of increasing polarity was used to elute the peptide materials from the column. Folin analysis did not permit determination of the number of peaks present but subsequent paper chromatography of the 35 fractions collected revealed at least 30 ninhydrin positive components. Pressor activity was absent in peak 16 but peak 21 gave approximately 2 U/mg. The column gave fairly good resolution of peptide components but required 5 weeks for development, making it unsuitable for preparation of labile biologically active materials.

Countercurrent distribution of a sample of methanol extract (CCD 6) in n-butanol-0.09 M p-toluenesulfonic acid at 25°C gave 11 overlapping peaks when analyzed by the Folin method; each fraction examined proved to be heterogeneous in subsequent paper chromatography experiments.

Polycarboxylic acid resins have been used effectively for the separation of protein and peptide mixtures (81). A sample of methanol extract chromatographed on IRC-50 resin (IRC-50 8) equilibrated with 0.2 M ammonium acetate at pH 4.69 and gradient eluted to 0.2 M ammonium hydroxide gave no homogeneous fractions.

Hyflo-supercel columns, under the conditions employed, also proved incapable of resolving adequately the peptide mixtures represented by protopituitrin, Kamm product, or methanol extract. It became obvious that no single fractionation technique would provide homogeneous material and that a combination of procedures would have to be used.

The Kamm procedure (57) used in the preparation of the Kamm fraction reported here, Protopituitrin or Pitressin (132) requires a heating step to 95°C and an acetone-desiccated posterior pituitary lobe starting material. The effect of heating and acetone extraction on the CRF activity was not previously investigated and the success of Acher et al. (4, 5, 6) in purifying oxytocin and vasopressin by a cold, dilute sulfuric acid extraction of acetone-desiccated posterior lobe tissue prompted the following experiments.

A yield of 48 g. of wet posterior pituitary lobe, obtained from about 1 lb. of whole glands was homogenized in 200 ml. of 0.25% acetic acid, filtered, and the acetic acid extraction and filtration repeated twice more on the residue.

The combined filtrate, concentrated to a volume of 150 ml., was made 5% in trichloroacetic acid and filtered. The precipitate resulting from the addition of 82.5 g. of ammonium sulfate was collected and dried and extracted three times with glacial acetic acid. The Kamm product finally obtained weighed about 50 mg. with a pressor activity of 4.0 U/mg. giving a weight yield of approximately 0.5% calculated on the basis of dry weight of tissue. A paper chromatogram of this material gave five ninhydrin positive areas, three of which were eluted and obtained as a lyophilized powder. The components at R_F 0.14 (LVP 40 U/mg.) and 0.17 (LVP 140 U/mg.) gave CRF responses of 390% and 344% respectively for a 60γ dose. In the light of present knowledge of the ACTH-releasing capabilities of vasopressin (30, 96, 133) the extremely large doses of 2400 mU and 8400 mU for the two assays would account for all the CRF response observed.

Hypothalamic tissue, treated similarly to the posterior pituitary lobe described above, gave 41.6 mg. of Kamm product from 29.3 g. of wet starting material. The Kamm product which had a pressor activity of 0.072 U/mg. and no CRF activity at a 600γ dose, gave four ninhydrin positive components when chromatographed in BAW (4:1:5). Materials obtained from the areas having R_F's at 0.15 and 0.19 gave no CRF activity at 80γ dose levels. The residue from the dilute acetic acid extraction gave a Kamm product having a pressor activity of

0.124 U/mg. when subjected to the regular Kamm procedure (57).

Since the cold, dilute acetic acid extraction did not yield any vasopressin-free CRF active material, an experiment using freshly obtained pituitary glands processed by the Kamm procedure was performed. Desiccation of 77.3 g. of wet posterior lobe tissue was accomplished by a once repeated addition of 6000 ml. of acetone at 3-5°C yielding 15.1 g. of dried glands. The tissue was homogenized in the recommended quantity of 0.25% acetic acid and subjected to the Kamm procedure (57). The final yield of methanol extract was 188 mg. which gave 7.5 mg. of D material. Fraction D had a pressor activity of 0.06 U/mg. and when injected at doses of 100 γ (6 mU LVP) and 25 γ (0.15 mU LVP) gave plasma steroid increases of 210% and 91% respectively. This activity cannot be attributed to the vasopressin present and must be due to a material having CRF- or ACTH-like activity. Unfortunately no ACTH assay was performed but similar material prepared by other investigators using an identical procedure showed no ACTH activity (33). Fraction D possesses MSH activity as reported here and elsewhere (33) and the possibility exists that the response in the CRF assay was due to α-MSH (124) or α-CRF (37, 109). To test this possibility, 5 mg. of the D material was distributed in a CCD for 59 transfers in a sec-butanol-0.5% TCA solvent system. The area including that known to contain α-MSH (K = 2.1) (63) and an adjacent area of a higher

K value gave no CRF activity when assayed. Therefore the CRF activity in the original D material could hardly have been due to α-MSH or α-CRF unless these were inactivated before assay. The total weight recovered was over four times that of the starting material indicating possible contamination with TCA which could have caused partial hydrolysis of the active peptide in the 15 day interim occurring between the completion of the CCD and the assay.

Six hundred and thirteen grams of brain tissue, including the hypothalamus, after desiccation with 22.5 l. of acetone at 3-5°C, blendoring and lyophilization gave 145.6 g. of tan powder. Seventy grams of the powder gave 0.20 g. of Kamm product (LVP < 0.02 U/mg.), a peptide mixture containing at least 13 ninhydrin positive components, which in turn yielded 60 mg. of methanol extract (LVP < 0.02 U/mg.). Fraction D prepared from the methanol extract, which gave no CRF activity when assayed at 100γ, showed 14 ninhydrin positive spots when fingerprinted. An aqueous extract of hypothalamic powder, having no MSH activity at doses of 2.5 or 5.0γ, contained 16 ninhydrin-staining spots.

Dilute acetic acid extractions of the residue remaining after evaporation of the acetone used in the hypothalamic desiccation procedure gave a material, consisting of at least eight ninhydrin positive components, having no CRF activity at a 100γ dosage. Rumsfield and Porter (99) were able to

obtain by a 0.9% saline extraction of an acetone extract of beef hypothalamic sections a CRF active fraction which was fractionated into 19 ninhydrin positive peaks. Observations here and by Rumsfield and Porter (99) disagree with those of Winnick et al. (134) that no significant peptide pool exists in the hypothalamus. Experiments indicate that the hypothalamus contains 4-5% of the peptides found in the posterior pituitary by weight and the mixture numbers a minimum of 14-19 different ninhydrin positive components.

A collection of 522 g. of posterior pituitary lobes weighing 90.3 g. after acetone desiccation yielded 7.13 g. of Kamm product having a pressor activity of 8.8 U/mg. The Kamm product gave 12 distinct spots and a large area of streaking in a fingerprint experiment. A methanol extract prepared from the Kamm product gave 0.53 g. (2.05 U/mg. of pressor activity); an aqueous extract of the residue amounted to 1.90 g. (5.0 U/mg. pressor activity) giving a weight recovery of 81% but only 42% of the original pressor activity. Although inconclusive evidence is available, it appears that pressor inactivation occurs during the methanol extraction. The CRF activity obtained in fraction D can be wholly explained by the presence of the lysine vasopressin present in the sample.

An aqueous extract performed on the original posterior pituitary powder according to the method of Landgrebe and

Waring (61) gave material having high MSH activity and a composition of 14 ninhydrin positive spots as revealed by fingerprinting.

In summary, no CRF activity was obtained from hypothalamic tissue by cold or hot 0.25% acetic acid extractions, or by acetone extraction of the frozen tissue when assayed at the 100 γ level. Aqueous extractions of hypothalamic tissue failed to show MSH activity at doses of 2.5 or 5 γ. Cold acetic acid extractions of posterior lobe tissue gave no CRF active fractions which could not be explained by the pressor activity present whereas there are conflicting data available for the results of hot extractions according to the Kamm procedure. The active material, FD (12-14-59) gave a 210% increase at 100 γ (6 mU LVP) whereas a similarly obtained fraction, FD (2-2-60) gave an 85% increase at 100 γ (30 mU LVP). However, the assay for the latter gave very high controls which makes it appear that all of the activity can be explained by the vasopressin contamination of the sample. The actual increase in compound B was 20.4 ± 2.8/100 ml. plasma for FD (12-14-59) and 17.4 ± 1.9/100 ml. plasma for FD (2-2-60); these values are probably not significantly different due to the large standard errors of the mean. Thus, a tentative conclusion from the results of these experiments include the probability that a CRF material other than vasopressin is present in fraction "D" of posterior lobe origin and because

of the known MSH activity of D, may be α-CRF.

The active CRF fractions were in such small quantity that in order to obtain amounts sufficiently large to perform many purification steps and degradation studies, larger amounts of starting material were necessary.

Ten grams of combined Kamm product (LVP 5.2 U/mg.) stored in a deep freeze for about one year, was distributed in n-butanol:acetic acid:water (4:1:5), 200 ml. per phase, for 14 transfers at 25-30°C (CCD 39). Most of the weight and pressor activity remained in the first four tubes; tube 0, 2.28 g. (LVP 8.0 U/mg.); tube 1, 2.15 g. (LVP 9.99 U/mg.); tube 2, 1.09 g. (LVP 9.62 U/mg.); and tube 3, 0.71 g. (LVP 4.22 U/mg.). An overall weight recovery of 75.8% was obtained with a complete recovery of pressor activity.

Continuous electrophoresis fractionation (Ce 208-2,3) were performed on CCD 39 peaks 2 and 3 using conditions similar to those employed by Guillemin et al. (34). Ce 208-2, using CCD 39 peak 2 as starting material, gave a weight recovery of 69% and a pressor recovery of 47% while Ce 208-3 with CCD 39 peak 3 as starting material gave a 66% weight recovery but a pressor recovery of 63%. Both electrophoresis experiments gave pressor peaks at fraction 15-16 and the extent of purification reflected the pressor activity of the starting material; in Ce 208-2, 15-16 assayed 50.0 U/mg. and in Ce 208-3, 15-16 assayed 35.8 U/mg. The fractions obtained

in the pressor active area showed heterogeneity in ninhydrin positive components. The CRF assays of Ce 208-2 indicated activity in fraction 15-16 at doses of 0.6 γ and in fraction 17-18 at doses of 2.4 γ. All other fractions tested gave CRF activity at doses of 45 γ. Almost all of the activity in fraction 15-16 can be accounted for by vasopressin, which also accounts for the entire activity of fraction 27-28. The activity exhibited by fraction 21-22 in the CRF assay can be explained in large measure (60%-75% of total) by the ACTH and vasopressin activity known to be present. Although not assayed for ACTH, the activity found in the CRF assay for the fractions adjacent to fraction 21-22, namely 19-20, 23-24 and 24-26, can be explained in the same fashion. Fraction 17-18 appears too far removed from the ACTH area and contains too little vasopressin to account for all of the CRF activity determined. Since fraction 15-16 of a later continuous electrophoresis (Ce 225, 226) was shown to contain MSH activity, the possibility that α-CRF was present can explain the response obtained from the CRF assay of fraction 17-18.

A preliminary CCD consisting of 59 transfers was conducted on 10.1 mg. of Ce 208-2 fraction 21-22 (CCD 42). The small amount of CRF activity found in peaks 0 and 2 can probably be attributed to pressor active contamination, although no pressor assays were conducted. However, an estimate of the vasopressin content can be attempted from the assay of

similar areas in CCD 44 conducted on the same starting material. Peak 1 is far too active in the CRF assay to be explained by the proposed pressor activity; thus CRF, probably as α-CRF which has been reported to move with α-MSH at a K value of 2.1 (5, 38), or ACTH must be responsible for the activity. Peak 1 gave three ninhydrin positive components upon paper chromatography in BAW (4:1:5) at R_F values of 0.176, 0.50 and 0.73. Schally et al. (110) report an R_F value of 0.62 for α-MSH in the same solvent system.

The preceding experiment was repeated with 32.2 mg. of Ce 208-2 (21-22) giving a weight recovery of 110% and a pressor recovery of 114%. The distribution was pooled into seven areas; fractions 2 to 6 gave CRF activity at injections of nearly equal pressor content but varying in amount from 10 to 50 γ. Fractions 3 and 5, corresponding to areas having K values approximating those of lysine vasopressin and α-MSH respectively, were further investigated for ACTH content. Two separate assays indicated no ACTH present in fractions 3 and 5 and a repeat of the CRF assay indicated a release of steroid nearly equal to the first assay, but higher control values resulted in a lower per cent increase. Shepherd et al. (117) employing a similar system obtained a slow and fast component having both corticotropin and intermedin activity. Fraction 1 was therefore assayed for ACTH and was found to be inactive. The ACTH known to be present in Ce 208-2 (21-22) was not found

in fractions 1, 3 or 5 and may be in the other fractions not investigated or inactivated during the experiment, a less likely possibility. CCD 44 revealed the presence of two separable CRF active materials; one moving with the vasopressin fraction and possibly chemically related to it and another in the α-MSH area. Unfortunately no MSH assays were performed on any of the seven fractions obtained or on the starting material. Approximately one-half of the CRF activity of peak 3 can be attributed to the vasopressin injected using the value obtained in the later assay (3-17-60) however only one-fourth of the CRF activity of peak 5 can be explained by vasopressin contamination.

None of the fractions obtained from CCD 44 were found to be homogeneous by paper chromatography in BAW (4:1:5). Fraction 3 contained six ninhydrin positive components whereas fraction 5 had only four.

A 50 transfer countercurrent distribution (CCD 41) was conducted on 1.5 g. of peak 1, CCD 39 (LVP = 9.99 U/mg.) in sec-butanol-0.5% TCA at 25°C. A pressor recovery of 57% was obtained with the vasopressin peak at tube 18 (K = 0.56) giving 0.1066 g. of fraction 3 having a pressor activity of 61.3 U/mg. This affords a rapid means of obtaining material of high pressor activity although only 57% of the starting pressor activity was recovered. Paper chromatography indicated all fractions to be heterogeneous. The ninhydrin posi-

tive area at a K of 0.25 in CCD 41 is believed to be due to ammonium salts, possibly ammonium sulfate. Fraction 5 of CCD 41 (30 mg.) was subjected to countercurrent distribution (CCD 45) in sec-butanol-0.5% TCA to determine the complexity of the peptide mixture and to test the reproducibility of the technique. The color analysis indicates the heterogeneity of the starting material by the unsymmetrical pattern obtained for the peak (K = 6.37). A shift of this magnitude in the K value range encountered here is not of great significance. The heterogeneity of the peak was further indicated by the six spots obtained on paper chromatography. No explanation of the poor ninhydrin analysis obtained in CCD 45 has been found.

Light et al. (68) were able to prepare vasopressin of a high degree of purity (240-280 U/mg.) by ion exchange chromatography on Amberlite IRC-50. A 98.8 mg. sample of CCD 41 peak 3 (LVP = 61.3 U/mg.) was chromatographed using the conditions reported by Light et al. (68) in the hope of obtaining vasopressin of very high purity and to determine the resolving power of the column when more complex starting materials than reported were employed. A weight recovery of 50% and a pressor recovery of 25% was obtained in contrast to the 75-100% activity recoveries reported in the published experiment. This discrepancy cannot be explained, although in our hands, this method never resulted in better than 30%

activity yields. The peak 2, eluted at approximately 2.5 hold-up volumes, gave 5 mg. of material with a pressor assay of about 220 U/mg. and three ninhydrin positive components upon paper chromatography in BAW (4:1:5) with the heaviest staining spot at an R_F of 0.29.

The most active CRF fractions obtained in the work reported thus far, albeit not the only ones, were located in the vasopressin area (CCD 44, Ce 208) which corroborated the published work of Schally et al. (115) and Guillemin et al. (34). In opposition to this view was the experimental evidence presented by Rumsfield and Porter (79), Royce and Sayers (97) and De Garihle et al. (18, 42).

It became imperative to conduct a series of experiments employing materials in quantities that would permit varied purification steps carefully followed by a sufficient number of assays which would enable an unequivocal conclusion to be drawn regarding the nature of the CRF(s).

To this end, 28.86 g. of Pooled Kamm product (LVP = 4.2 U/mg.) was submitted to a 14 transfer distribution (CCD 47) in BAW (4:1:5), a procedure identical to CCD 39. Results of pressor, weight and Folin analysis almost identical to CCD 39 were obtained with a final weight recovery of 87% and a pressor recovery of 91%.

Schally et al. (115) reported that CRF had an R_F value slightly higher than lysine vasopressin in a BAW (4:1:5) paper

chromatogram, therefore the leading edge of the pressor peak of CCD 39, tubes 2 and 3, were used for subsequent purification procedures. The lysine vasopressin contamination would also be lessened and more easily removed if the starting material were less active in that hormone. The continuous electrophoresis step, although useful, was of limited resolving ability and time consuming when large samples were to be fractionated. Therefore, CCD 47 was followed by another CCD in a different solvent system which would yield a smaller quantity of more active material to be subjected to continuous electrophoresis.

A total of four countercurrent distributions were conducted on peaks 2 and 3 of CCD 47 in sec-butanol-0.5% TCA at 3-5°C. The pressor peaks of CCD 48, 49 and 50 were located by assay of the distribution tubes. Again the area on the descending part of the pressor curve, fraction 2, was pooled and isolated for further purification. The weight recovery was about 75-85% and the pressor assay of fraction 2, CCD 48, indicated a 3-fold vasopressin purification.

It was believed the presence of ammonium sulfate caused 1) the very high partition coefficients found for lysine vasopressin in CCD 48, 49 and 50; 2) the intense ninhydrin color encountered at a K of about 0.7 in CCD 48 and 49, the two experiments analyzed; and 3) the unusual loss of lower phase found in each distribution nearly mirroring the ninhydrin

peak.

Two continuous electrophoresis experiments using conditions identical to Ce 208-2,3 were conducted on peak 2 of CCD 48, 49, 50 and 51. Ce 225 used 0.3 g. of fraction 2 from CCD 48 (LVP = 9.7 U/mg.) and 0.3 g. of fraction 2 from CCD 49; Ce 226 was run on 0.35 g. of CCD 50 and 0.19 g. of CCD 51. The pressor recovery of Ce 225 amounted to 68% compared to a weight recovery of 67% for the combined experiments.

A CRF assay was performed on Ce 225 and a pressor assay was conducted on both Ce 225 and Ce 226. Fraction 13-14 of Ce 225 was assayed at doses of 0.9, 1.8, 3.6 and 7.2 γ and the elicited response was used as a standard of activity arbitrarily based on one unit per microgram as indicated by the increase in compound B per 100 ml. of plasma. This unit was designated a Ce U and was the unit used for graphing CRF activity in Ce 225, PC 120, IRC-50 19, PC 121 and CCD 52. Comparisons made between the CRF response of Ce 225, 13-14 and synthetic lysine vasopressin indicate the plasma compound B levels at injections of 0.9 and 1.8 γ of Ce 225, 13-14 were probably due to vasopressin present while the greater responses at the higher doses were due to contamination with α-CRF. De Garihle et al. (18), Guillemin et al. (37) and Schally et al. (109) reported an MSH activity for α-CRF equal to $5\text{-}6\times10^{8}$ U/g. for the former and $0.1\text{-}0.3\times10^{10}$ U/g. for the latter two groups of investigators or 1-2% and 10-20%

of the activity of α-MSH respectively. In a later continuous electrophoresis experiment not reported here, Miss Evelyn Weber found the greatest MSH activity in fractions 11-12 and 13-14. It is not unlikely that fraction 13-14 of Ce 225 would contain α-CRF since it is a known contaminant of α-MSH and active at minimum doses of 1-2 γ (13, 37, 109).

The LVP and CRF peaks were superimposable as indicated in their separate assays in Ce 225. The LVP peak of Ce 226 was found to occur in the identical position as in Ce 225 and an approximate 5-fold vasopressin purification was found in both experiments.

Considerable CRF activity occurred in fraction 17-18 (LVP 12.4 U/mg.) of Ce 225 and a preparative paper chromatogram was conducted with a 10 mg. sample employing BAW (4:1:5) at 3-5°C. The eluates from 3 mm. segments of the developed chromatogram were analyzed by the Folin and ninhydrin methods and certain selected segments were assayed for LVP and CRF activity. Although the pressor peak was displaced to a higher R_F, 0.25, than the 0.11 and 0.12 reported previously (7, 99), both activities were superimposable with no separation evident. The higher R_F value may be a temperature dependent phenomena.

The total material obtained at the LVP and CRF peak comprised of 47.5 mg. of Ce 225, 15-16 (LVP 50 U/mg.) and 26.9 mg. of Ce 226, 15-16 (LVP 80 U/mg.) was subjected to chroma-

tography on a column of Amberlite IRC-50 resin buffered at pH 6.41 with 0.5 M ammonium acetate. This technique was known to produce a highly purified lysine vasopressin in our hands (see IRC-50 18) and was based essentially on the method of Light et al. (68) except that the temperature of the experiment was lowered to 3-5°C. The Folin analysis revealed a large peak with a small shoulder on the descending slope occurring at the hold-up volume and a well-defined symmetrical peak at 3.2 hold-up volumes. Weight recovery of the column was 67% whereas the pressor recovery was about 30%. Fraction 3, 8.3 mg., gave a pressor activity of 160 U/mg. and an excellent CRF response at 0.17γ. Fraction 1 gave a CRF response at 60γ which could not be explained by the vasopressin injected. An MSH assay on that fraction showed strong activity present which may reveal the presence of α-MSH and α-CRF. A similar MSH assay on fraction 3 gave a response equal to 62.5 U/mg., comparatively small and perhaps due to a tailing off of peak 1. The tubes selected for CRF and LVP assays in peak 3 indicated no separation of these activities; however, the activity curve did not coincide with the Folin analysis, showing heterogeneity of the material. This was further corroborated by paper chromatography, which showed the presence of four ninhydrin positive components.

Paper chromatography followed by electrophoresis showed a large ninhydrin positive area at an R_F of 0.2 and a distance

of 18.8 cm. The lower third of this spot was a yellow color when sprayed with ninhydrin, which could be due to proline containing peptide contaminants besides lysine vasopressin. Another light spot appeared at R_F 0.16 and 6.9 cm.

Schally et al. (115) used an acetone:water (3:2 v/v) paper chromatography system in their preparation of a highly purified CRF fraction. A 2 mg. quantity of IRC-50 19 peak 3 was streaked on a paper chromatogram (PC 121) and developed in the Schally system with the exception that urea was not used (108). A ninhydrin-sprayed center strip showed a predominant area at R_F 0.73 and a very lightly stained area at 0.54. The chromatogram was cut into 1 cm. segments which were eluted; the eluant was then used for LVP and CRF assays as well as for ninhydrin and Folin analysis. The CRF and LVP activity curves coincide with each other and with the ninhydrin and Folin curves within experimental error; therefore, no separation of the two activities was observed. Inactivation of the pressor and CRF activity occurred to the extent of 85% in this experiment, the identical percentage of pressor inactivation obtained when a sample of the same starting material was allowed to remain dissolved in an acetone:water solution for the same time and temperature. Schally (108) also noted this inactivation; it is difficult to suggest reasons other than possible oxidation effects or disruption in the tertiary structure of the molecule (93). Schally et al.

(115) reported an R_F of 0.69 for lysine vasopressin and a value of 0.35-0.46 for CRF. The vasopressin peak in this experiment had an R_F of 0.73 and the CRF activity found at an R_F of 0.47 could be explained by vasopressin contamination.

A countercurrent distribution (CCD 52) was conducted on 2.5 mg. of IRC-50 19, peak 3 in sec-butanol-0.5% TCA for 130 transfers at 3-5°C. The greatest amount of material, as revealed by Folin and ninhydrin analysis, occurred at a K value of 0.646. Pressor and CRF assays were performed on the lower phase of tubes selected from this area. Two separate CRF assays indicated that no observable separation of CRF activity from the pressor activity curve was obtained. Further, the vasopressin curve fitted a theoretical curve calculated for a K of 0.646 precisely for the descending slope but with slight deviation on the lower arm of the ascending slope.

Unfortunately, such poor results were obtained in the ninhydrin and Folin analysis that very little correlation between them could be made. The entire peak covered a range of roughly 0.1 on the optical density scale for both analyses and the variations in the base lines were great enough to give results of dubious significance. However, the analyses indicate the presence of very little material in this area other than the CRF and LVP active component or components.

Recovery of CRF activity calculated by summation of the assay curve equalled 66% in the 7-29-60 assay and 100% in the 8-19-60 assay. The pressor recovery amounted to 94%.

The material isolated from tubes 46-59, assayed for pressor activity, gave a response of 120-150 U/mg. This value should probably be higher since the accuracy of the amount weighed for assay was questionable. At any rate it indicates a material of pressor content nearly equal to the starting material, IRC-50 19, peak 3 (LVP = 160 U/mg.).

An amino acid analysis was performed on fraction 2 of CCD 52 using the procedure of Spackman *et al.* (123). Such a small amount of material was used that the limits of precision of the method were approached; a precision of 100±3% for loads from 0.1 to 3.0 micromoles of each amino acid is possible with the automatic recording apparatus. The manual operation employed here had greater inherent technical errors and the precision would probably be less. The size of the sample calculated from the amino acid analysis was about 0.25 mg. A weight of approximately 1 mg., obtained on a humid day with a microbalance, was recorded for the sample; however, moisture and probably TCA from the solvent employed in the countercurrent distribution may have been present.

The amino acids found are those contained in lysine vasopressin in approximately equimolar quantities with the exception of serine. The tyrosine peak was contaminated and the value reported was only an approximation and should probably be higher. When based on a milliliter sample of hydrolysate, the LVP amino acids appear in 0.12 μM quantities while

serine was 0.041. If, as the pressor assay indicates, lysine vasopressin comprised 57% of the analyzed sample then a peptide containing serine and all of the other amino acids except cystine found in 0.04 micromolar quantities would explain the results obtained. The contaminant, probably CRF, would contain the following amino acids: aspartic acid, serine, glutamic acid, proline, glycine, phenylalanine, lysine and possibly tyrosine. Schally et al. (115) reported the same amino acids for their CRF plus cystine, alanine and histidine but no tyrosine. Schally (108) also reported the destruction of CRF activity by thioglycolate treatment which is known to reduce the disulfide bridge of vasopressin.

It appeared unlikely that the CRF of Schally et al. (115) could be identical to the serine containing contaminant found in CCD 52, peak 2. It is more likely that peak 2 of CCD 52 was highly purified lysine vasopressin with a small peptide contaminant containing serine and perhaps proline and glycine in small amounts.

If the weight and pressor content of the sample analyzed for amino acids were more accurately known then the molar quantities of the amino acids present in the lysine vasopressin could be calculated. Also if the tyrosine content of the sample were less doubtful a more definitive statement of the nature of CCD 52, peak 2 could be made. However even with these uncertainties taken into consideration, it seems un-

likely that any but the amino acids present in lysine vasopressin were contained in the CRF active material of that fraction.

Schally et al. (115) used a m-cresol:water solvent system to separate lysine vasopressin from CRF by paper chromatography. A 2-dimensional paper chromatogram using BAW (4:1:5) followed by m-cresol:water (95:5) gave three distinct ninhydrin positive spots for IRC-50 19, peak 3. The R_F's were 0, 0 for one spot; 0.30, 0 for the second; and 0.35, solvent front (with tailing) for the third in the two systems.

Since two of the spots did not move in the m-cresol:water direction, this solvent system was employed in an attempt to separate the CRF and lysine vasopressin activities as reported by Schally et al. (115). These investigators reported R_F values of 0.85 and 0.46-0.64 for lysine vasopressin and CRF respectively at room temperature. The remaining sample of IRC-50 19, peak 3 was dissolved in 50 λ of water and a 10 λ aliquot was set aside for a pressor assay. The remainder was chromatogrammed (PC 122) in m-cresol:water (95:5) for 13.5 hours at 3-5°C. The paper was divided into three segments having R_F areas of 0-0.33 (fraction 1), 0.33-0.66 (fraction 2) and 0.66-1.0 (fraction 3) which were extracted with cold dilute acetic acid. The extracts were lyophilized, re-extracted and centrifuges to remove the paper and lint and relyophilized. A pressor assay showed 66.5% of the activity

in fraction 1, 9.7% in fraction 2 and 23.8% in fraction 3 based on an overall recovery of 54.8% of the original activity. Schally (108) and his co-workers (115) do not mention this splitting of the pressor activity. The pressor content of fraction 2 probably resulted from the long tailing observed for the material in fraction 3. Aronoff (8) found a plurality of spots resulting when lysine was chromatographed in a saturated phenol:water system depending on the pH of the aliquot of the sample spotted. The possibility that lysine vasopressin may behave in a similar fashion in m-cresol:water exists but unfortunately no further experiments have yet been conducted to check this observation or to examine the phenomena more completely.

The CRF assay of the three fractions was conducted at a constant pressor level of 10 mU. Fraction 2 gave a 107% increase at 10.2 mU of lysine vasopressin while fraction 1 and 3 at 10 mU and 9.9 mU gave 56.4% and 30% respectively. In every case, the elicited response is too great for the vasopressin injected but peaks 1 and 3 may be within experimental error of the assay which is further complicated by extremely low controls. The activity found for fraction 2, if not due to a poor assay, may be due entirely to vasopressin within the experimental variation of the assay. It could also indicate the presence of a CRF, probably the β-CRF of Guillemin et al. (37) and Schally et al. (109). It will be

recalled that the starting material, IRC-50 19, peak 3, contained MSH activity of approximately 25 U/mg. Guillemin et al. (37) reported an MSH activity of $0.1\text{-}0.3 \times 10^7$ U/mg. for α-CRF. If one assumes that all of this MSH activity resulted from the presence of α-CRF, then approximately 25 γ of α-CRF was in the 2 mg. of starting material employed on the chromatogram. Also, if all of the α-CRF was in fraction 2, the quantity of α-CRF injected would be about 0.02 γ which is far below the minimal effective dose of 1-2 γ reported for this material (19, 37, 109). De Garihle et al. (19) gave a lower value of $5\text{-}6 \times 10^5$ U/mg. of MSH activity in their CRF fraction, however this would result in only 0.08 γ injected which is still too small a dose to give a response.

If the CRF assay was reliable, PC 122 revealed the presence of a highly active CRF material which is not lysine vasopressin or α-CRF and which agree with the published data on β-CRF (37, 108, 109, 115).

In only one instance after a continuous electrophoresis purification step was there found a response of over 185% increase for unknown samples at injections of 30 mU of vasopressin. This strange fact coupled with the inability to achieve a separation of CRF and pressor activity led to an investigation of the effects of synthetic lysine vasopressin in the CRF assay at various dose levels. Injections of 33.5 mU of synthetic lysine vasopressin, 262 U/mg., gave a response

of 14.7±1.4 γ of compound B per 100 ml. of plasma compared to 8.1±0.8 γ of compound B per 100 ml. of plasma in the controls. The response was an 81±18% increase over the non-injected animals.

Table 1 gives a compiled listing of the results of CRF assays performed on various fractions during the purification procedure from Ce 226 to CCD 52. The response of IRC-50 19, peak 1 is undoubtedly due, in part, to the presence of α-CRF for the reasons previously discussed. The percent increase reported for PC 121, tube 14, can be attributed solely to the vasopressin injected. Except for those two fractions, the response varied from 38.2 to 185% for injections of 30 mU of pressor activity. An interesting fact arises from the assays conducted approximately 3 weeks apart on selected tubes of CCD 52. The CRF assay of 8/19/60 gave percent increases of 98.5, 175, 152 and 148 for tubes 43, 47, 51 and 55 respectively while the 7/29/60 assay resulted in values of 82, 92.5, 86.5 and 66.8 for the same tubes. The absolute values of these figures may be questioned because the control reported for the assay of 7/29/60 was the result of averaging three previous controls obtained in prior assays to the one used here. However such a divergence of values exist that variation of the controls would probably not explain the discrepancy. Since the later assay gave the higher values, arguments of possible inactivation were ruled out and it is unlikely

Table 1. CRF assay of fractions obtained in purification procedure

Fraction or tube	γ/inj.	LVP U/ml. or mg.*	LVP mU/inj.	B control	No. of rats	B response	No. of rats	ΔB	% inc.
Ce 226									
13-14	1.8	17.4*	31	6.3±1.5	(4)	15.3±1.8	(5)	9.05	145
15-16	0.6	50.0*	30			15.5±2.3	(5)	9.2	150
PC 120									
14		10.5	25	7.3±0.5	(5)	10.8±.4	(5)	3.5	50
IRC-50 19									
1	60	0.48*	30	4.6±0.6	(5)	24.6±1.3	(5)	20.0	435
2	3	8*	24	5.7±0.85	(3)	11.75±0.3	(3)	6.05	108
3	0.17	160*	27	4.6		13.1±2.5	(5)	8.05	185
IRC-50 19 Peak 3									
86		49	29	6.0±0.9	(4)	12.3±2.0	(4)	6.3	105
91		66	31.5	6.0±0.9		11.1±1.2	(4)	5.1	85
96		92	30	6.0±0.9		8.3±2.8	(4)	2.3	38.2
96		91	30	4.6±0.4	(5)	9.0±1.3	(5)	4.75	95
PC 121									
14		0.2	17.1	6.4±0.6	(2)	8.4±0.8	(5)	2.0	31.3
20		0.7	30	7.6±0.4	(4)	12.25±0.9	(4)	4.65	61
22		4.3	30	6.4		18.0±2.5	(4)	11.6	181
24		0.2	30	7.6		11.8±1.6	(3)	4.2	55

Table 1. (Continued)

Fraction or tube	γ/inj.	LVP U/ml. or mg.*	LVP mU/inj.	B control	No. of rats	B response	No. of rats	ΔB	% inc.
CCD 52									
7/29/60									
43		10.6	30	6.6(calc.)		12.0±1.65	(3)	5.4	82
47		16	29	"		12.7±1.3	(4)	6.1	92.5
51		22	28	"		12.3±1.4	(3)	5.7	86.5
55		15	30	"		11.0±2.8	(3)	4.4	66.8
59		4.4	30	"		11.6±0.6	(4)	5.0	76
8/19/60									
43		10.6	30	7.1±1.0	(4)	14.1±2.4	(3)	7.0	98.5
47		16	30	"		19.5±2.3	(3)	12.4	175
51		22	30	"		17.9±2.4	(4)	10.8	152
55		15	31	"		17.6±4.7	(4)	10.5	148

that potentiation could have occurred. A variation of the same order was found in the assay of IRC-50 19, tube 96. If the assay has a variation of the magnitude cited here then it would appear that the responses obtained for IRC-50 19, fraction 3 and subsequent isolation procedures would be due solely to lysine vasopressin unless inactivation of a CRF active material occurred; if so, β-CRF does not exist. It is interesting to find that an average value obtained for the controls was 6.5, with an average response of micrograms of plasma compound B of 13.12 giving an average increase of 105% for the 21 assays used in the computations. These very closely approximate the values obtained for synthetic lysine vasopressin.

The investigations reported here showed 1) a peptide fraction containing lysine vasopressin has strong CRF activity at doses of 0.17γ ; 2) a less active peptide, α-CRF, possessing MSH activity also exists; 3) β-CRF, if it exists, could not be separated from lysine vasopressin when subjected to ion-exchange column chromatography, paper chromatography, countercurrent distribution and paper electrophoresis; 4) none of the fractions obtained here, and elsewhere (19, 37, 74, 97, 99, 109) have demonstrated a minimal effective dose equal to the 30-40 millimicrograms (10 mU) found for synthetic lysine vasopressin; and 5) lysine vasopressin appears to be as active as, if not more so, than the reported β-CRF (37, 109) and since it occurs in greater quantity is probably

"the" CRF physiologically important.

From an anatomical consideration, vasopressin fits the needs of a neurohumor in higher vertebrates, being synthesized in the supraoptic and paraventricular nuclei of the hypothalamus and moving down the nerve tract to the posterior lobe of the pituitary (116). However, recent elegant studies by Sachs (101, 102) present data which appear to be inconsistent with a simple homogeneous two-compartment system as a theory of neurosecretion. Daniel and Pritchard (17) reported observation of short portal vessels which arise in the neural lobe and drain blood to the pars distalis and more recent work by Holmes and Zuckermann (52) showed a vascular connection between the neural process and the pars distalis in the female rhesus monkey. Metuzals (78) in a detailed neurohistological investigation of the cat observed numerous nerve fiber bundles in the external layer of the median eminence oriented perpendicular to the pars tuberalis. It was concluded that the hypothalamic final common path is constructed:

> (a) of neurons which extend into the adenohypophysis and terminate probably among the gland cells, and (b) of neurons which terminate in the external layer of the eminentia and establish contact with the gland cells of the adenohypophysis through the hypophysical portal vessels. (78, p. 123)

The last three works cited indicate the ease with which vasopressin might reach the adenohypophysis and act to stimulate corticotropin release.

If vasopressin is to be "the" corticotropin-releasing factor, then instances of neurotropic stress should be accompanied by responses associated with that hormone, that is, antidiuretic activity and increase of blood pressure should occur. Mirsky et al. (80), after showing the antidiuretic activity of the plasma of normal rats exposed to a variety of noxious stimuli, found a marked increase in the antidiuretic activity of the plasma in adrenolectomized and hypophysectomized rats exposed to identical noxious stimuli implying that neither gland is the source of the responsible factor. Casentini et al. (13) in their study of corticotropin release in 1959, review the role of ADH in the "stress" reaction, and suggest ADH as the neurohumoral substance which stimulates the release of ACTH. McDonald et al. (75) investigated the effects of fluid deprivation, hypertonic saline, nicotine, hand immersion in ice water and insulin induced hypoglycemia as stressing conditions in human subjects. An antidiuresis occurred under the first four conditions without observation of a concomitant release of ACTH. In the five subjects selected for insulin induced hypoglycemia, a maximum fall of blood sugar (54 mg./100 ml.) occurred after an intravenous injection of 0.1 U/kg. body weight. One subject gave a concurrent rise in urine osmolality and plasma hydrocortisone, three subjects gave a rise in plasma hydrocortisone but no increase in osmolality while the remaining subject gave an

increased osmolality and no rise in plasma hydrocortisone. The data were interpreted as indicating an ACTH release without an increase in ADH. However, Ito and Machido (56) reported a decrease of ADH content in rat pituitary gland and an increase of the antidiuretic substance content in the serum after an insulin injection. Miller and Bogdonoff (79), in their investigation of the antidiuresis induced by insulin injection (20 U i.v.) into human males (45-75 years), concluded that insulin, either directly or indirectly, stimulates the renal tubule to reabsorb filtrate hypotonic to the plasma and that the antidiuretic effect is not mediated through the pituitary-ADH-distal tubule cycle. In his review, Wirz (135) proposes a) the descending limb of the loop; b) the distal convoluted tubules; and c) the collecting ducts, as the sites of antidiuretic hormone action in the mammalian kidney.

The discrepancy in the experimental evidence cited (56, 79) for the action of insulin upon antidiuresis places the observation by McDonald et al. (73) in some doubt and further investigations into the mechanism of the antidiuresis in normal and hypophysectomized animals should be conducted. Perhaps the phenomena may be partly due to the difference in the insulin dose level employed by these investigators.

The experiments conducted by McDonald et al. (73) and Nichols and Guillemin (84) which show a release of anti-

diuretic hormone and no concomitant release of ACTH as measured by an increase in plasma free blood steroids can probably be explained by a difference in the sensitivity of the target sites. Nichols and Guillemin (84) argue that a factor of 3.5×10^3 to 7.1×10^3 exists between maximal antidiuresis and the threshold dose of lysine vasopressin for ACTH release. Recent studies in this laboratory (133), also reported here, indicate that 10 mU of synthetic lysine vasopressin is the minimal effective dose necessary for ACTH stimulation in the nembutal-morphine blocked rat. Vasopressin in the rat is usually measured by the blood pressure response elicited by doses between 4 and 8 mU (20). Nagareda and Gaunt (83) found that 5 mU of pitressin injected intraperitoneally into rats had no effect on adrenal ascorbic acid but did exert definite physiological actions such as antidiuresis, chloruresis and natriuresis. Therefore, there is sufficient experimental evidence (20, 83, 133) to show that the antidiuretic and pressor activity of vasopressin occur at lower dose levels than those required for ACTH release.

Nichols and Guillemin (84) also measured the 17-hydroxycorticosteroid levels of blood samples from dogs in various stages of hydration and diuresis. They reported an inhibition of the release of ADH simultaneously with evidence of stimulation of ACTH release. Of the approximately 60 experiments cited only six show a maximal diuresis (4-10 ml. urine/min.)

during some of the highest levels of 17-OHC (8-18 γ/100 ml.) observed. The control values obtained on approximately 150 single determinations for normal (resting) concentrations of 17-OHC were 0 to 8 γ/100 ml. of plasma. The observations of Nichols and Guillemin may be explained in part by the findings of Nagareda and Gaunt (83) in the rat. They suggest that a small amount of body water may be eliminated by a suppression of the secretion of ADH but a larger water load causes an increased adrenal cortical hormone secretion which augments the diuretic response. Verification and further investigation of the observations reported by Nichols and Guillemin (84) would be desirable.

Recently Kwaan and Bartlestone (60) found a definite increase in adrenal 17-hydroxycorticosteroid output in dogs with intraventricular injections of 2 mU of synthetic arginine vasopressin. A similar injection of oxytocin or the vehicle alone failed to increase adrenal corticosteroid output. Since an intravenous injection of 20 mU of AVP did not affect the hydroxycorticosteroid level, the increase of corticosteroid level due to the 2 mU of AVP placed in the third ventricle was not caused through a peripheral action. Intraventricular injections of 5 micromicrograms of either arginine vasotocin (arginine oxytocin) or oxypressin (leucine vasopressin) did not produce a significant increase in hydroxycorticosterone output.

If a pressor activity of 480 U/mg. is assumed for the AVP used in the study by Kwaan and Bartlestone (60), then a dose of 4.17 millimicrograms was used in the injection. This is far more active than any of the partially purified CRF preparations assayed *in vitro* (36) and as active as a highly purified CRF reported by Schally *et al.* (115). It also far exceeds the activity of the purest preparation of α-CRF (M.E.D. 2-3 γ *in vivo* or 0.5 γ *in vitro*) and β-CRF (M.E.D. 0.1 γ *in vivo* or 0.05 γ *in vitro*) reported from the Guillemin laboratory (37, 109).

In conclusion, it would appear that vasopressin satisfies almost all of the physiological evidence encountered in experimental observations of the neurotropic stressing stimuli and exhibits properties consistent with a role as "the physiological corticotropin-releasing factor".

An Evaluation of the Published Data

Comparisons and reconciliation of the experiments performed in this field are frustrated by the many different assays employed to measure CRF activity and antidiuretic activity. Species differences of starting material used for preparation of CRF, and the use of preparations of widely varying degrees of purification also complicate the problem.

Schally (115) was not able to confirm work reported by Slusher and Roberts (121). However, if the CRF active lipid

or lipoprotein material reported by Slusher and Roberts (121) were contaminated with peptides, then the ACTH-releasing, ninhydrin-positive substance found in an acetone extract of beef hypothalamus by Rumsfield and Porter (99) may have contained the same substance. A D fraction prepared from porcine hypothalamus gave CRF active ninhydrin-positive material as reported in this thesis.

The purification studies of Saffran and his coworkers (103, 104, 105, 108, 115) resulted in a CRF active at 1 millimicrogram as determined by the in vitro assay; that is more active than any other CRF preparation reported since (36, 60, 109, this thesis). Schally (115) reported synthetic lysine vasopressin active at a dose of 0.07μg. whereas Guillemin and Ward as quoted by Guillemin and Schally (36, p. 560), using the same in vitro assay method, report a minimal active dose of 1.0μg. for lysine vasopressin and 2.0μg. for arginine vasopressin. The CRF activity of the vasopressins found in the recent in vitro studies contradict the observations of Guillemin and Hearn (32) and cast doubt on the results obtained in their early tissue culture studies in which a 4-day incubation was employed.

Fortier and Ward (26) claimed a ready inactivation of ACTH on incubation and suggested that the increased release of ACTH may reflect a leakage and not a true secretion of the hormone. Therefore results based on the rate of ACTH release

in the *in vitro* assay "should be interpreted with extreme caution". Roberts (94), in his study of the corticosteroid-releasing activity of blood concluded that the *in vitro* assay for ACTH may be both "non-specific and non-responsive in the presence of interfering protein or protein-bound substances". Having found evidence that ACTH-release by pituitary tissue may also be non-specific, he questioned the significance of the work on CRF using the *in vitro* assay. Guillemin and Schally (36), in answer to these objections, found no detectable destruction of ACTH activity, no potentiation of ACTH or inherent ACTH activity by CRF preparations, a high specificity and complete agreement of CRF characterization between data obtained by *in vivo* and *in vitro* assay systems. These investigators admitted limitations of the method in its inability to show linear responses to increasing doses of purified CRF. The *in vitro* assay of Swingle *et al.* (128) was admittedly found to be non-specific and irreproducible by the authors themselves in an addendum to their reprints, reported by Guillemin and Schally (36).

Since Kwaan and Bartlestone (60) were able to show a CRF activity for 2 mU (*ca.* 0.004 μg.) of synthetic arginine vasopressin injected intraventricularly, it would be expected that the *in vitro* assay should approximate this activity. The fact that it does not suggests either an inactivation of the hormone during incubation or an inability of the hormone

to reach the secretory cells of the adenohypophysis concerned with the synthesis of ACTH. Experiments designed to measure the inactivation of vasopressin alone and as its neurophysine complex (7) in the *in vitro* system would be very valuable in testing this possibility.

Guillemin *et al*. (34) reported that their CRF active fraction D contained MSH activity and a pressor activity of 43 mU/mg. If it is assumed that D contained at least 62.5 U of MSH activity in the 2-4 γ usually tested for in the Landgrebe and Waring assay, and if it is also assumed that this activity was due to α-CRF (MSH activity $0.1\text{-}0.3 \times 10^7$ U/mg.) (37, 109), D could contain approximately 1 γ of α-CRF in a 100 γ dose which would explain the CRF activity of this material. It was possible that the DΔ fraction contained all of the vasopressin and α-CRF contained in the D material since Sideman and Sobel (120) reported the rapid mobility of lysine vasopressin on paper electrophoresis and Schally *et al*. (110) found the vasopressin and α-MSH active areas at nearly the same mobility in zone electrophoresis. Lee (62) also indicated a rapid mobility for α-MSH in zone electrophoresis on starch. It is possible therefore, for the DΔ material reported in the publication (34) to contain 1-5.25 mU/γ of lysine vasopressin and a fair amount of α-CRF. If the *in vitro* assay is 2-4 times more sensitive than the *in vivo* assay as reported by Guillemin and his co-workers (37,

109) for the α- and β-CRF, then the activity of D and DΔ can be easily explained by the presence of α-CRF and lysine vasopressin.

The early work performed by Porter and Rumsfield (73, 90, 100) on portal vessel plasma and the protein fraction III_o can probably be explained by the presence of vasopressin and its protein complex (7). No pressor assays were obtained on their active fractions but chemical inactivations performed are consistent with the known behavior of vasopressin (120). The active fraction prepared from an acetone extraction of beef hypothalamus followed by column chromatography (99) gave two spots of R_F 0.41 and 0.64 in BAW (4:1:5). Schally et al. (110) reported an R_F of 0.61 for α-MSH in that system and because α-CRF contaminates α-MSH and has its partitioning characteristics (37, 38, 109), Porter and Rumsfield (99) probably have isolated α-CRF. An MSH assay and amino acid analysis of the CRF active material would conclusively prove the identity of the R_F 0.61 material and its relationship to α-CRF. There is no doubt of the presence of α-MSH in bovine and its identical structure to the α-MSH of porcine source (27).

The activity of the beef stalk-median eminence extracts found by Royce and Sayer could not be attributed to ACTH or AVP (96) or a potentiation of ACTH by vasopressin (95). A curious result unexplained in the published report (96) was

obtained in the pepsin inactivation experiment. Table 2 shows the assay results reported. It will be noted that in SME extract, A-23, there are 42 mU (Exp. 6a) of pressor activity which should give an approximate response of 50 mg./100 gm. of AAAD (Exp. 3) in the ME lesioned rat. The hypophysectomized rat shows 39±12 mg./110 gm. of AAAD due to ACTH contamination (Exp. 6a), therefore not more than 30-40% of the ME lesioned rat response could have been due to the CRF present in SME extract A-23. Royce and Sayers (97) reported that an intravenous dose of 0.025 stalk-median eminence unit contained 24-53 γ of protein. Experiments 6c,d,e indicate that neither the pressor or corticotropin activity were destroyed by pepsin. If the assays are to be consistent, the ME lesioned rat should give a response approximating the sum of those two activities, or about 80 mg./100 gm. of AAAD. SME extract, A-23, after pepsin treatment gives only 49±9 mg./100 gm. of AAAD in Exp. 6c. The CRF activity of extract A-23 was probably due to α-CRF although no MSH assays were performed. The hormone, α-MSH, is known to be present in beef (27) and is probably susceptible to hydrolysis by pepsin since Harris and Roos (46) used that enzyme to cleave β-MSH, a structural congener. At the present time, α-CRF is thought to resemble α-MSH because of the difficulty in separation, high inherent MSH activity and amino acid content and sequence (113). Royce and Sayers (98) further purified

Table 2. Adrenal ascorbic acid depletion in acute median eminence (ME) lesioned rats following ACTH, pitressin or stalk-median eminence (SME) extracts

Exp. no.	Material injected	Dose in fraction of SME unit	Pressor activity, mU	Adrenal ascorbic depletion (mg./100 g.) Hypox.	ME lesioned
2	a. ACTH, .5 mU			58±6[a](20)[b]	50±12 (13)
	b. ACTH, 2.0 mU			139±5 (14)	150±7 (13)
3	a. Pitressin		5000	77±11 (9)	172±8 (7)
	b. Pitressin		500		144±11 (5)
	c. Pitressin		100	5±13 (5)	90±15 (8)
	d. Pitressin		20		14±8 (9)
6	b. SME extract, A-23	0.025	42 (35 to 50)[c]	39±12(11)	117±6 (15)
	c. Idem + pepsin	0.025	46 (34 to 63)c	31±10 (6)	49±9 (11)
	d. Idem + ACTH, 1.0 mU	0.025		141±14 (5)	
	e. Idem + pepsin	0.025		171±7 (5)	

[a]Mean and standard error.

[b]Number of animals.

[c]Potency with 95% fiducial limits.

their SME extracts on a carboxymethyl cellulose column employing stepwise gradient elution. Corticotropin-releasing fractions were obtained at fractions corresponding to 0.01 and 0.10 M ammonium acetate buffer. The dose of each fraction used in the assay systems was equivalent to 0.3 SME or approximately 4-5 γ of protein for the 0.1 M fraction. The criticisms of Schally et al. (109) of Royce and Sayers (98) and the operation of their column are generally agreed with, although if the 0.01 M fraction resulted from overloading the column it should have contained pressor activity which was shown to be absent. Royce and Sayers (96) also found no difference in the sensitivity of the lesioned and hypophysectomized rat to ACTH contrary to the published results of Guillemin et al. (30) and McCann and Haberland (74); the latter two groups disagree on the magnitude of the difference, however.

McCann and Haberland (74) repeated the work of Royce and Sayers (96) and were in essential agreement with the published data except for their inability to repeat the oxycellulose behavior of their CRF active extract. They attribute 96% of the activity of Royce and Sayers (98) purified 0.1 M fraction to a corticotropin-releasing factor other than vasopressin. The purified CRF was inactive in the Porter and Jones assay (89) which might have been due to the demonstrated decreased sensitivity of this assay (21, 74).

There appears to be little doubt that De Garihle and his coworkers (18, 19, 42) were isolating the α-CRF (109).

The β-CRF prepared from a highly purified lysine vasopressin (LVP ≥ 300 mU/mg.) by Guillemin et al. (37) and Schally et al. (109) by paper chromatography employed a m-cresol:water solvent system. The minimal effective dose of this material in the in vivo assay was found to be 0.1γ. Weber and Hearn (133) have found that synthetic lysine vasopressin (LVP = 262 U/mg.) had a minimal effective dose of 10 mU (0.04γ) in the identical in vivo assay. In the publication in which Guillemin et al. (30) reported the CRF activity of lysine vasopressin to be ineffective below a "threshold of approximately 60-80 mU", a purified natural lysine vasopressin prepared by CMC chromatography and paper chromatography in m-cresol:water assaying 287 U/mg. was used. It is difficult to understand why β-CRF was found in the more highly purified preparation (37, 109) and not in the preparation of lower pressor activity.

Schally (108, p. 102) found a 40% increase of ACTH release by a lysine vasopressin preparation (LVP = 200 U/mg.) at a level of 0.5γ which was not considered statistically significant. After chromatography in the m-cresol:water system, no CRF activity was detected at doses of 0.2 and 0.5γ. He reported (p. 120) that synthetic lysine vasopressin (LVP = 300 U/mg.), which was active at 0.067γ in the in vitro

assay, lost all of the CRF and most of the pressor activity after an attempted purification in m-cresol. The work reported here (PC 122) indicated a strange splitting of the pressor activity of a purified vasopressin preparation (LVP = 160 U/m.g) in the same m-cresol:water solvent system.

Hilton and his coworkers (48) found that vasopressin lost most of its cortisol stimulatory activation of the isolated adrenal gland of the dog after 2 hours at room temperature in saline. However the pressor activity was maintained and no explanation of this activity separation was offered.

Therefore, it is possible that the m-cresol chromatography employed by Schally (108, 115) and the Schally and Guillemin laboratory (37, 109) may produce a pressor active but CRF inactive artifact of vasopressin or even the converse, a pressor inactive and CRF active material.

MATERIALS AND METHODS

Materials

Armour posterior pituitary powder

A gift of one pound of hog posterior pituitary lobe (Armour 851-25) was obtained through the courtesy of Dr. S. Steelman in 1956. This material had the appearance of a tan lyophilized powder. It was not clear from correspondence with Armour that the fresh tissue had been acetone desiccated; therefore, 100 g. of this material was extracted three times at 3-5°C with 1500 ml. portions of anhydrous acetone, each extraction being carried out with constant stirring for two hours. A weight loss of only 8 g. indicated that the material had probably been acetone extracted prior to shipping.

Princeton hog posterior pituitary powder

Several kilograms of material were donated by the Princeton Laboratories Inc. of Princeton, New Jersey, over the period of 1957-1958. This material had a darker brown color than the Armour product, and was described in correspondence as acetone-desiccated, homogenized, lyophilized hog posterior pituitary lobe powder. The label on the powder indicated an oxytocin and pitressin potency of about 1500 U/g. This preparation served as a starting material for most of the succeeding experiments.

Kamm extraction product

The initial extraction employed was essentially that of Kamm et al. (57) with minor modification. To 7 l. of 0.25% aqueous acetic acid (17.5 ml. glacial acetic acid and 6982 ml. distilled water) in a 12 l. round bottom Pyrex flask, was added 70 g. of posterior pituitary powder. The mixture was stirred by means of a motor-driven teflon paddle while being heated to 95° during a 30 minute period by means of a Glas-col 12 l. heating mantle with both Variacs set at 120 volts. After the heating period the flask was transferred from the heating mantle to a sink filled with cold flowing tap water; stirring was continued until the mixture had cooled. After filtration with suction the solid residual was washed on the filter with 700 ml. of 0.25% acetic acid. The combined amber filtrate and washings were concentrated at reduced pressure to 700 ml. in a 12 l. round bottom flask heated to approximately 50-60° by the heating mantle (Variacs at 70-90 v); foaming was prevented by addition of one or two drops of Dow antifoaming agent B. Three hundred eighty-five g. of C.P. ammonium sulfate was dissolved in the cloudy concentrate with stirring and the solution allowed to stand in the cold for almost 24 hours. The resulting precipitate was collected by suction filtration, pressed dry on the filter, and then dried further over calcium chloride in a vacuum desiccator. The thoroughly dried precipitate was powdered in a mortar and

pestle and divided into four equal parts. Each portion was placed in a 250 ml. centrifuge bottle and extracted with 44 ml. of glacial acetic acid by vigorous stirring, centrifugation and careful decanting of the deep amber colored supernate. This process was repeated once on each portion and all of the supernates were combined in a 4 l. Pyrex beaker. Addition of 875 ml. of ethyl ether and 1750 ml. of Skelly A to the glacial acetic acid extract produced a white flocculent precipitate. This precipitate was allowed to settle out in the cold and the greater part of the supernate was decanted before filtration. The precipitate collected on the filter was dissolved in 300-400 ml. of ion-exchange water and the solution concentrated in vacuo on the rotary evaporator and lyophilized. Approximately 85 such extractions were performed to give yields of Kamm product (corresponding to Fraction B of Waring and Landgrebe (132)) ranging from 0.37 g. to 8.7 g. with an average of about 4.7 g. This material assayed 4-5 units/mg. of pressor activity and had the appearance of a light tan powder.

Methanol extract of Kamm product

Three grams of the Kamm product was put into a 250 ml. centrifuge tube, to which was added 7.5 ml. of ion-exchange water and enough glacial acetic acid, up to 30 drops, to dissolve the solid. To the dark amber solution was added 75 ml.

of absolute methanol; the white precipitate which formed was stirred well, centrifuged, and washed twice with 15 ml. portions of methanol;water (10:1) before discarding. The original supernate and washings were combined, concentrated on the rotary evaporator, and finally lyophilized in a 250 ml. round bottom flask. Yields from about 100 extractions ranged from 0.3 to 1.4 g. with an average yield of 0.8 g. or about 26.7%. One experiment on recovery of pressor activity after methanol extraction of Pooled Kamm (10/9/59) gave the following results. A sample of 2.0 g. of Kamm product gave 0.45 g. of methanol extract and 1.22 g. of a water extract of the methanol-insoluble residue. The original Kamm product had a pressor potency of 5.2 U/mg., the methanol extract assayed at 6 U/mg. and the water extract assayed at 12 U/mg. The total weight recovery was thus 83% and the pressor recovery 166%. A more accurate recovery experiment is reported in the section on Fresh Tissue Posterior Pituitary (Jan.).

Analytical Methods

Ninhydrin spray reagent

A 0.1% (w/v) ninhdrin (triketohydrindene hydrate) solution in water-saturated n-butanol was sprayed on paper chromatograms with an atomizer. The paper was hung in a 100-110° drying oven for approximately five minutes. Ninhydrin-positive spots were preserved by further spraying with a solution

of dilute copper nitrate (1 ml. sat'd aq. $Cu(NO_3)_2$ + 0.2 ml. 10% v/v HNO_3 diluted to 100 ml. with ethanol) (11), converting the rapidly fading purple spots to more stable pink spots. Intensities of the ninhydrin positive areas were indicated by assigning an arbitrary unit of +4 to the spot of highest color and in descending order to +1 for the spot having the least color.

Ninhydrin analysis of solutions

Countercurrent distribution fractions The methods of v. Hofsten (131) and of Stein and Moore (126) were both employed during the course of this work. The Hofsten method was preferred under the conditions of analysis reported here. The sample size was determined by the concentration of material in the CCD machine; usually 1.0 ml. of each phase was taken from tubes in the machine and 1.0 ml. of the upper phase from tubes of the withdrawal segment of the distribution. In the latter case, an equal volume of lower phase was added to the sample being analyzed. One ml. of fresh or stored ninhydrin reagent was pipetted with a pipette, an automatic syringe or a pipetting machine into the sample contained in an 18 x 150 mm. lipless test tube. The tubes in a test tube rack were covered with aluminum caps and heated in a covered boiling water bath for 15 minutes, cooled in running tap water and diluted with 3 ml. of an ethanol:

water (1:1) solution. Absorbancy at 570 mU was determined within one hour in a Bausch and Laumb Spectronic 20 against a distilled water flask with frequent checking of the instrument because of a tendency to drift. Attempts to obtain a precise Beer-Lambert curve from peptide sample FD 126, regardless of the conditions employed, proved fruitless. In distributions in solvents containing high acid concentrations (CCD 39 and CCD 47), the samples were first neutralized with the calculated amount of 6 N sodium hydroxide. Whenever the absorbance was too high, further dilutions were made until valid spectrometer readings could be obtained.

Papergram fractions Usually the unsprayed paper chromatograms were dried and cut into segments which were placed in individual tubes containing a measured volume of either 0.25% acetic acid or ion exchange water. An aliquot from each tube, the volume of which varied with the load placed on the paper, was pipetted into an 18 x 150 mm. lipless test tube to which was added 1.0 ml. of the Hofsten (131) ninhydrin reagent. Treatment of the sample from this point was identical to the procedure described above for countercurrent distribution samples.

Folin analysis

Ammonium acetate buffer column fractions The method of Lowry et al. (70) was used, usually with 1.0 ml. of sample

but sometimes with smaller volumes. Four ml. of the Folin-Lowry Reagent A was added to the sample and the contents of the tube mixed by shaking and let stand. The tube was shaken vigorously immediately after further addition of 0.4 ml. of Reagent E. After 30 minutes the absorbancy was determined with a Klett-Summerson colorimeter using a No. 66 filter. Fig. 4 shows the result of a Folin analysis of peptide sample FD 126, in 1.0 ml. of ion-exchange water and also 1.0 ml. of a 0.5 M ammonium acetate solution, pH 6.4; the buffered sample gave less color than the aqueous sample.

Countercurrent distribution fractions Attempts to employ the procedure described above with samples containing alcohol solvents failed because of precipitation of a white solid. This precipitate would not always settle sufficiently to permit measurement of the absorbancy of the supernate. However, the method of Shepherd et al. (117) could be used directly as described; dilutions, when necessary, being made with water. As in ninhydrin analysis, equal volumes of both phases in a tube were taken, or an equal volume of lower phase was added to the sample from a withdrawl series tube. Fig. 4 shows a curve prepared from a sample of FD 126 using the Shepherd technique, which shall be referred to as the cyclohexane-Folin method.

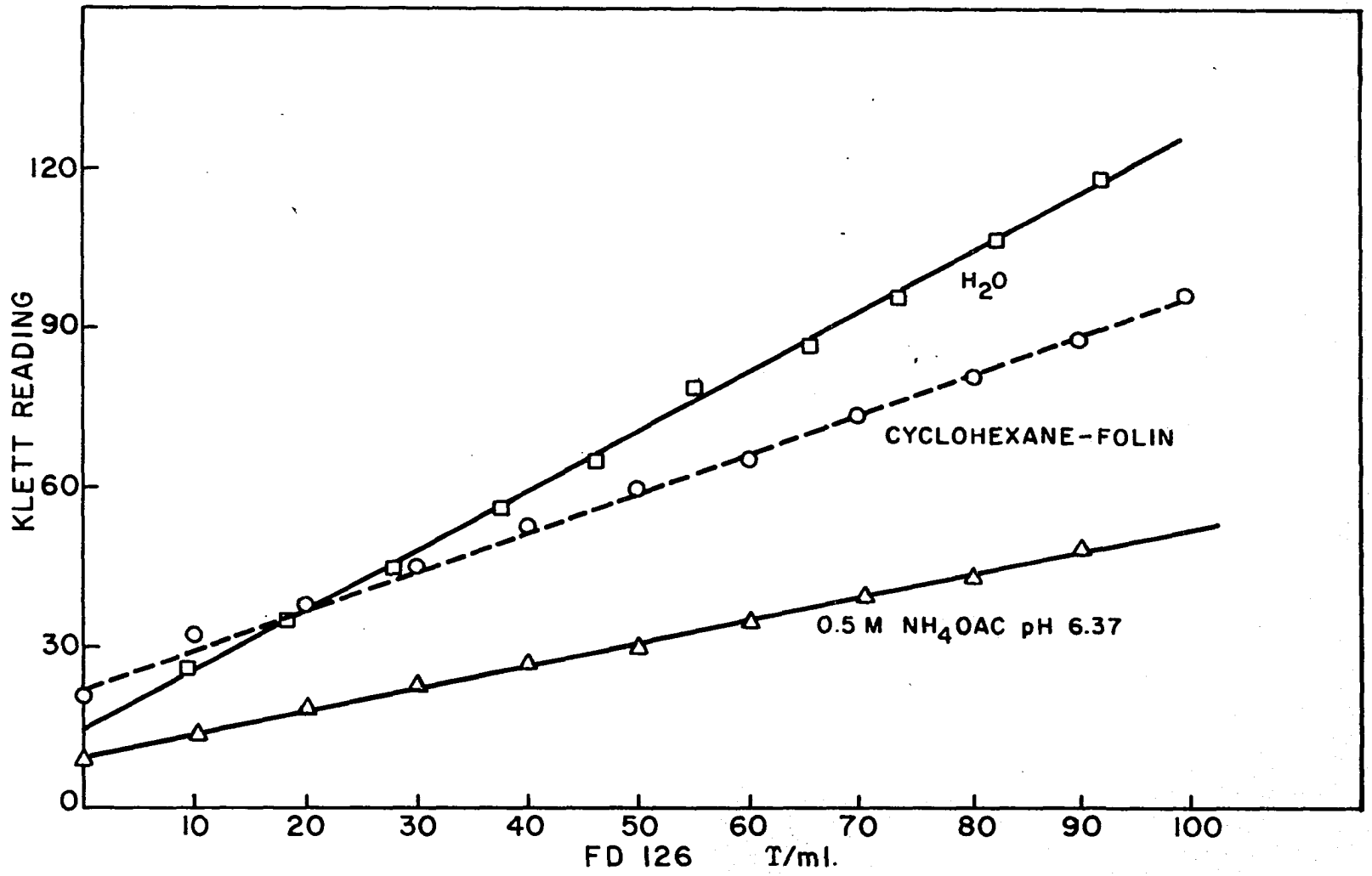


Fig. 4. Folin analysis of FD 126 using buffered alcohol and aqueous solvents

Weight analysis

All freshly lyophilized fractions were weighed on either a single-pan Sartorius Selector automatic balance or a chainomatic analytical balance. The hygroscopic character of peptide fractions, especially during the humid summer months, made accurate weightings difficult. Some assay samples of less than 500 μg. were weighed on an Ainsworth micro-balance, type FDJ.

Paper chromatography

Paper chromatography was used routinely to follow fractionation progress by revealing the complexity of fractions with regard to the number of ninhydrin positive components. Samples were applied to Whatman No. 1 paper of an appropriate size with a glass capillary tube drawn to a fine tip. Papergrams were developed using the ascending technique with either of two solvent systems: 1) n-butanol:acetic acid:water (BAW 4:1:1) or 2) n-butanol:acetic acid:water (BAW 4:1:5). Solvent BAW (4:1:1) is completely miscible and papers could be placed in the tank, a cylindrical Pyrex jar with a light-fitting, greased glass lid, after a short period of equilibration of solvent vapors in the tank. For solvent BAW (4:1:5), the two phases were allowed to equilibrate at the temperature of the room in which the chromatograms were to be developed and the

upper layer was added to the chromatography tank. A separate container of lower phase was not placed in the tank in this work. Most of the chromatography was conducted at approximately 25° which permitted a solvent ascent of about 30 cm. in 15 hours. To remove metal ions from the paper in some experiments, the paper was placed flat in a large bakelite photography tray, washed three times with a solution of 1.0% EDTA (disodium dihydrogen Ethylenediaminetetraacetate dihydrate, "Titraver", Hach Chemical Company, Ames, Iowa) in ion-exchange water, and rinsed three times with distilled water and three times with ion-exchange water stored in a polyethylene bottle. To avoid tearing, the later washings were performed with the aid of a plexiglass support cut to fit the tray and drilled with large holes sufficient to allow the free passage of the solution. The plexiglass shield was then used as a drying rack until the paper regained enough strength to remain hanging unsupported.

Countercurrent distribution procedure

Three all-glass machines were employed in the course of this work. A small "microdistribution" manually operated apparatus based on the design of Bell et al. (10) consisting of 60 tubes was constructed by the glass shop at Iowa State University and mounted on a frame designed and manufactured by the Chemistry shop. Special acknowledgement is given to

Mr. E. Moore and Mr. Ray Seamore for construction of this apparatus, which is operated with 1 ml. of each phase per tube.

A second manually operated machine, made previous to this work and based on the Craig-Post (15) design consisted of 53 tubes with a capacity of 31 ml. for each phase.

A third countercurrent distribution apparatus used was the "Automatic Countercurrent Fractionator", 200 tube Extraction Train manufactured by the E-C Apparatus Company, 538 Walnut Lane, Swarthmore, Penn. This machine was equipped with an automatic filling device and a fraction take-off. When the single withdrawal technique was employed, the upper phase of the last tube siphoned into a funnel leading to an automatic fraction collector. The collector was synchronized with the Fractionator on a time basis and when once adjusted, both gave excellent results and could be allowed to operate unattended. The screw caps for each tube of this machine were lined with Parafilm (E. H. Sargent and Co., Chicago, Illinois) to give a better leak-proof seal, but the Parafilm may have been slightly soluble in some of the solvents employed in this work.

The solvent system to be employed was first equilibrated at the temperature of the room in which the distribution was to be carried out. Lower phase was added to the microdistribution machine with a 2 cc. syringe equipped with a long

needle. A graduated cylinder was used to fill the large manual machine and the automatic filling device was used for the automatic machine. During the duration of the distribution the two phases were always stored in the presence of each other. After the lower phase had been distributed throughout the apparatus, an additional amount was added to the beginning tubes and the machine was run through 10 to 15 cycles to insure that lower phase filled the tubes completely. The sample to be distributed was placed in the first tube by removing the solvent, dissolving the sample in a portion of it, returning the solution to the tube, and rinsing the container into the tube with the remainder. Upper phase was added to the first tube with a 2 cc. syringe, a graduate cylinder, or the automatic filling device, respectively, for the three machines. A complete transfer cycle, consisted of addition of upper phase, 50 to 100 oscillations manually or 25 with the E-C apparatus, a five minute settling out period, and transfer of upper phase to the next tube. Addition of fresh upper phase to the first tube was accomplished simultaneously with the transfer in the case of the automatic machine. After the desired amount of transfers and analysis conducted as previously described, contents of the tubes were pooled and stored in a refrigerator until processed further. For systems containing volatile components, the organic solvent was stripped off in a rotary evaporator with a temper-

ature bath never above 40°C and the aqueous layer either further concentrated or lyophilized immediately. For TCA (trichloroacetic acid)-containing systems, the organic solvent was stripped off and the aqueous-TCA layer was extracted with freshly distilled ether to remove TCA. The remaining aqueous layer was again concentrated in the rotary evaporator to one-half its volume and again ether extracted. This process was repeated until a volume suitable for lyophilization was obtained. A preliminary experiment with a TCA solvent system indicated that all of the TCA could be removed by 36 hours of lyophilization without ether extraction, but ether extraction was employed in experiments reported here.

Ion-exchange chromatography

Amberlite IRC-50, a polycarboxylic weak cation exchange resin, was treated according to the method of Hirs et al. (49). The resin was titrated to the pH desired in either of two ways: by increment addition of ammonium hydroxide, and stirring for periods of at least 30 minutes, lengthening the equilibration period as the pH approached the desired value, or by repeated addition of the buffer at the desired pH and molarity with lengthy equilibrations followed by a redetermination of the pH of the supernatant solution. In either case, the resin and buffer to be employed were stirred for extended periods, usually 8 hours, until the proper pH

was obtained; the supernates and fines were then decanted and fresh buffer added to the resin. Sufficient buffer, prepared according to either Hirs et al. (49) or Acher et al. (7), was usually made up in advance to insure that equilibration and elution of the column could be conducted with the identical buffer. Stepwise elution procedures were employed for some columns.

All columns were packed under gravity-flow, usually as a slurry, with care to avoid channeling or uneven packing. Flow rates of the earlier columns were controlled by a stopcock or pinch clamp; later columns were fitted with a constant head device employing the "chicken feeder" principle. A closed buffer reservoir was attached through a 10 mm. glass tube to an Erlenmeyer flask fitted with a siphon leading to the column. The level of buffer in the Erlenmeyer remained constant because of the continued addition of buffer from the reservoir above it as buffer siphoned out to the column. This constant head, once adjusted to the proper height, maintained a steady flow through the column, providing no further packing of the column resin occurred. With gradient buffers of increasing pH, repeated adjustments had to be made because of the pronounced swelling of IRC-50 in basic solution. Flow rates were always maintained at 1.5 to 2.0 ml./ $cm.^2$/hr. or less, to insure complete internal equilibration of the material being chromatographed with the resin. All

resin and buffer pH's were measured at room temperature even for columns conducted in the cold room at 3-5°C.

"Fingerprinting" by paper chromatography and electrophoresis

This two-dimensional technique was utilized successfully both as a qualitative method for determining the complexity of peptide fractions and as a means of obtaining individual peptides in sufficient quantity for qualitative amino acid analysis. Essentially the method of Katz et al. (59) was used as modified by Pfuderer (86). A sheet of Whatman No. 3 or 3 MM, 22.5 x 18 1/2 in., was ruled in pencil with three lines parallel to the long edge at distances of 2, 6 and 12 cm. from the edge and a single line 10 cm. from the short edge. About 1 mg. of sample was pipetted at the intersection of the 12 cm. and 10 cm. lines to form a spot less than 1 cm. in diameter. The paper was creased upward at the 2 cm. line and downward at the 6 cm. line with the aid of a straight-edge. The chromatogram was developed with BAW (4:1:5) in the descending manner at 25°C in a Chromatocab (Research Specialties Co., Berkeley, California) with equilibrated lower phase in the bottom tray. The solvent front moved 30 to 33 cm. in about 12 hrs. depending upon the type of paper used. After the paper was dried, it was trimmed on one side to remove the "trash" line at the solvent front, usually a distance of 30 cm. from the 12 cm. line, and on the opposite

side at about 9.5 cm. from the edge, leaving the 12 cm. line. A pyridine-acetate buffer (PYR:HOAC:H_2O, 1:10:289 v/v), pH 3.55, was then carefully pipetted along either side of the 10 cm. line, with the paper elevated above the glass-topped bench by a large glass rod, so that the buffer spread toward the line from both sides. After the entire paper had been wet with buffer and excess buffer removed by blotting with clean paper tissue, it was carefully placed on a glass rack and inserted into the kerosene-cooled electrophoresis apparatus. The paper was placed so that the side having the 10 cm. line was at the anode. Electrophoresis was conducted at 2400 volts for 90 min. at 55-65 milliamps and a temperature of 20-22°C. After the kerosene and water had been thoroughly removed by drying at room temperature or in a 100° oven, the papers were sprayed with a 0.01% alcoholic ninhydrin solution and heated to bring out the color of the spots.

In experiments conducted to determine the amino acid content of the ninhydrin positive components, the spots were cut out, eluted into small tubes with 1 to 2 cc. of 6 N hydrochloric acid, sealed in vacuo and hydrolyzed for 18 hrs. at 110°C. Elution was carried out by placing the cut-out, further divided into pieces about 2 mm. square in a small funnel made from a section of 6 mm. Pyrex tubing with one end drawn down to a fine capillary, through which 1 ml. of 6 N hydrochloric acid was allowed to percolate into a hydrolysis

tube; alternatively and preferably, the hydrochloric acid eluant flowed over the cut-out in the form of a wick, dripping into the hydrolysis tube. The hydrolysates in the opened tubes were dried *in vacuo* over sodium hydroxide and the light yellow residue spotted on an 8 x 8 inch Whatman No. 1 paper which was subjected to two dimensional chromatography according to Hardy *et al.* (43).

Continuous flow electrophoresis

A number of fractionations were conducted by Miss Evelyn J. Weber with the continuous flow Paper Electrophoresis Cell Model CP (Beckman Instruments, Inc., Spinco Division, Belmont, California) using the Spinco Cons-stat constant current supply. The electrolyte, 0.5 N acetic acid, was recirculated from a bottle immersed in an ice-bath, and tap water was run through the cooling plate at a valve-regulated constant flow rate. The sample, made up to a 1% solution in the electrolyte, was fed continuously on the proper tab nearest the anode; collected fractions were numbered from No. 1 at the cathode to No. 32 at the anode. Fractions were stored at 3-5°C until the solvent could be removed *in vacuo* in the rotary evaporator and/or by lyophilization.

Preparation of "D"

The solvent system used in the preparation of "D" material by paper chromatography was essentially that of Guillemin

et al. (34). Solvent I as employed here consisted of a freshly made solution of acetone:water:diethylene glycol (6:3:1) without the addition of urea. An aqueous solution of 50 mg. of methanol extract was streaked across the origin line of a sheet of 22 1/2 in. by 18 in. Whatman No. 1 chromatography paper and dried at room temperature. The sheet was formed into a cylinder with the use of glass or polyethylene hooks and developed by the ascending technique for about 15 hours or to a height of approximately 30 cm. at a temperature of 25°C. After drying the paper at room temperature, a vertical center strip was cut and sprayed with ninhydrin. Usually a one inch horizontal strip about three-quarters of an inch below the solvent front was taken and eluted with water in a water-saturated atmosphere enclosed by a bell jar. This region of R_F 0.9 to 1.0 had been reported (34) to contain the greatest corticotropin-releasing activity. The eluant from 20 papers was lyophilized to a dark yellow oil, to which was added 3 ml. of 90% aqueous methanol. The yellow solution was transferred to a 30 ml. centrifuge tube and the clear supernate decanted from any residue into another 50 ml. centrifuge tube. The flask and residue from the centrifugation were rinsed with another ml. of 90% aqueous methanol and the supernate from this washing added to the original supernate. Addition of 10 volumes of dry ethyl acetate to the combined supernate produced a flocculent, light-yellow pre-

cipitate, which was centrifuged and washed twice with ethyl ether. The air-dried or lyophilized tan powdery precipitate was stored in a refrigerated desiccator. Twenty paper chromatograms, representing 1.00 g. of methanol extract starting material, gave an average yield of 0.049 g. of this "D" with a range of 0.0142 g. to 0.1224 g. A pooled sample of 588 mg. of this material was labeled FD 126 and stored over calcium chloride in the deep freeze. A paper chromatogram of this material developed in BAW (4:1:1) gave ninhydrin positive spots at R_F's of 0.07^{+3}, 0.16^{+4}, 0.22^{+2}, 0.28^{+3}, 0.32^{+2}, 0.44^{+2}, 0.55^{+4} and 0.74^{+4}.

Material from the area of R_F 0.5-0.75, corresponding to the pressor active fraction reported by Guillemin et al. (34), was recovered from the same procedure outlined for D material.

Assays

Corticotropin-releasing factor (CRF)

In vitro method Several assays performed for us by Dr. Guillemin at Baylor University College of Medicine employed the short term pituitary and adrenal incubation technique originally developed by Saffran and Schally (103) and modified as described in detail by Guilleman et al. (34). In this assay the estimation of the release of (ring A-Δ4, 3 keto) steroids by fragments of rat adrenal glands is taken as the criterion of adrenocorticotropin hormone (ACTH) activ-

ity which in turn is a measure of corticotropin-releasing activity of the sample incubated with the anterior lobe of the pituitary. The data are expressed in "potency" of the stimulated pituitary as related to the amount of corticotropin released by the unstimulated pituitary to which was attributed a value of 1 or 100%.

In vivo method In this laboratory, Miss Evelyn J. Weber and several assistants carried out bioassays based on the later method of Guillemin et al. (28, 30, 41), in the nembutal-morphine blocked rat. The sample was administered intravenously and release of ACTH was assessed by the concentration of the plasma free corticosteroids using a fluorometric method. Activity was expressed by either the percentage increase of corticosteroids over the level found in control animals or the increase in micrograms (γ) of Compound B (ΔB).

As an aid in determining the efficacy of each purification step, it was decided to use Ce 225 peak 13-14 as a standard for CRF activity. This was selected because of its immediate proximity to the CRF activity peak in a continuous electrophoresis experiment. Table 3 shows the results of an ACTH and CRF assay on that material. Also included are the results obtained for a sample of synthetic lysine vasopressin (JM V 68/16, LVP 258 U/mg.) generously donated by Dr. Vincent du Vigneaud.

Table 3. Assay of a continuous electrophoresis fraction for CRF and ACTH activity and a CRF assay of synthetic lysine vasopressin

Sample	γ/inj.	LVP U/mg.	LVP mU/inj.	B control	B sample	ΔB	% inc.
Ce 225 13-14	0.9	17.7	15.95	4.25±1.2 (4)[a]	8.3±1.7 (4)	4.05	95
	1.8	17.7	31.90		11.6±1.3 (4)	7.35	170
	3.6	17.7	63.7	5.25±1.1 (4)	21.8±1.3 (4)	15.55	315
	7.2	17.7	127.3		30.4±1.6 (4)	25.15	480
ACTH	1.0 mU			2.85±0.3 (3)	23.4±1.6 (5)		723
	0.25 mU				13.6±3.1 (5)		378
Ce 225 13-14	0.9	17.7	15.95		2.9±0.2 (5)	0	0
	3.6	17.7	63.7		4.0±0.4 (8)	1.15	40.1
Syn.LVP			8.4	8.0±0.8 (7)	8.5±1.1 (10)	--	--
			16.8	8.2±0.7 (18)	11.4±0.7 (19)	3.2	39
			33.5	8.1±0.8 (10)	14.7±1.4 (9)	6.6	81
			67	8.1±1.4 (7)	18.2±1.4 (10)	10.1	125

[a]The number in parenthesis in this and the following assay results indicates the number of rats used in the assay.

Figure 5 shows a graph of the increase of B due to a log dose of Ce 225, 13-14 as indicated by Table 3. An arbitrary assignment of 1 unit per microgram (1 Ce U/γ) was made for this material and is the CRF unit referred to in later experiments. Figure 5 also shows the response of steroid release due to synthetic lysine vasopressin as reported in Table 3. The low controls found for Ce 225, 13-14 give a much greater elevation tha might be expected. It is believed that the increase at 0.9 γ and 1.8 γ is due to vasopressin within the limitations of the assay. At the higher doses, the response is much too great to be explained by the vasopressin and ACTH known to be present. The assays conducted on a chromatography column (IRC-50 19) using Ce 225 15-16 for starting material revealed the presence of melanocyte-stimulating-hormone (MSH); therefore it is possible that the activity of Ce 225 13-14 at the higher doses may be due to α-CRF which is known to contaminate α-MSH.

Melanocyte-stimulating-hormone (MSH)

Assays for MSH performed on the intact frog by Miss Weber and her assistants used the semi-quantitative method of Landgrebe and Waring (61). Standard preparations made by a water extraction of posterior pituitary lobe were assigned a value of one unit (1 u) per 0.04 γ of material injected (119). The usual injection of 2.5 γ (62.5 u) gave a response of 3-4 on

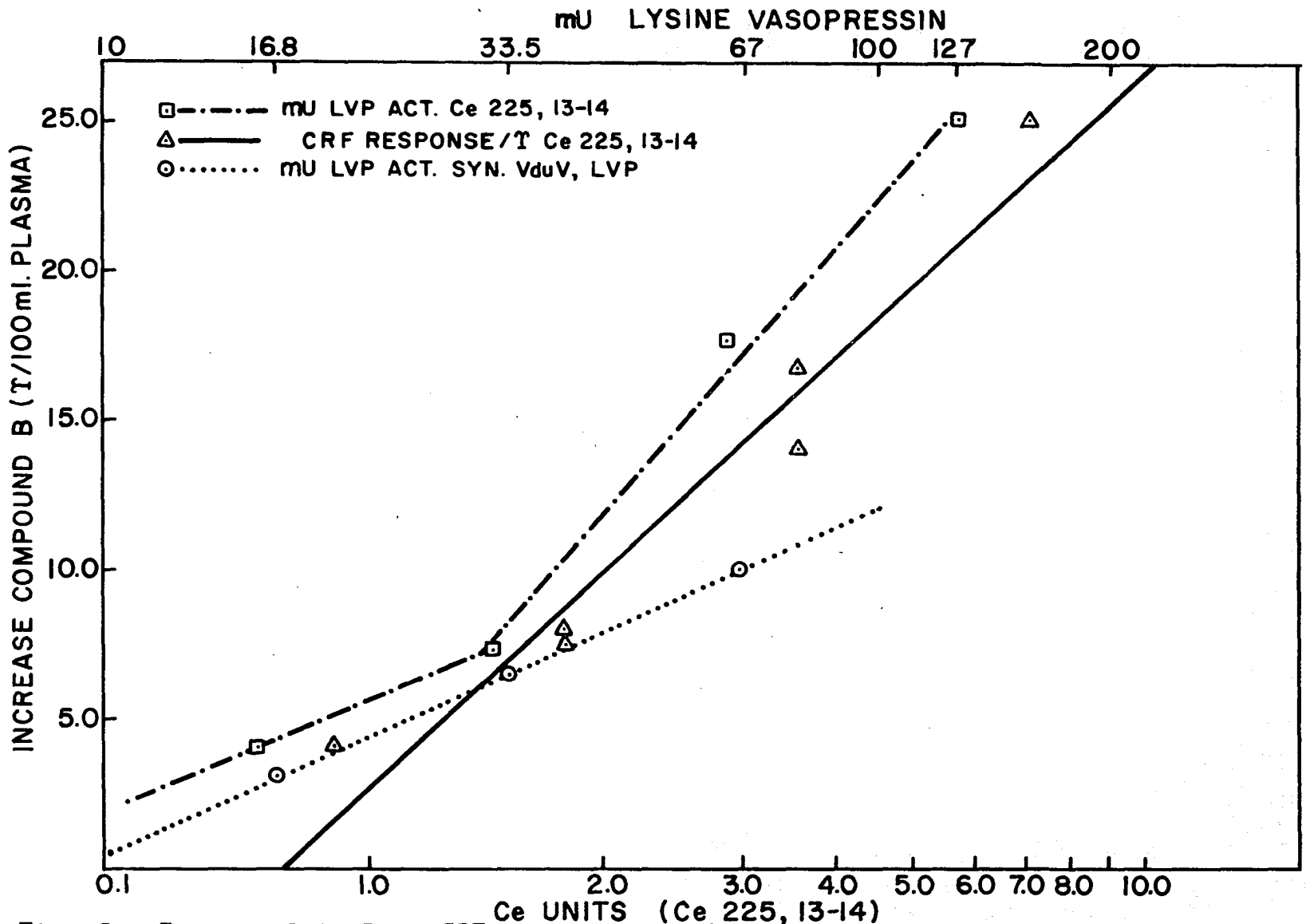


Fig. 5. Increased in B in CRF assay due to Ce 225, 13-14 and lysine vasopressin

the Hogben Melanophore Index scale, a criterion dependent on the shape of the melanocytes. The MSH activity of an unknown sample was evaluated in its relation to the standard response.

Adrenocorticotropic hormone (ACTH)

Miss Weber and assistants used the 24 hour post-hypophysectomized rat for this assay based on the method of Guillemin et al. (29). Samples of either the ACTH USP Standard or the unknown material were administered intravenously in physiological saline and ACTH activity was maeasured by determining the levels of plasma free corticosteroids. Results are reported in terms of units of ACTH as referred to the USP standard preparation.

Vasopressin (LVP)

Miss Weber and assistants determined pressor activity of isolated fractions in rats anaesthetized with methane and treated with dibenamine according to Dekanski (20). Blood pressure in the carotid artery was measured by direct cannulation with a polyethylene tube leading to a Statham pressure transducer connected to a Sanborn amplifier and recorder. Samples were injected intravenously and assayed against USP Posterior Lobe Reference Standard.

Fresh tissue preparations

Fresh posterior pituitary (7/20/59) A day's collection of whole hog pituitaries was purchased from the Iowa Packing Company, Des Moines, Iowa. The glands were collected approximately 45 minutes after the animals were slaughtered and stored in a tin can at room temperature for the entire working day. Approximately 1 pound of the wet glands, stored overnight in a deep freeze, yielded 48 g. of wet, posterior pituitary lobe. The posterior lobes were placed in a Waring Blendor to which was added about 200 ml. of 0.25% acetic acid at room temperature. Five minutes of blending produced a thick, pink foam which threatened to overflow. This mixture was filtered without suction and the extraction and blending repeated twice more in like manner. The final combined filtrate, about 700 ml. of clear, strawberry-red solution, was concentrated to 150 ml. on the rotary evaporator with the bath temperature kept below 40°C and foaming prevented through use of Dowex antifoaming agent B. The concentrated extract was made 5% in trichloroacetic acid (TCA) by addition of 7.5 g. of the Mallinkrodt Analytical Reagent. The precipitate which formed was removed by filtration, leaving a light yellow, clear solution. As described in the Kamm procedure (57), 82.5 g. of ammonium sulfate was added to the solution to give a precipitate which was allowed to stand before collecting on a filter. The dried precipitate, weighting 1.1235 g., was

extracted three times with 30 ml. portions of glacial acetic acid. Completion of the Kamm procedure on this material yielded slightly over 50 mg. of product having a pressor assay of about 4.0 U/mg. The entire product was streaked on a Whatman No. 1 paper which was then developed in BAW (4:1:5) using the ascending technique (PC-7). Ninhydrin revealed five positive areas on a center strip at R_F's of 0^{+2}, 0.064^{+}, 0.14^{+2}, 0.19^{+2}, 0.74^{+4}. The fractions at R_F's 0.14, 0.19 and 0.74 were recovered by cutting horizontal strips, elution of the segments and lyophilization of the final solutions. The material at R_F 0.14 (1.7 mg.) gave a pressor assay of 40 U/mg.; the material at 0.19 (0.6 mg.) assayed at 140 U/mg. Lysine vasopressin has an R_F of 0.11 (68, 99) in this solvent system and CRF has been reported as found in the area of R_F 0.05 to 0.20 (115). The eluate from R_F 0.14 gave a CRF response of 390%, and the eluate from R_F 0.19 a response of 344%, when both were assayed at 60 γ, the dose level at which D is usually assayed. The eluate from R_F 0.74 weighed 7.0 mg., giving a total weight recovery of 18.6% but a pressor recovery of 76% for the three areas extracted.

Cold acetic acid extraction of fresh hypothalamus tissue (7/20/59) A small cube of brain tissue adjacent to the pituitary, roughly 1 cm. on each side, was dissected from the brain of about 100 hogs 45 minutes after slaughter and frozen on dry ice immediately. The entire 29.3 g. of tissue was ex-

tracted at room temperature three times with 300, 250 and 200 ml. of 0.25% acetic acid for 5 minutes each time in a Waring Blendor. The filtrate resulting from the first homogenization was a light pink color; subsequent filtrates were water clear. The solid residue was saved for a usual Kamm extraction. The combined filtrates were concentrated to 85 ml. on the rotary evaporator at temperatures below 40°C. Proteins were precipitated by addition of 4.3 g. of TCA and the solution was filtered to give a light yellow filtrate. Addition of 49.5 g. of ammonium sulfate to the filtrate produced a white gelatinous precipitate which floated to the top. After standing, the precipitate was collected by filtration and dried in a vacuum desiccator. The resulting 0.16 g. of material was extracted twice with 4 and then with 2 ml. of glacial acetic acid and the extract diluted with water, lyophilized and finally re-lyophilized. The white powder from the second lyophilization weighed 41.6 mg., gave a pressor activity of 0.072 U/mg. and no CRF activity at levels of 600γ. The remaining 35 mg. of this material was streaked on a Whatman No. 1 paper and developed in BAW (4:1:5)(PC-5). Four ninhydrin positive areas designated O, A, B and C at R_F's 0.0, 0.15, 0.19 and 0.68 were recovered with weights of 4.5, 1.0, 0.5 and 1.7 mg. respectively. Areas A and B gave no CRF activity at levels of 80γ. The weight of material in the areas eluted amounted to ≈82% of the material chromatographed.

The residue from the original extraction was extracted with the usual Kamm procedure as a check on the cold acetic acid extraction. The white solid was extracted with 750 ml. of hot 0.25% acetic acid yielding a cloudy light yellow filtrate which was concentrated to a cloudy suspension about 75 ml. in volume. Addition of 413. g. of ammonium sulfate gave a white precipitate weighing 1.02 g. after drying. Extraction of this precipitate with 20 and then 10 ml. of glacial acetic acid and further treatment as described in the Kamm procedure gave a white powder with a pressor activity of 0.124 U/mg.

Kamm extraction of fresh posterior pituitary tissue (12/59)

Whole pituitary glands, purchased and handled in the manner previously described gave 77.3 g. of wet posterior lobe tissue (representing about 1500 hogs). Desiccation (57) of the tissue with 6000 ml. of acetone (commercial, not redistilled) in the cold room, 3-5°C, once repeated gave 15.1 g. of dry light pink solid residue. The desiccation process took about seven days to complete. The desiccated tissue was pulverized in a mortar and pestle and stored for a short period in the deep freeze. About 3 g. of this powder was added to a Blendor containing 100 ml. of 0.25% acetic acid and homogenized for 5 minutes. This homogenization procedure was repeated on the remaining powder in four 3 g. batches and

the combined suspension of 15 g. in 1500 ml. was placed in a large round-bottomed flask and subjected to the Kamm procedure. Roughly 1.0 g. of Kamm product was obtained which gave 188 mg. of methanol extract. Three paper chromatograms each using 50 mg. of methanol extract gave 7.5 mg. of D material (R_F 0.90-1.0 in Solvent I). This material gave the assay results shown in Table 4.

Table 4. CRF assay of D prepared from fresh posterior pituitary (12/59)

	γ/ inj.	LVP U/mg.	LVP mU/ inj.	B control	B sample	ΔB	% inc.
FD fresh post. pit. (12/59)	100	0.06	6	6.6±0.85(4)	20.4±2.8(4)	13.8	210
	25		0.15		12.6±2.2(5)	6.0	91

The remaining material, approximately 5 mg., was subjected to countercurrent distribution in the microdistribution machine (CCD 43). Fifty-nine transfers employing the solvent system sec-butanol-0.5% trichloroacetic acid at 25°C were performed. The distribution was conducted in 56 hours. A cyclohexane-Folin analysis was conducted on 0.5 ml. of each phase of every third tube. Ninhydrin analysis of similar samples failed to yield color; the cause of this failure is unknown. The pooling scheme, weights of the fractions and

assays on two of the fractions are reported in Table 5. Figure 6 shows the results of the cyclohexane-Folin analysis. The total weight recovered was obviously much too high; this discrepancy could have resulted from inadequate extraction of TCA, inadequate drying of the samples, or the presence of solvent impurities. Since fractions 4 and 5 failed to show any steroid increase in the CRF assay, even at an extremely high level, the discrepancy in weight was not investigated further.

Table 5. Pooling scheme, weights and CRF assays of CCD 43

Peak	Tubes	Wt. (mg.)	T/inj.	B control	B sample	ΔB	% inc.
1	0-13						
2	14-25	5.4					
3	26-35	4.1					
4	36-45	5.2	230[a]	7.8 (5)	6.5 (4)	--	0
5	46-59	6.3	262.5[a]		8.0 (3)	--	0

[a]The distribution was conducted on Dec. 20, 1959; assays were performed on Jan. 5, 1960.

D preparation from fresh hypothalamic tissue (1/20/60)

Hog heads, obtained about 45 minutes after slaughter minus the lower jaw and most of the flesh, were split by an automatic knife to expose the entire brain (Fig. 7). Brains

Fig. 6. CCD 43 distribution of fresh posterior pituitary D (12/59) 2° BuOH-TCA

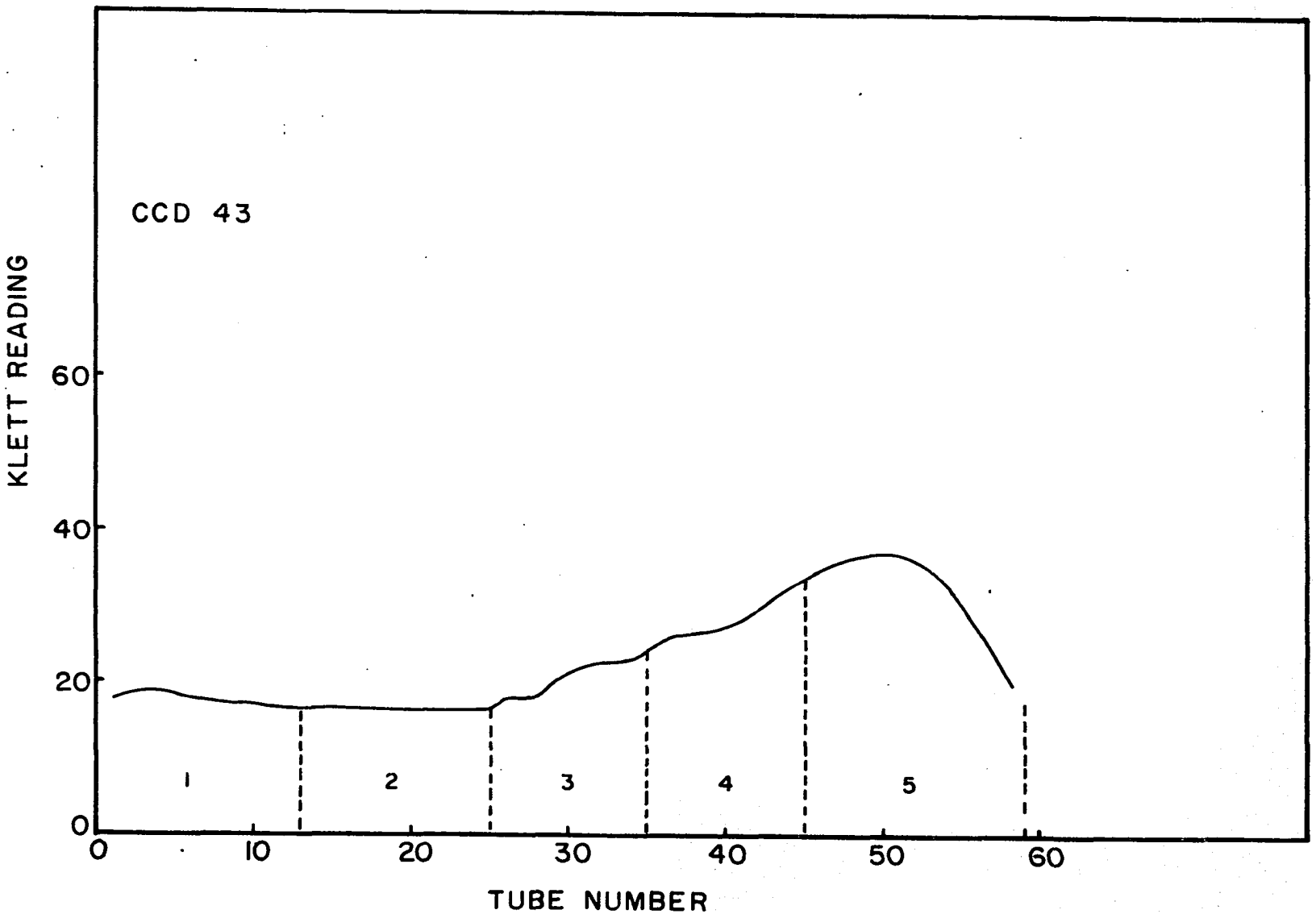

CCD 43
KLETT READING
0
20
40
60
0
10
20
30
40
50
60
TUBE NUMBER
1
2
3
4
5

Fig. 7. A. Longitudinal section of hog brain along the long axis

T Thalamus

M.B. Mammillary body

O.C. Optic chiasma

Hyp. Hypothalamus

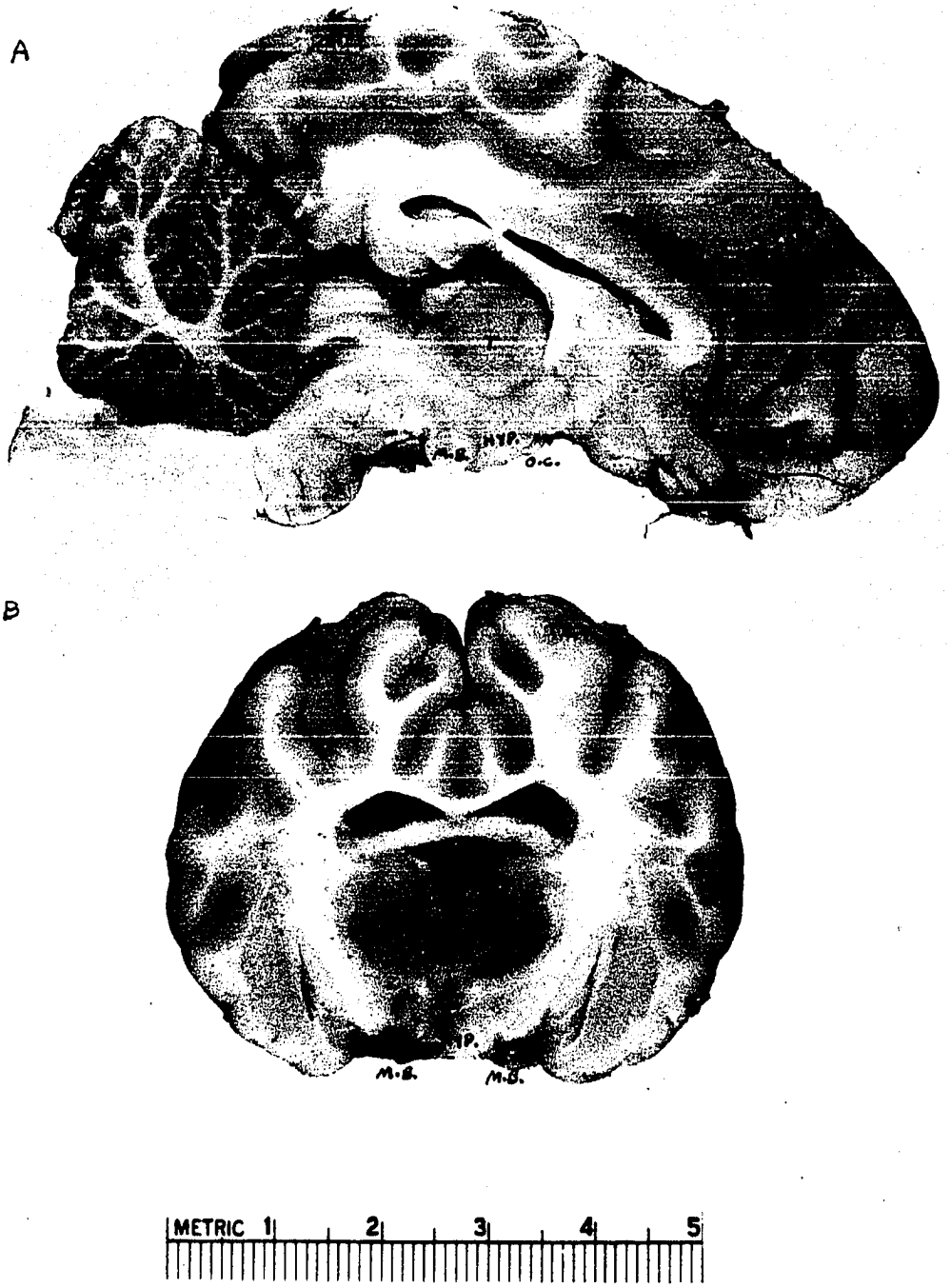
B. Cross section through the region of the thalamus viewed from an anterior position

III Third ventricle

M.B. Mammillary bodies

T Thalamus

Hyp. Hypothalamus

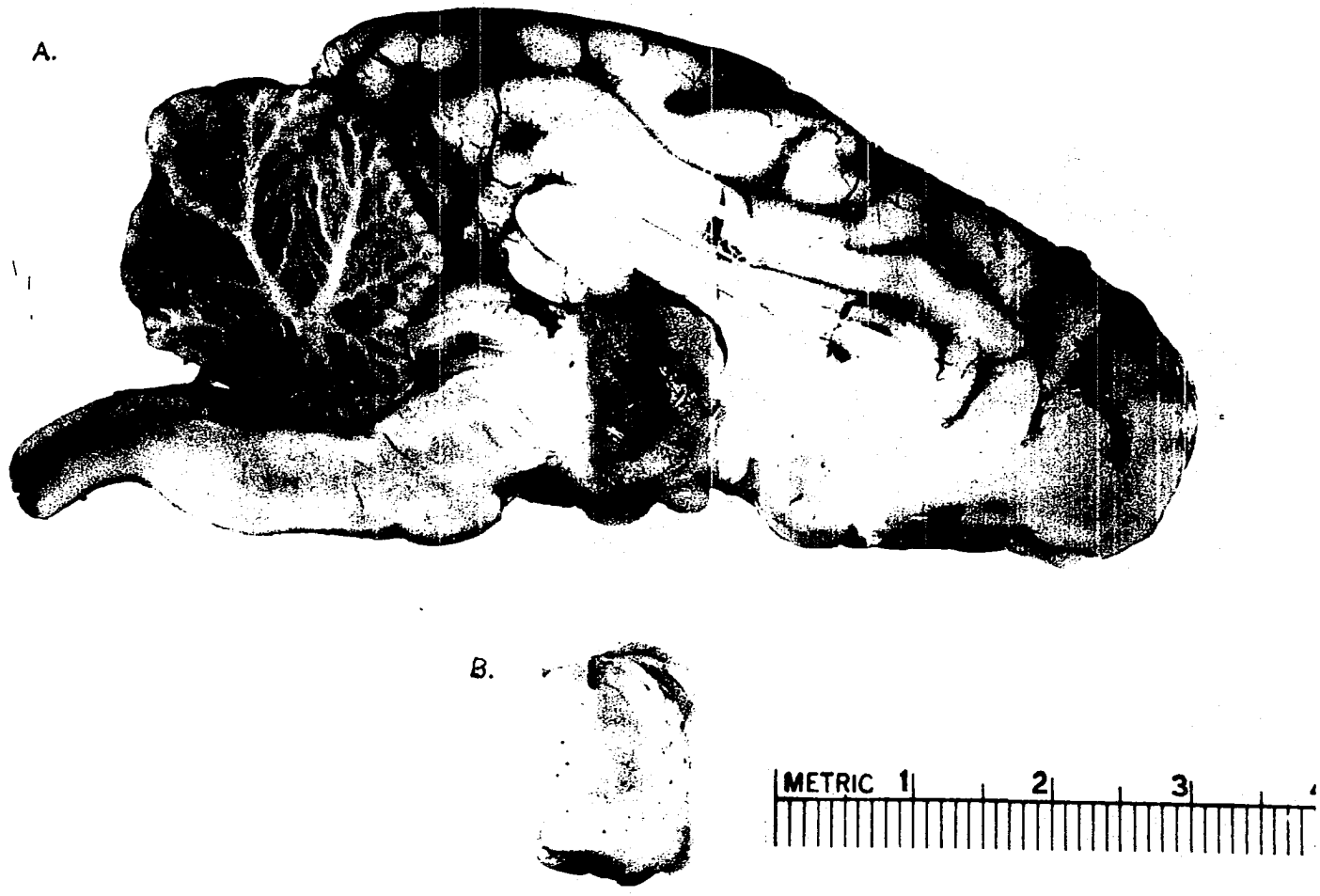

A
M.B.
HYP.
O.C.
B
M.B.
M.B.
METRIC 1 2 3 4 5

were removed from the Dura Mater membrane, leaving the pituitary gland intact when possible. A No. 8 brass cork borer was placed posterior to the optic chiasma and centered either on the pituitary gland or, in case the pituitary stalk had been severed, on the opening of the ventricular system to the base of the stalk. The borer was pushed into the brain until a core about 2 cm. in length could be lifted out (Fig. 8); this core, which was immediately frozen in dry ice, probably included part of the mammillary body, the medial tip of the cerebral peduncle, the medial half of the hypothalamus, and areas of the thalamus. If the borer remained centered upon the third ventrical, the core probably contained areas of the pineal body to the floor of the lateral ventrical. In some cases the borer may also have entered the meningiis.

Frozen wet tissue (from about 600 animals) weighing 613 g. (from about 600 animals) was put into 22.5 l. of cold acetone contained in a large glass tank in the cold room at 3-5°C. The acetone desiccated tissue was collected, homogenized in deionized water in a Waring Blendor, lyophilized, and weighed. The 145.6 g. of tan powder obtained represented 24% of the original wet tissue; 70 g. of this powder processed according to the Kamm procedure gave 6.0 g. of ammonium sulfate precipitate and 0.20 g. of Kamm product, assaying less than 0.02 U/mg. of pressor activity. The Kamm product gave 60 mg. of methanol extract (< 0.02 U/mg. pressor act.). A

Fig. 8. A. Brain with core removed

B. Core taken for extraction


A.
B.
METRIC 1
2
3

"D" preparation from the methanol extract gave no CRF response when assayed at 100 γ. An aqueous extract according to the method of Shizume (119) of 0.5 g. of the acetone desiccated lyophilized powder gave 0.14 g. of product after lyophilization which showed no MSH activity at does levels of 2.5 or 5.0 γ.

The acetone used in the desiccation process was evaporated in the rotary evaporator (bath temperature 35-45°C) and the solid recovered. This amounted to 8.16 g. of a cream-colored, flaky material. In an attempt to duplicate an experiment by Rumsfield and Porter (99), 1.0248 g. of this material was dissolved in 200 ml. of freshly distilled ether, and extracted three times with 200 ml. portions of 1% acetic acid. Dilute acetic acid was employed here rather than 0.9% sodium chloride (99) because of its ease of removal by lyophilization. The waxy residue from the lyophilized acetic acid extract, weighing about 10 mg., gave no CRF activity at 100 γ, but some indication of activity at the 400 γ level in an inconclusive assay. In a paper chromatogram employing BAW (4:1:5) as the solvent, ninhydrin positive components were revealed at R_F's 0.0278^{+1}, 0.0874^{+3}, 0.131^{+3}, 0.214^{+3}, 0.282^{+4}, 0.377^{+}, 0.489^{+} and 0.544^{+}.

D preparation from fresh posterior pituitary tissue (1/20/60)

Immediately upon collection of the entire pituitary gland from hogs as performed routinely by the Iowa Packing Company, Des Moines, Iowa, approximately 45 minutes after slaughter, the posterior lobe was removed and frozen on dry ice. A week's production was stored frozen after collection in this manner and when 522 g. of the wet tissue had accumulated, the entire block of frozen tissue was dropped into 26 l. of cold acetone. The published directions (57) recommended 200 cc. of acetone for each 50 posterior pituitary lobes, or approximately 2.6 g. of wet tissue; for the amount of material used here a volume of 40 l. would have been required, but no convenient container of this size was immediately available. The acetone-dried, homogenized, and lyophilized powder weighed 90.3 g. or 17% of the wet weight.

When 70.0 g. was processed according to the Kamm procedure (57), 30.7 g. of ammonium sulfate precipitate was obtained which yielded 7.13 g. of Kamm product assaying at 8.8 U/mg. of pressor activity. Three g. of Kamm product gave 0.53 g. of methanol extract (2.05 U/mg. pressor activity) and the methanol-insoluble residue yielded 1.90 g. of aqueous extract (5.0 U/mg. pressor activity). The weight recovery was thus 81% but the pressor recovery only 42%. The D prepared from the methanol extract gave the CRF assay reported

in Table 6. When 0.51 g. of the original acetone-dried, homogenized, lyophilized powder was extracted with water according to the method of Shizume (119), 0.14 g. of a white powder was obtained by lyophilization of the aqueous extract; this powder gave melanophor indices of 5^+ at levels of 2.5 and 5 γ, compared to indices of 3-3.5 for 1.25 γ and 4-4.5 for 2.5 γ of the standard MSH preparation (119).

Table 6. CRF and pressor assay results of posterior pituitary D (1/20/60)

Sample	γ/ inj.	LVP U/mg.	LVP mU/ inj.	B control	B sample	ΔB	% inc.
FD	100	0.4	30	9.4±1.4(4)	17.4±1.9(4)	8.0	85±20
2/2/60	50	0.3 (0.2,0.4)	15		14.4±1.7(5)	5.0	53±20

Chromatopile of protopituitrin (chromatopile No. 2)

Four chromatopile experiments were conducted. A sample of protopituitrin (Parke-Davis, 12.5 U/mg. pressor activity, 12 U/mg. oxytocic activity) weighing 1.07 g. gave 0.51 g. of methanol extract which was dissolved in water and pipetted onto 11 circles of 7 cm. Whatman No. 1 filter paper. The papers containing sample were air dried, then placed in a stainless steel chromatopile at a position of 20 papers from

the upper end of a 1000-paper pile. The papers were aligned and clamped in the apparatus, which was then placed in a covered glass chromatography tank containing Solvent 1 to saturate the atmosphere. Solvent 1 (containing urea) was applied to the chromatopile through a tygon syphon. Development was stopped after 9.5 hours and the chromatopile permitted to air dry overnight. Every 50th paper was removed, cut into small segments, and extracted with 10 ml. of the Folin alkaline-copper reagent; 4 ml. of this extract, filtered through sintered glass, was used for Folin analysis. Results are shown in Fig. 9. Papers were grouped as indicated in Table 7, extracted with 0.5% aqueous acetic acid in a Waring Blendor; the extracts were suction filtered through a sintered glass funnel and concentrated in a rotary evaporator. The resulting viscous concentrates were treated as D fractions for removal of diethylene glycol.

Bioassay for CRF in the <u>in vitro</u> system (34) at doses of 100 γ gave a potency of 2.0 for fraction 400-550, and 2.1 for fraction 550-750. Fraction 750-950 showed no activity at that level.

The green color of fraction 400-550 was found to be due to copper salt contamination from contact with metal parts of the Waring Blendor. Chromatopile experiments showed low resolving power for the time and effort required, and offered no advantages over paper chromatography for preparation of D

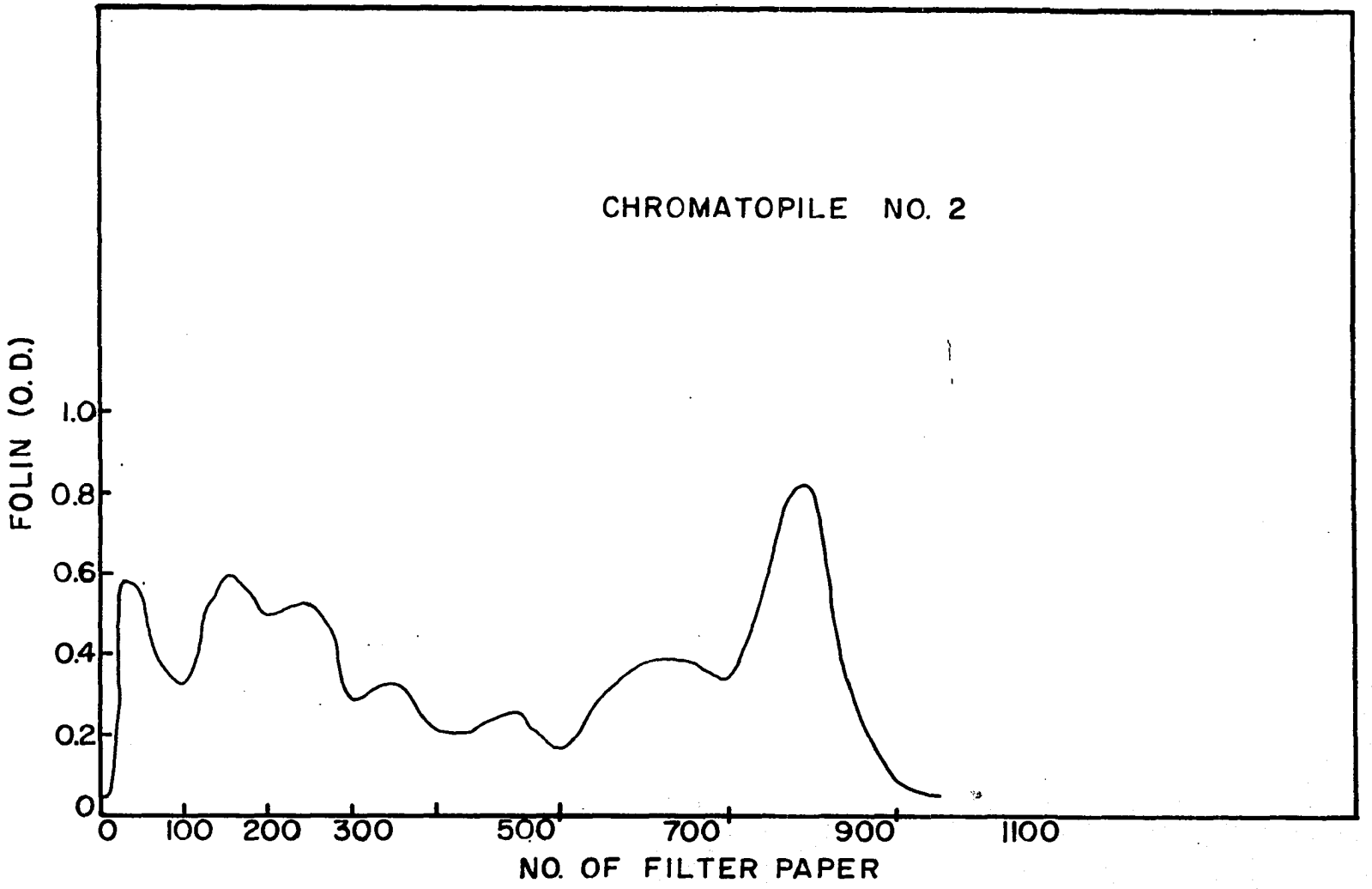


Fig. 9. Chromatopile of Parke-Davis protopituitrin using Solvent 1

Table 7. Weight and chromatography behavior of chromatopile 2 fractions

Fraction	Wt. (mg.)	Description	R_F (Solvent 1)
20-100	68	Fluffy brown	Red spot 0.1 streaking to purple spot 0.25
101-200	67	Fluffy brown	Purple spot 0.30, 0.45 streak at 0.70
201-300	67	Brown	Brown streaking centered at 0.51
301-400	36	Fluffy	Streaking 0.64 to 0.75
401-550	43	Green gum	Streaking 0.64 to 0.75
550-750	77	Fluffy	Streak centered at 0.70
750-950	115	Green ppt.	Streak from 0.7 to solvent front

material.

Powdered cellulose columns (Cell. No. 5)

Some preliminary experiments were conducted using dry-packed powdered cellulose columns irrigated with Solvent I to fractionate methanol extract. Difficulty was encountered in obtaining the optimum degree of packing by tamping with a steel rod; in loosely packed columns, peptide material came through with the hold-up volume and in tightly packed columns solvent could not be forced through the column.

However, local success in separation of carbohydrates on

this packing material (129) led to a further attempt using a different packing technique. A column, 47.5 cm. by 4.5 cm., was prepared by packing a heavy slurry of Whatman Cellulose Powder in n-butanol under 60 cm. of positive air pressure. A sample of 754.6 mg. of Kamm product (6/18/58) was dissolved in n-butanol containing a little water and acetic acid. This solution was placed on the column, and additional cellulose packed to a height of 2 cm. above the resulting yellow band. Anhydrous n-butanol was continued as the eluant, until tube 109, each tube containing 5 ml. fractions; the eluant was then changed to n-butanol:acetic acid:water (16:1:1 v/v). Other solvent changes were made at tube 309 (BAW 8:1:1), tube 689 (BAW 4:1:1), tube 1323 (ethanol), and tube 1560 (water). A maximum flow rate of 1 drop/3.9 sec. was maintained throughout operation of this column. The contents of every third tube were concentrated to dryness in a rotary evaporator, extracted with 2.0 ml. of water, and 1 ml. of this solution was taken for folin analysis. Results are given in Fig. 10. Tubes 973 to 982 were inadvertantly overlapped into tubes 1044 to 1053. Material in the pooled fractions indicated in Table 8 was recovered by concentration of the solvent on the rotary evaporator and lyophilization.

Weight recovery was 195.7 mg. or 38.7% of the starting material corrected for the amount taken for folin analysis. Pressor assays showed that peak 16 had no pressor activity

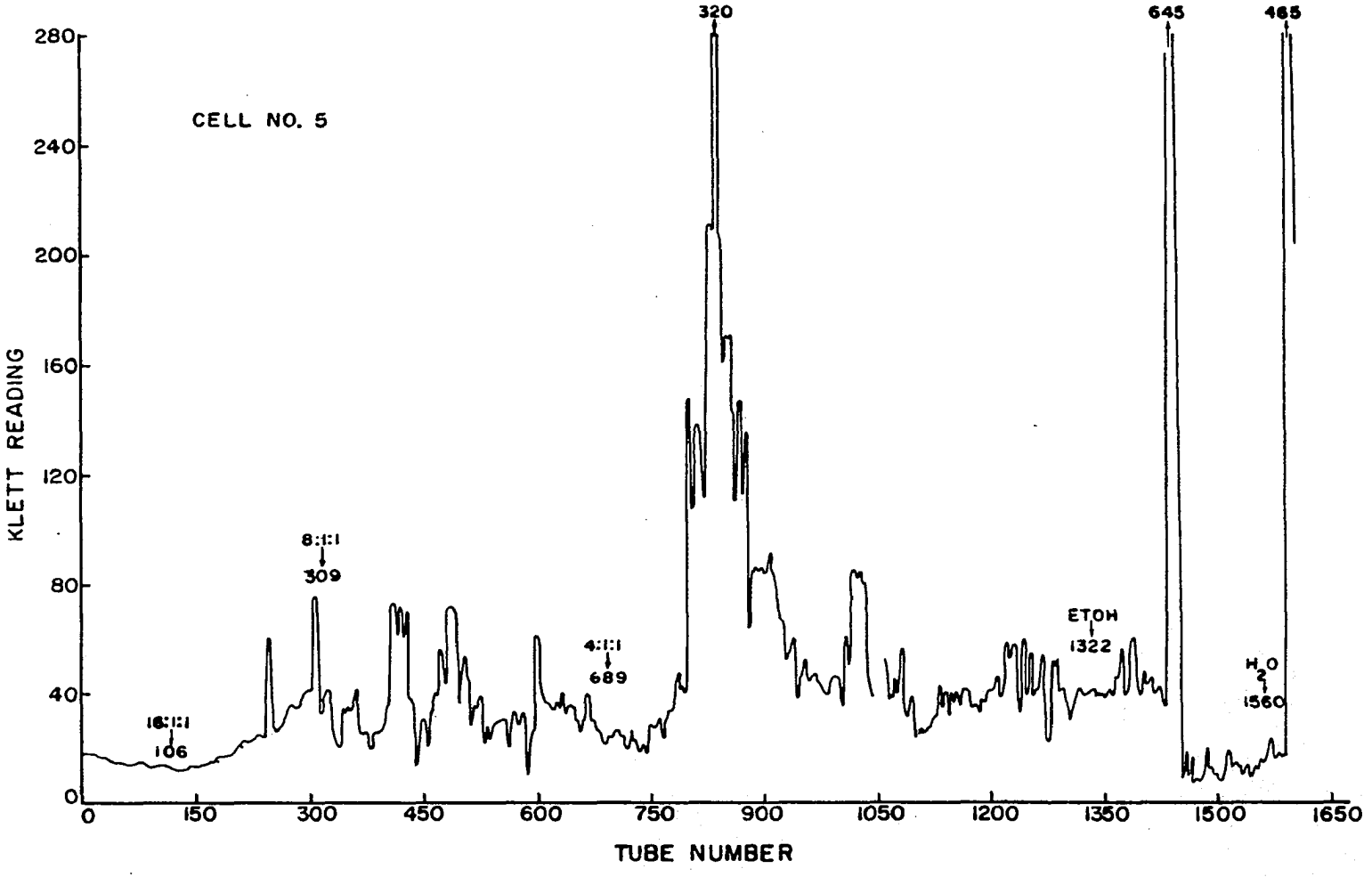


Fig. 10. Column chromatography of Kamm product on cellulose powder, BAW

Table 8. Weight and paper chromatography results of Cell. 5

Peak	Tubes	Wt.(mg.)	R_F's (BAW 4:1:5)
1	190-224	7.3	0^{+3} streak to 0.13^{+}, 0.16^{+}, 0.23^{+}, 0.38^{+}, 0.46^{+}
2	225-253	0.5	0.18^{+}, 0.4^{+}
3	254-280	2.4	0.14^{+}, 0.38^{+}, 0.59^{+}, 0.68^{+}, 0.77^{+}
4	281-300	1.1	0.14^{+}, 0.22^{+}, 0.28^{+}, 0.60^{+3}, 0.64^{+}, 0.69^{+}, 0.77^{+}, 0.59^{+4}
5	301-312	1.7	0.59^{+4}
6	313-336	1.9	Lt. streaking from 0.33^{+} to 0.59^{+4}
7	337-378	1.1	0.15^{+}, 0.25^{+}, 0.29^{+}, 0.53^{+}, 0.62^{+4} streak to 0.89^{+}
8	379-420	3.7	0^{+}, 0.07^{+}, 0.16^{+4}, 0.21^{+}, 0.26^{+}, 0.41^{+}, 0.51^{+3}, 0.55^{+} streak to 0.81^{+2}
9	421-441	1.1	streak to 0.13^{+}, 0.22^{+2}, 0.39^{+4}, 0.46^{+}, 0.54^{+}, 0.6^{+}, 0.68^{+}
10	442-465	1.5	0.14^{+}, 0.24^{+}, $0.35(yel)^{+2}$, 0.40^{+}, 0.50^{+}, 0.60^{+}
11	466-495	3.1	0.93^{+}, 0.16^{+}, 0.19^{+}, 0.26^{+3}, $0.29(yel)^{+}$, 0.35^{+3}, 0.48^{+}, 0.62^{+}
12	496-534		0.14^{+}, 0.25^{+4}, 0.33^{+2}, 0.40^{+}, 0.59^{+}, 0.64^{+}
13	535-585	2.4	0^{+2}, 0.09^{+}, 0.14^{+}, 0.25^{+3}, 0.33^{+3}, 0.46^{+}, 0.60^{+}, 0.66^{+}
14	586-621	4.5	0.08^{+}, 0.22^{+2}, 0.29^{+}, 0.60^{+}, 0.64^{+}
15	622-657	4.3	0.14^{+4}, 0.37^{+2}, 0.64^{+}, 0.71^{+}
16	657-717	5.5	0.15^{+4}, 0.23^{+}, 0.60^{+}, 0.63^{+}, 0.67^{+}
17	718-762	5.9	0.16^{+3}

Table 8. (Continued)

Peak	Tubes	Wt.(mg.)	R_F's (BAW 4:1:5)
18	763-792		0.14^{+4}, 0.29^{+}, 0.41^{+2}, 0.51^{+2}, 0.62^{+2}
19	793-804	2.6	0.14^{+4}, 0.28^{+}, 0.41^{+2} lt. streaking to 0.69
20	805-822	4.2	0.13^{+4}, 0.26^{+}, 0.41^{+2} lt. streaking to 0.68^{+}
21	823-849	9.0	0.11^{+2}, 0.39^{+3} streaking to 0.48^{+2}
22	850-882	9.9	0.11^{+2}, 0.21^{+2}, 0.40^{+2}
23	883-912	4.1	0^{+2}, 0.10^{+2}, 0.18^{+2}, 0.38^{+}
24	913-945	2.9	0^{+2}, 0.09^{+1}, 0.13^{+2}, 0.34^{+}
25	944-981		0^{+2}, 0.07^{+}, 0.14^{+2} lt. streaking to 0.34^{+2}
26	982-1044	5.6	0^{+2}, 0.02^{+2}, 0.04^{+}, 0.09^{+}, 0.14^{+} streaking to 0.27^{+}, 0.32^{+}
27	1045-1099	2.8	0^{+3}, 0.20^{+}, 0.05^{+}, 0.10^{+} lt. streaking to 0.25^{+}
28	1100-1147	2.6	0^{+2}, 0.01^{+3}, 0.06^{+} lt. streaking to 0.23^{+}
29	1148-1211	3.7	0^{+2}, 0.01^{+2}, 0.08^{+}, 0.12^{+} lt. streaking to 0.25^{+}
30	1213-1274		0^{+}, 0.01^{+2}, 0.08^{+3}, 0.15^{+}, 0.20^{+2} lt. streaking to 0.30^{+}
31	1275-1382	9.7	0^{+2}, 0.02^{+3}, 0.09^{+4}, 0.14^{+2}, 0.21^{+2}, 0.29^{+}
32	1383-1430	9.5	0^{+3}, 0.07^{+2}, very lt. streaking to 0.22^{+}
33	1431-1454	27.1	0^{+4}, 0.05^{+2}
34	1455-1593		0^{+3} lt. streaking above
35	1594-1606	53.5	0^{+4}

but peak 21 had approximately 2 U/mg. pressor activity. Apparently well over 30 different peptides were revealed in Kamm product. An earlier experiment, employing the two dimensional paper chromatography system of Hardy et al. (43), showed no free amino acids in the Kamm material.

Supercel columns

Early in the work, four columns packed with an unidentified batch of supercel were employed to fractionate methanol extract by the method of Condliffe (14) for separation of oxytocin and arginine vasopressin; the complex mixture of peptides in methanol extract was poorly resolved. Substitution of n-butanol saturated with 0.5% aqueous acetic acid for the sec-butanol 0 1% aqueous acetic acid solvent of Condliffe gave equally poor resolution. Difficulty was encountered in the Folin analysis of the fractions due to precipitation of the reagent.

Carboxymethyl cellulose columns

Carboxymethyl cellulose was prepared according to the method of Peterson and Sober (85). The only variation in procedure was in substitution of Whatman cellulose powder dry-sieved at 200 mesh for the 325 mesh material recommended. A potentiometric titration of the final product gave a curve similar to that reported by Peterson and Sober.

A 1.0 cm. diameter column was prepared by pouring a slurry of the carboxymethyl cellulose and 0.2 M ammonium acetate buffer, pH 4.5, and applying pressure of approximately 5 psi from a nitrogen tank until the height of ion-exchange adsorbent remained at 35 cm. A sample of 60 mg. of D-62A (some of which failed to dissolve in 8 ml. of buffer) was applied to the column set up in the cold room, 3-5°C, and elution begun with the equilibrating buffer. A maximum flow rate of 3.5 ml./hr. was maintained and 7.0 ml. of effluent collected per tube. The buffer was changed to pH 4.95 at tube 50, to pH 5.5 at tube 112, and to 0.2 M ammonium hydroxide at tube 222. Effluent pH measurements were 4.32 at tube 50, 4.88 at tube 104, 5.26 at tube 156 and 9.86 at tube 281. Folin analysis of 1.0 ml. of sample from every other tube showed color development only from tube 7 to tube 22, corresponding to the calculated hold-up volume. A weight of only 24.8 mg. was recovered in that peak which showed almost the same pattern in a paper chromatogram using BAW (4:1:1) as the starting material. Ninhydrin positive spots having R_F's of 0, 0.045, 0.109, 0.168, 0.227, 0.304, 0.417, 0.568 and 0.728 were found.

The larger mesh size and lower packing pressure than used by Peterson and Sober (85) could account for the apparent lack of adsorbtion of peptide material on the column.

Another column 0.9 cm. x 17.5 cm. of carboxymethyl

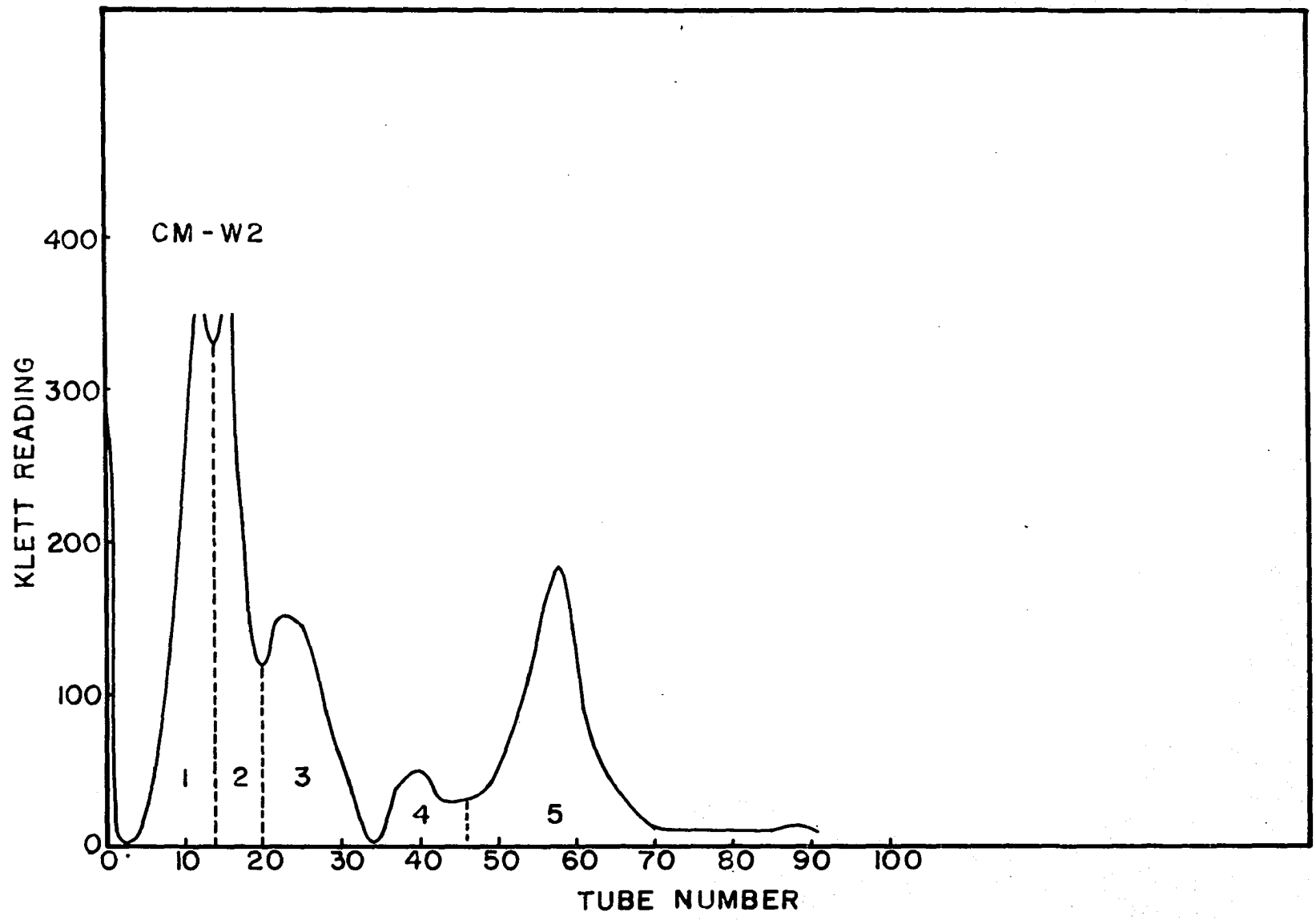
cellulose in 0.2 M ammonium acetate, pH 4.5, was packed under 57 cm. of mercury using air pressure. Flow rate was approximately 3 min./drop and each effluent fraction contained 15 drops (about 1 ml.). The material obtained from the experiment described above (24.8 mg.) was placed on the column in the cold room, 3-5°C. Folin analysis of every third tube is reported in Fig. 11. Paper chromatography patterns in BAW (4:1:1) of pooled fractions is shown in Table 9.

Table 9. Paper chromatography results of fractions from CM-W2

Peak	Tubes	R_F's BAW (4:1:1)
1	0-13	0, 0.0664, 0.113, 0.27, 0 407
2	14-20	0, 0.0746, 0.133, 0.186, 0.262, 0.34, 0.532, 0.631
3	21-33	0, 0.029, 0 0746, 0.124, 0.153, 0.257, 0.332, 0.469, 0.577, 0.801
4	34-46	0.0211, 0.076, 0.127, 0.198 (insufficient material)
5	46-90	0.0126, 0.0632, 0.143, 0.30, 0.481

Carboxymethyl cellulose did not have the resolving power, under the conditions employed, to fractionate completely the peptide mixtures obtained from paper chromatography of methanol extract of Kamm product.

Fig. 11. Chromatography of a D extract on carboxymethyl cellulose


CM - W2
KLETT READING
400
300
200
100
0
1
2
3
4
5
0
10
20
30
40
50
60
70
80
90
100
TUBE NUMBER

Carboxylic acid ion-exchange resin columns

Many columns using varying pH and elution schemes were employed in attempts to fractionate both Kamm product and methanol extract. Results obtained in a typical experiment, IRC-50 8, are presented. A 1.0 g. sample of methanol extract was applied to a column 4.3 cm. x 34.8 cm. equilibrated with 0.2 M ammonium acetate at pH 4.69 and set up in the cold room, 3-5°C. Effluent fractions collected varied between 2.8 ml. and 4.2 ml. but were mostly 3.5-3.8 ml. in volume. Folin analysis of 1.0 ml. samples of every second tube is shown in Fig. 12. Change of effluent pH resulting from change of buffer to 0.2 M ammonium hydroxide is also indicated. R_F's for the various pooled fractions in two solvent systems are given in Table 10.

No bio-assays were available for any of these fractions so no estimate of enrichment of biological activity can be given. The chromatograms of the fractions indicate that all are complex mixtures consisting of many ninhydrin positive components.

Countercurrent distribution of methanol extract (CCD 6)

A sample of 108 mg. of methanol extract was distributed in the 60 tube, 10 cc. manually operated countercurrent distribution apparatus in an n-butanol-0.09 M p-toluenesulfonic

Fig. 12. Column chromatography of methanol extract on Amberlite IRC-50, 0.5 M NH_4OAC

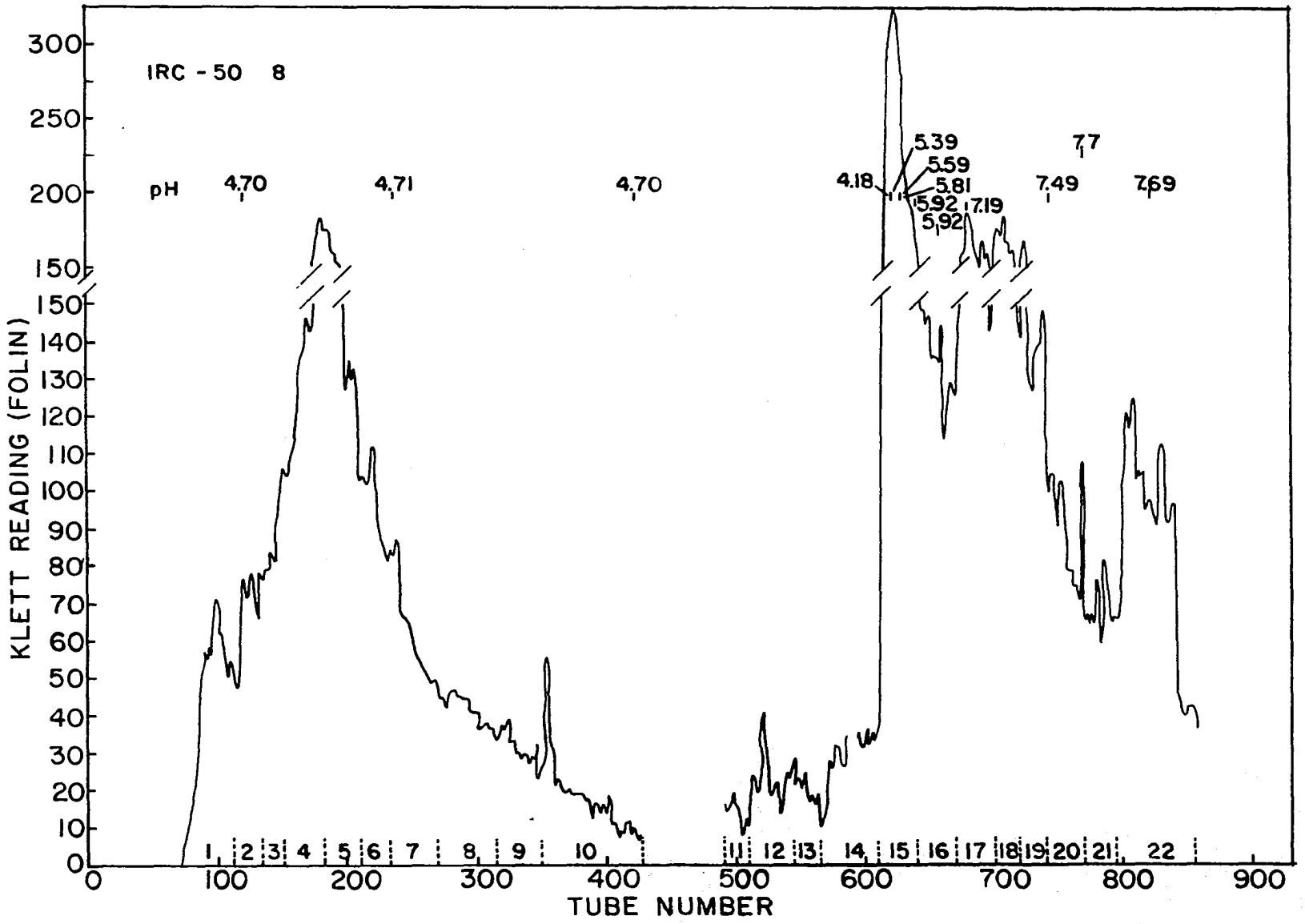

IRC - 50 8
pH 4.70 4.71 4.70 4.18 5.39 5.59 5.81 5.92 5.92 7.19 7.49 7.7 7.69
KLETT READING (FOLIN)
TUBE NUMBER
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

Table 10. Paper chromatography results of IRC-50 8 fractions obtained from methanol extracts

Fraction	Tubes	R_F (4:1:1)
1	74-112	0(heavy), 0.09, 0.18, 0.31
2	113-135	0(v.lt.), 0.074, 0.18, 0.29
3	136-151	0(v.lt.), 0.17, 0.28, 0.34
4	152-185	0, 0.077, 0.15, 0.22, 0.32, 0 42
5	186-210	0.165, 0.35
6	211-232	0.17, 0.35
7	233-270	0.06, 0.11, 0.30, 0.35, 0 59
8	271-315	0.42
9	316-350	0.42
10	351-428	0.42
11	492-510	--
12	511-534	0, 0.05, 0.124
13	535-566	0, 0.05, 0.124
14	567-610	--
15	611-640	--
16	641-670	solvent front
17	671-700	solvent front
18	701-720	solvent front
19	721-740	--
20	741-770	0, 0.65, 0.73, 0.79, solvent front
21	771-795	--
22	796-856	--

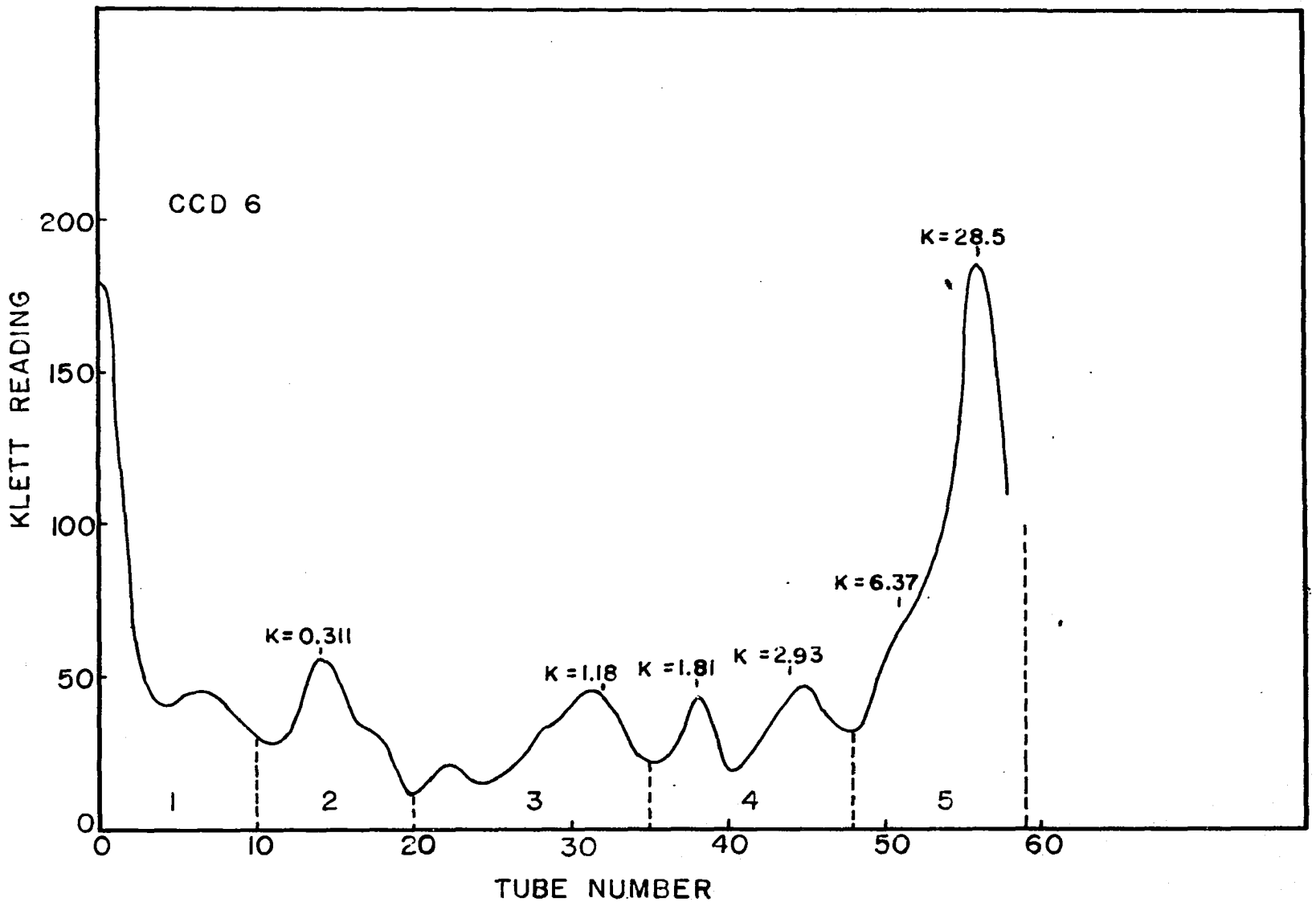
acid solvent system at approximately 25°C for 59 transfers. Cyclohexane-Folin analysis of 1.0 ml. of each phase from every second tube is shown in Fig. 13. Each of the five pooled fractions taken showed several ninhydrin positive components on paper chromatography using either Solvent I or a methyl ethyl ketone:propionic acid:water (120:40:48%) solvent system.

Countercurrent distribution of D (CCD 24)

A sample of 210 mg. of FD 126 was dissolved in 10 ml. of the lower phase of an equilibrated sec-butanol-0.5% trichloroacetic acid solvent contained in the first tube of the Automatic Countercurrent distribution apparatus and subjected to 450 transfers at 25°C. Fig. 14 shows results of ninhydrin analysis on 1.0 ml. of each phase of every other tube and also the pooling scheme employed. Paper chromatography of the first four fractions only was carried out because at that time published data (34, 115) indicated that lysine vasopressin and corticotropin-releasing-factor should occur in these areas. Table 11 summarizes the results obtained from this distribution.

The number of peaks indicated the presence of at least eight components in D and paper chromatography showed all peaks to be heterogeneous.

Fig. 13. Distribution of methanol extract, n-BuOH-0.09 M tosyl acid


CCD 6
KLETT READING
TUBE NUMBER
K=0.311
K=1.18
K=1.81
K=2.93
K=6.37
K=28.5
1
2
3
4
5
0
50
100
150
200
0
10
20
30
40
50
60

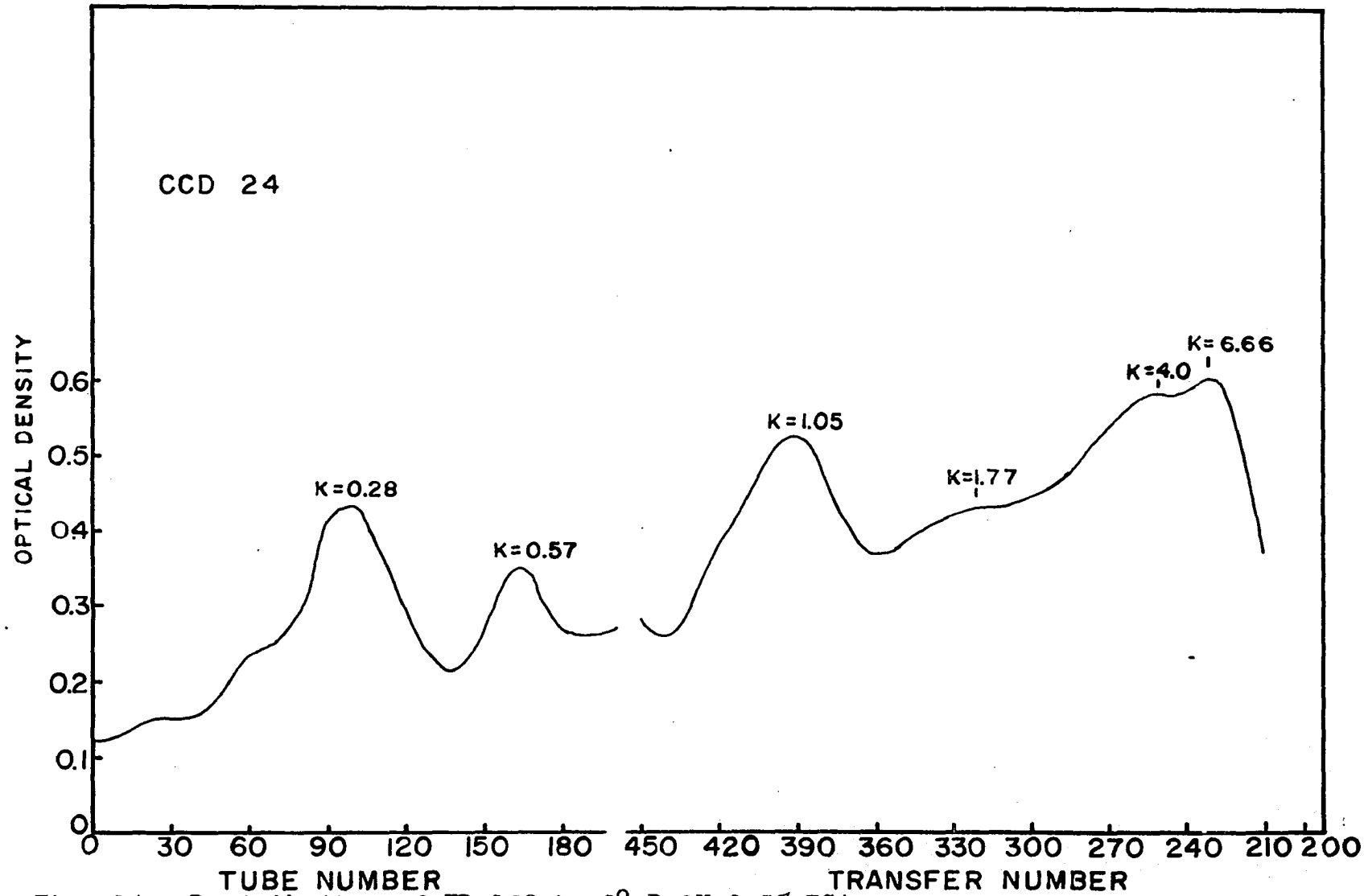


Fig. 14. Distribution of FD-126 in 2° BuOH-0.5% TCA

Table 11. Weight, paper chromatography and partition coefficients of fractions obtained from a CCD of FD 126

Fraction	Wt.(mg.)	K	R_F's BAW (4:1:1)
1	28.5	0.142	0.064, 0.11, 0.70
2	38.0	0.223	0.071, 0.17, 0.70
3	21.4	0.332	0, 0.07, 0.11, 0.272
4	21.3	0.574	0, 0.08, 0.12, 0.26, 0.42, 0.54
FD 126			0, 0.08, 0.15, 0.26, 0.33, 0.47, 0.62, 0.73

"Fingerprinting" by paper chromatography and electrophoresis

Five "fingerprinting" experiments (59, 86) on 1 mg. samples of FD 126 on Whatman No. 3 chromatography paper gave the typical results shown in Fig. 15. Elution of ninhydrin positive spots with 6 N hydrochloric acid and hydrolysis in sealed tubes at 100°C showed the following amino acids as revealed by paper chromatography (43):

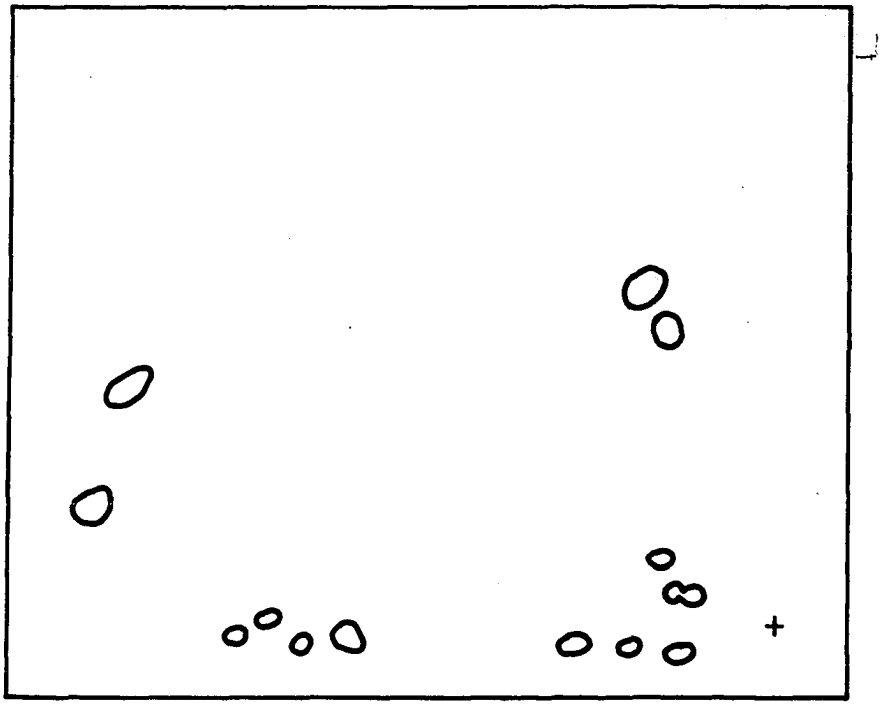
D-1 arg, lys, glu, gly, his, ala, val, leu

D-2 arg, gly, his, ala (trace ileu/leu)

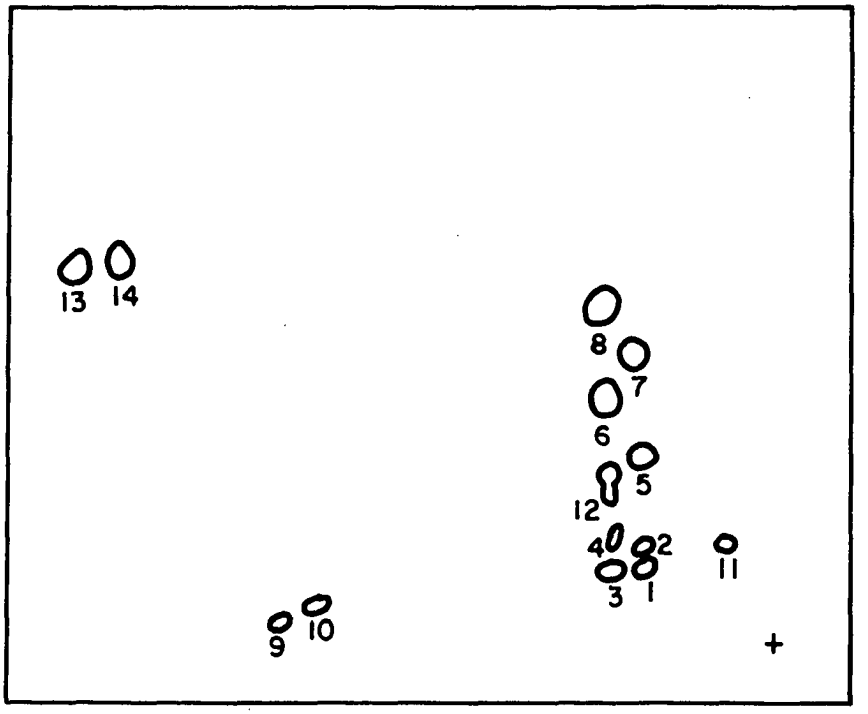
D-3 arg, asp, lys, gly, his, glu, ala, val, ileu/leu, tyr, phe (trace cys, thr)

D-4 arg, gly, ala, his, val, leu

D-5 arg, lys (or gly), his, glu, leu/ileu (trace val, thr)



b. HYPOTHALAMIC D



a. POSTERIOR PITUITARY D

Fig. 15. Two dimensional paper chromatography and electrophoresis of pituitary and hypothalamic extracts

D-6 arg, asp, lys, his (or ser), glu, val, leu/ileu

D-7 arg, gly, his, glu, ileu/leu (trace val, asp)

D-8 arg, gly, his, glu (trace val, ileu/leu)

D-9 arg, lys, gly, glu, his, ileu/leu

D-10 arg, gly, glu, his, val, ileu/leu

An area of the fingerprint paper taken as a blank and treated identically showed spots in areas corresponding to glycine, and arginine, and a light spot at alanine. Thus it is unknown whether all of the peptides actually contain arginine and glycine or whether some of these are artifacts. The isoleucine and leucine spots could not be distinguished in the concentrations employed. A different elution procedure was used in a repetition of this experiment. Ninhydrin positive areas were cut into smaller, 2 mm. segments and placed in glass funnels with fine capillary tips. Hydrochloric acid was permitted to percolate through the paper into hydrolysis tubes. The following results were obtained after hydrolysis and paper chromatography:

D-1 gly (trace asp, ala)

D-2 gly, ala, pro (trace lys, ser, val, ileu/leu)

D-3 gly, ala (trace lys, ser, val, ileu/leu)

D-4 gly, lys, thr

D-5 --

D-6 gly, ala, val, asp, ileu/leu (trace his)

D-7 gly (trace asp, ala, ileu/leu)

D-8 gly

D-9 gly (trace lys, ala)

D-10 gly, asp, ala (trace his, leu/ileu)

D-11 gly (trace lys, ala)

D-12 gly, ala, pro (trace lys, ser, thr, val, leu/ileu)

D-13 (trace gly, lys)

D-14 gly, ala, pro (trace arg, lys, ser, val, leu/ileu)

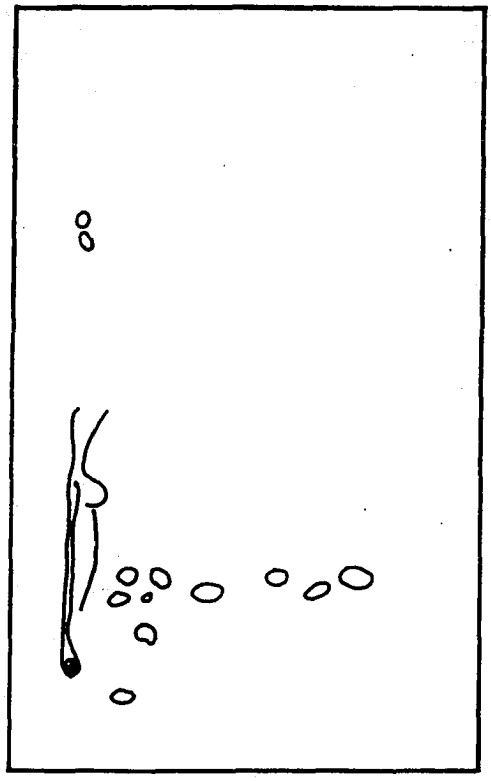
Better results were obtained with the first method and greater reliability was placed on that experiment.

The "fingerprinting" technique was employed to determine the number of ninhydrin positive components in various other extracts. Fig. 16 shows the pattern obtained for D material obtained from hypothalamic tissue. Fig. 16a shows the result from Kamm product of posterior pituitary tissue and Fig. 16b an aqueous extract of this same tissue. Fig. 16c and 16d show the patterns derived from Kamm product and aqueous extracts of hypothalamic tissue respectively.

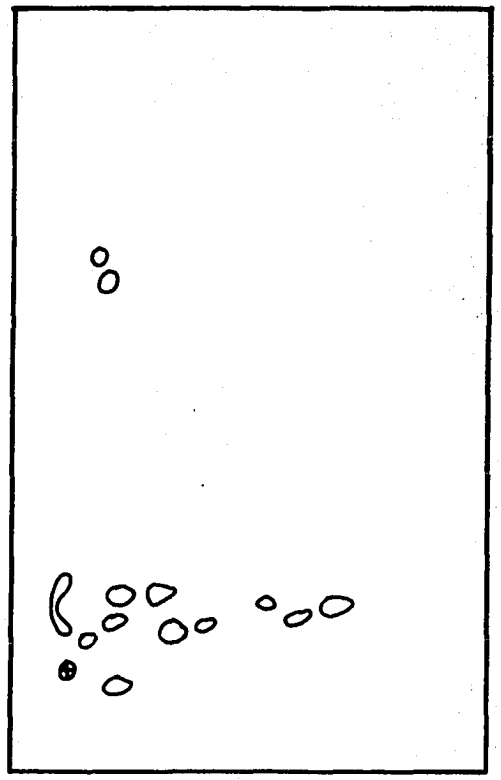
Countercurrent distribution of pooled Kamm product (10/9/59) (CCD 39)

Two hundred milliliters of the lower phase of a solvent equilibrated at 25-30°C consisting of n-butanol:acetic acid: water (4:1:5) was added to each of 15, 500 cc. separatory funnels. Ten grams of combined Kamm product (LVP = 5.2 U/mg.) made up of material collected on 6/18/58, 7/24/58 and 6/16/58 were added to the first tube. The distribution was conducted

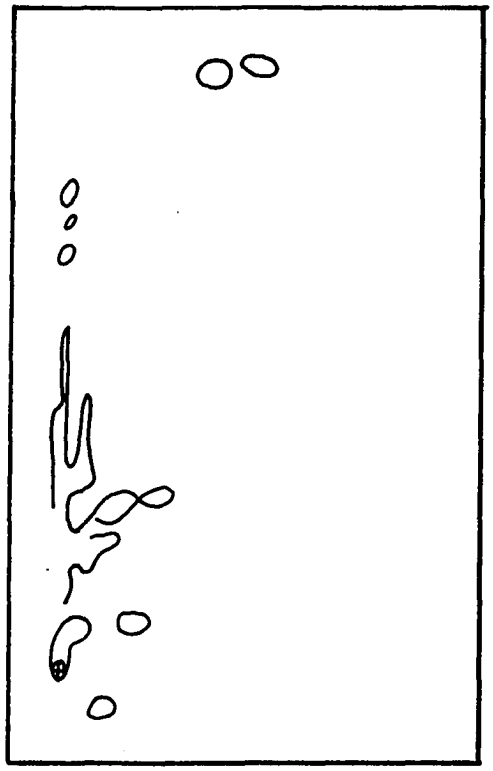
Fig. 16. Two dimensional chromatograms of pituitary and hypothalamic extracts



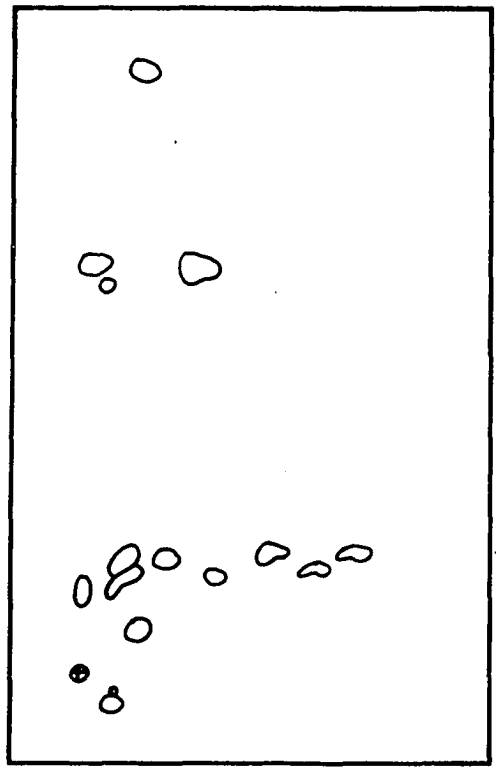
A. KAMM FRESH POSTERIOR PITUITARY



B. AQ EXTRACT FRESH POSTERIOR PIT.



C. HYPOTHALAMUS KAMM



D. AQ. EXTRACT HYPOTHALAMUS

with 200 ml. of upper phase for 14 transfers; emulsion formation was encountered in the first three tubes. The upper phase was transferred by means of a siphon attached to a water aspirator. The cyclohexane-Folin analysis reported in Fig. 17 was carried out by addition of 0.25 ml. of 6 N NaOH to samples of 0.5 ml. of each phase from each tube, followed by the usual procedure. Samples were treated in the same manner for ninhydrin analysis. The material from each tube was recovered by evaporating the organic solvent in a rotary evaporator at bath temperatures of 40-45°C and lyophilizing the aqueous solution remaining. Information obtained from this experiment is compiled in Table 12.

The highly amber colored material which moved rapidly through the distribution gave very little Folin or ninhydrin color. Although weight recovery from this experiment amounted to only 80.8%, pressor recovery of 112.2% was obtained. This is probably within the experimental error of the assay.

Continuous electrophoresis of CCD 39, peaks 2 and 3 (Ce 208-2,3)

Guillemin et al. (34) employed paper strip electrophoresis in their preparation of a peptide material having a high corticotropin-releasing activity. The supporting electrolyte they used was employed in this work on the Beckman continuous electrophoresis apparatus, a 0.5 M acetic acid solution. Flow rate settings were 6.5 on the left and 7.0 on the right.

Fig. 17. Distribution of Kamm product in BAW (4:1:5)

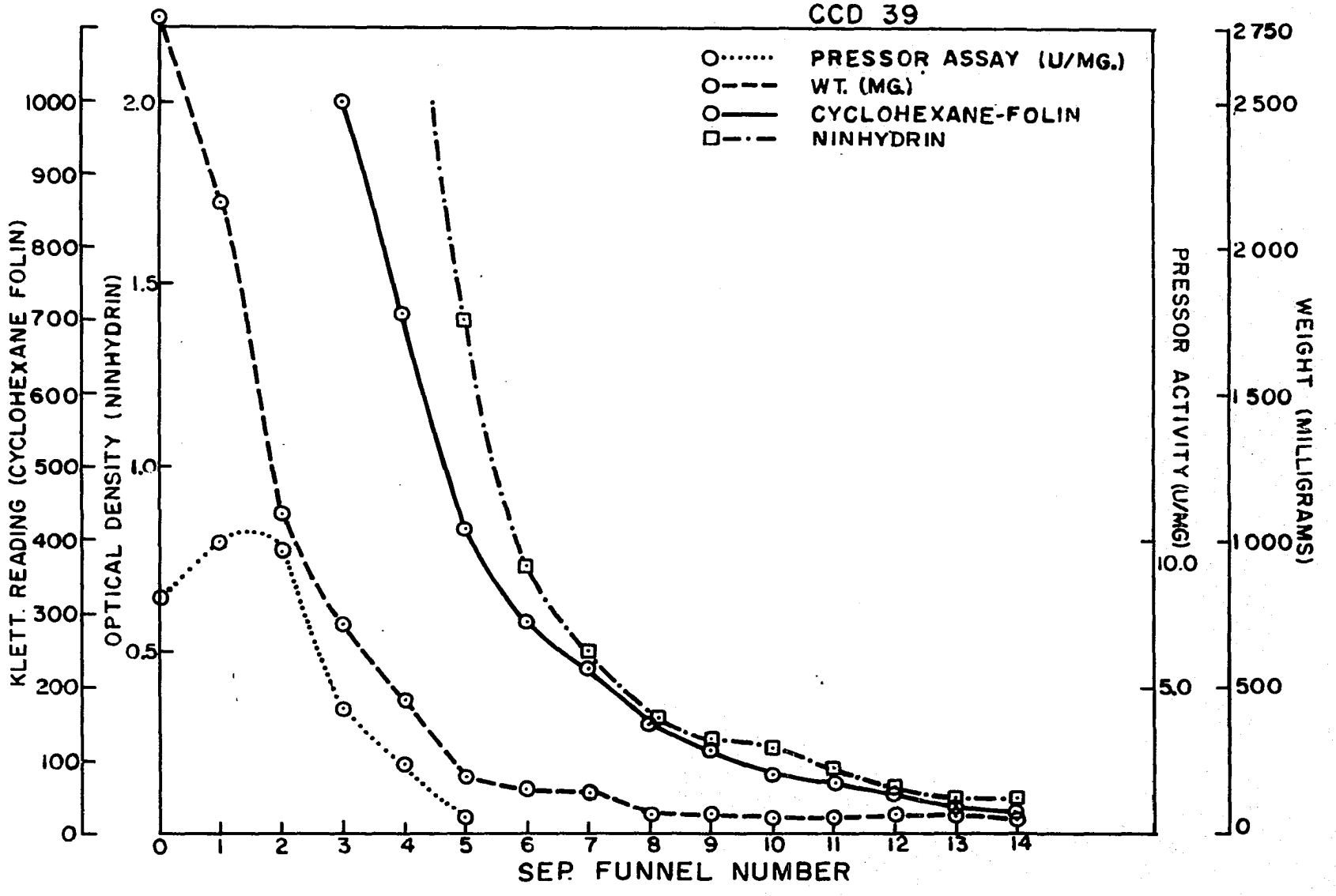

CCD 39
PRESSOR ASSAY (U/MG.)
WT. (MG.)
CYCLOHEXANE-FOLIN
NINHYDRIN
KLETT. READING (CYCLOHEXANE FOLIN)
OPTICAL DENSITY (NINHYDRIN)
PRESSOR ACTIVITY (U/MG)
WEIGHT (MILLIGRAMS)
SEP. FUNNEL NUMBER

Table 12. Weight, pressor and paper chromatography results of CCD 39

Tube	Wt. (g.)	LVP U/mg.	R_F's (BAW 4:1:5)
0	2.7770	8.0	0^{+4} streaking to 0.14^{+2}
1	2.1462	9.99	0^{+4}, 0.04^{+2}, 0.08^{+2}, 0.13^{+2}, 0.18^{+}
2	1.0881	9.62	0^{+2}, 0.06^{+3}, 0.11^{+4}, 0.14^{+3}, 0.19^{+2}, 0.27^{+2}
3	0.7120	4.22	streaking from 0 to 0.3, similar pattern to tube 2
4	0.4457	2.56	0^{+2}, 0.08^{+3}, $0\ 13^{+3}$, 0.19^{+4}, 0.26^{+3}, 0.29^{+2}, 0.33^{+}, 0.40^{+}, $0\ 53^{+}$, 0.60^{+}
5	0.1974	0.64	0.15^{+2}, 0.20^{+2}, 0.26^{+3}, 0.31^{+3}, 0.35^{+4}, 0.44^{+2}, 0.52^{+2}, 0.58^{+}
6	0.1533		0.13^{+} streaking to 0.29^{+2}, 0.37^{+3}, 0.47^{+3}, 0.53^{+4}, 0.68^{+}
7	0.1411		streaking to 0.38^{+2}, 0.48^{+3}, 0.56^{+4} streaking above
8	0.0720		streaking to 0.37^{+2}, 0.50^{+3}, 0.59^{+4} streaking above
9	0.0706		lt. streaking to 0.37^{+2}, 0.46^{+}, 0.54^{+3}, 0.63^{+}, 0.75^{+3}
10	0.0577		0.38^{+}, 0.49^{+}, 0.61^{+2}, 0.67^{+}, 0.79^{+4}
11	0.0513		lt. streaking of yellow material to 0.63^{+}, 0.79^{+4}
12	0.0640		mostly yellow material moving at high R_F
13	0.0592		"
14	0.0436		"

Voltage indicated on the power supply was 510-530 at 30 milliamps. Feed control was set at 5.5 giving a flow rate of 0.88 ml./hr. with the 15 mm. tube containing a 1.0% solution of the material being fractionated. Results of the pressor assay and paper chromatography are given in Table 13. The results of CRF and ACTH assays on the fractions obtained from Ce 208-2 are reported in Table 14.

The continuous electrophoresis was continued with a sample of 1.0688 g. of CCD 39 peak 2 (LVP = 9.62 U/mg.) using the same conditions noted for the fractionation of peak 3. Results of this experiment are given in Table 15.

Ce 208-3 gave a weight recovery of 295.9 mg. (66%) and a pressor recovery of 62.5%. However Ce 208-2 gave a weight recovery of 733.7 mg. (69%) but only a 46.6% pressor recovery. The reason for this difference in pressor recovery is unknown since both experiments were conducted similarly and inactivation of the vasopressin does not seem a likely explanation.

A rough calculation of the amount of CRF material in Ce 208-2 (21-22) was made in the following manner. The two different responses reported for the ACTH assay were labelled A and B as reported in Table 16 and graphed as shown in Fig. 18. An average figure for ACTH-like activity was obtained for Ce 208-2 (21-22) from A and B and subtracted from the CRF-like and ACTH-like activity given by CRF assay. A correction in milliunits of ACTH was made for the amount of pressor

Table 13. Pressor, weight and paper chromatography results of Ce 208-3

Peak	LVP (U/mg.)	Wt. (mg.)	R_F's (BAW 4:1:5)
1-2		--	0.28^{+3}
3-4		--	0.12^{+}, 0.25^{+3}
5-6		--	0.0, 0.01^{+2}, 0.08^{+}, 0.13^{+3}, 0.26^{+}, 0.34^{+} (streak to 0.34)
7-8		16.5	streaking 0.13^{+3}, 0.16^{+4}, 0.22^{+3}, 0.40^{+4}, $0\ 58^{+3}$
9-10	0.065	65.9	0.0^{+3}, 0.01^{+3} streaking to 0.07
11-12	1.03	84.8	0.0^{+2}, 0.02^{+3}, 0.04^{+2}, 0.20^{+3}, 0.23^{+} streaking
13-14	19.3	35.8	0.02^{+2}, 0.04^{+3}, 0.09^{+2}, 0.13^{+4}(yel.), 0.17^{+2} streaking to 0.26^{+2}
15-16	28.2	12.9	0.0^{+2}, 0.02^{+2}, $0\ 04^{+4}$, 0.07^{+2}, 0.09^{+3}, 0.11^{+4} streaking to 0.19^{+2}
17-18	0.53	12.9	streaking to 0.12^{+4}, 0.15^{+4} and to 0.28^{+2}
19-20	0.44	17.9	0.12^{+4}, 0.19^{+5} clear spots, some streaking beyond
21-22	0.64	19.5	0.11^{+4}, 0.14^{+4}, 0.20^{+3}, 0.39^{+3}, 0.56^{+3}
23-24	0.03	21.1	0.12^{+2}, 0.16^{+6}, 0.32^{+3}, 0.38^{+2}, 0.50^{+}
25-26		0.4	0.05^{+2}, 0.14^{+5}, $0\ 18^{+4}$, $0\ 25^{+2}$, 0.30^{+2}, $0\ 34^{+2}$
27-28	(yel.)	8.2	0.04^{+2}, 0.07^{+2}, 0.11^{+3}, 0.17^{+3}
29-30			0.07^{+}, 0.12^{+3}

Table 14. CRF and ACTH assays of fractions obtained from Ce 208-2

Date of assay & sample	γ/inj.	LVP U/mg.	LVP mU/inj.	B control	B sample	ΔB	% inc.
2/16/60 13-14	0.6	17.4	10.43	8.1±0.5 (5)			
2/16/60 15-16	0.6	50	30		19.4±2.1 (5)	11.3	140±25
2/8/60 17-18	2.4	6.74	16.1	7.4±2.1 (4)	25.1±4.8 (3)	17.7	240±65
2/10/60 19-20	2.4	1.06	2.54	5.8±1.5 (4)	4.8±0.9 (4)	--	0
2/8/60	45		47.8	7.4±2.1 (4)	35.4±2.8 (5)	28.0	380±35
1/18/60 21-22	45	1.25	56.3	6.7±1.7 (3)	31.8±1.3 (3)	25.1	375±20
2/10/60 23-24	45	0.44	19.8	5.8±1.5 (4)	18.2±2.4 (5)	12.4	210±40
2/10/60 25-26	45	1.10	49.5		27.9±4.0 (4)	22.1	380±70
2/16/60 27-28	45	0.79	35.6	7.95±0.5	12.7±1.4 (5)	4.75	60±15

Table 14. (Continued)

Date of assay & sample	γ/inj.	LVP U/mg.	LVP mU/inj.	B control	B sample	ΔB	% inc.
			ACTH assay for Ce 208-2 (21-22)				
A							
ACTH	1.0 mU			3.45±0.3 (5)	18.1±0.5 (3)	14.6	426
2/12/60	0.25 mU				5.1±1.0 (3)	1.65	47.8
21-22	90	1.25	112.5		29.4±1.8 (4)	25.9	752
	22.5		28.1		9.7±0.9 (3)	6.25	181
B							
5/10/60	1.0 mU			2.4±0.1 (4)	26.7±2.6 (4)	24.3	1025
ACTH	0.25 mU				8.1±2.4 (4)	5.7	238
21-22	112.5	1.25	140.5		23.6±5.8 (3)	21.2	884
"	28.1		35.1		13.1±3.2 (4)	8.7	446

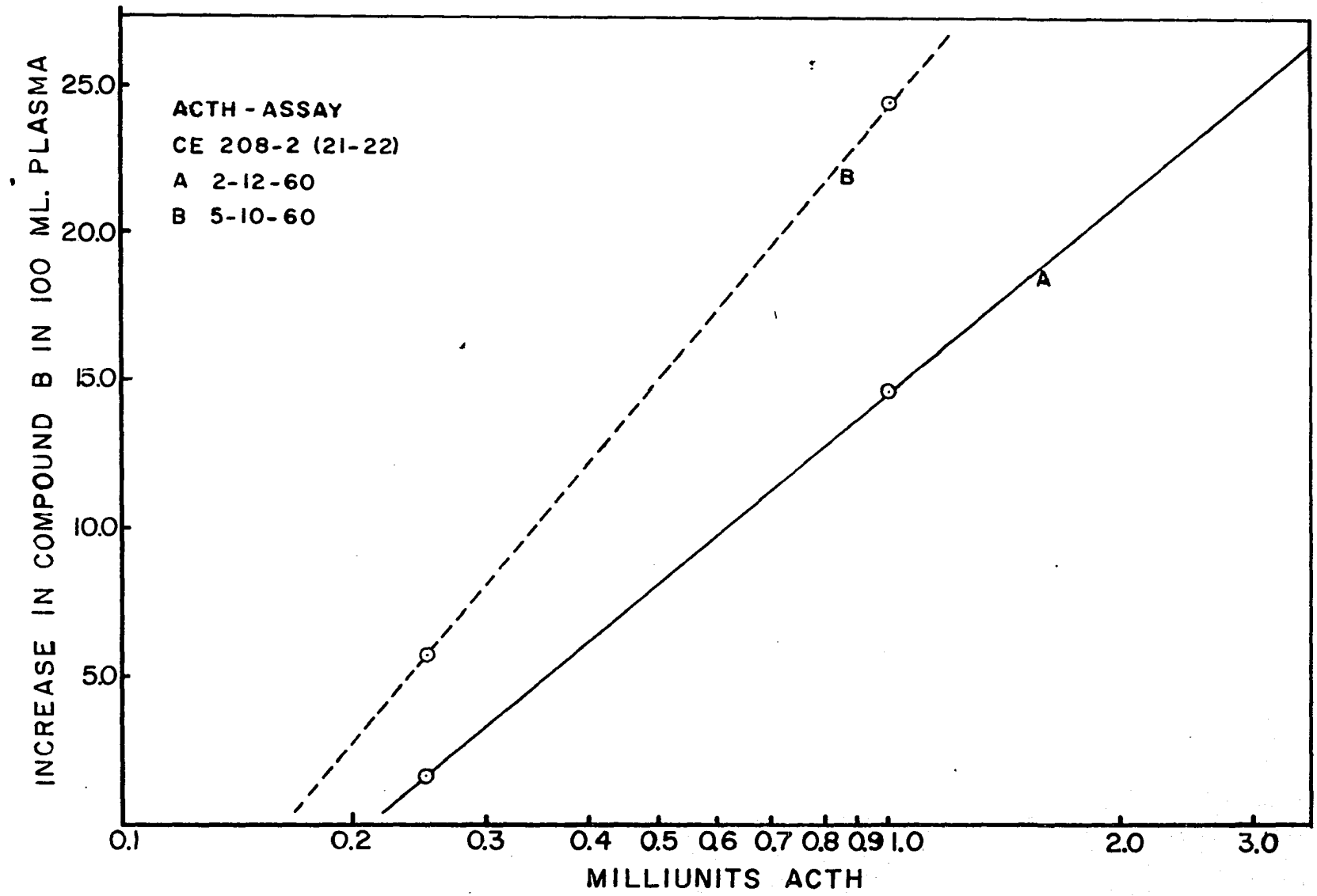
Table 15. Pressor, weight and paper chromatography results of Ce 208-2

Fraction	LVP (U/mg.)	Wt. (mg.)	Appear. on Ce sheet	R_F's (BAW 4:1:5)
1-2		1.7	--	
3-4		5.3	--	
5-6		6.1	--	
7-8	0.02	38.1	(8)+2	
9-10	0.02	124.6	+3	
11-12	0.5	169.6	+3	
13-14	17.4	92.0	+2	0.0^{+}, 0.06^{+2}, 0.09^{+3}, 0.13^{+2}, 0.15^{+2}, 0.18^{+4}, 0.20^{+3}, 0.30^{+2}, 0.38^{+} streaking to 0.47
15-16	50.0	52.3	(15)+2	0.0^{+2}, 0.07^{+2}, 0.11^{+3}, 0.15^{+4}, 0.18^{+5}, 0.23^{+4}, 0.29^{+2}, 0.34^{+2}, 0.37^{+2} streaking
17-18	6.7	46.3	--	0.0^{+3}, 0.02^{+2}, 0.04^{+3}, 0.07^{+2}, 0.11^{+4}, 0.22^{+2} lt. streaking to 0.54^{+}
19-20	1.1	42.8	(20)+2	0.0^{+2}, 0.02^{+2}, 0.04^{+3}, 0.10^{+4}, 0.18^{+5} little streaking
21-22	1.25	42.9	+2	0.0^{+2}, 0.03^{+2}, 0.07^{+2}, 0.11^{+4}, 0.16^{+4}, 0.24^{+5}, 0.48^{+3}, 0.65^{+3}
23-24	0.6	43.3	+3	0.0^{+}, 0.11^{+3}, 0.15^{+2}, 0.19^{+5}, 0.22^{+4}, 0.27^{+2}, 0.31^{+}(yel.), 0.36^{+}, 0.50^{+}, 0.60^{+}, 0.70^{+}
25-26	1.1	17.1	(25)+3	
27-28	0.8	20.6	--	
29-30	0.02	13.3	--	
31-32	7.9			

Table 16. CRF, pressor and ACTH assays of Ce 208-2, 21-22 expressed in mU ACTH

Experiment	τ/inj.	LVP mU/inj.	ΔB	ACTH mU/inj.	ACTH act. mU/τ	sample
A						
ACTH 1.0 mU			14.65			
0.25 mU			1.65			
21-22	90	112.5	25.95	3.39	0.0377	
21-22	22.5	28.1	6.25	0.408	0.01815	0.05585/2 = 0.02792
B						
ACTH 1.0 mU			24.3			
0.25 mU			5.7			
21-22	112.5	140.5	21.2	0.795	0.00706	
21-22	28.1	35.1	8.7	0.313	0.01115	0.01821/2 = 0.00911
CRF assay						
A 21-22	45	56.3	25.1	3.09	0.0687	
B	45	56.3	25.1	1.06	0.0235	
Syn LVP						
A		67	10	0.6	0.0133	
B		67	10	0.35	0.0078	

Fig. 18. ACTH assay results from two separate experiments


ACTH - ASSAY
CE 208-2 (21-22)
A 2-12-60
B 5-10-60
B
A
INCREASE IN COMPOUND B IN 100 ML. PLASMA
25.0
20.0
15.0
10.0
5.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
2.0
3.0
MILLIUNITS ACTH

activity injected in the CRF assay but neglected in the ACTH assays because of its small amount of ACTH activity. Lysine vasopressin has been shown to have CRF (133) and ACTH (48, 95) activity. No corrections were made for any possible potentiation effects (95).

The results of Experiment A show that 40% of the activity could not be explained by the known amount of ACTH and vasopressin present in the sample; only 28% remained in Experiment B. It appears that only a very small amount of corticotropin-releasing factor, if any, could be present in Ce 208-2 (21-22).

Countercurrent distribution of CCD 39 peak 1 (CCD 41)

The first tube of the all-glass 53 tube, 30 ml. per phase, manual countercurrent distribution machine was charged with 1.5 g. of peak 1, CCD 39 (LVP = 9.99 U/mg.). The machine contained the lower phase of a sec-butanol-0.5% TCA solvent system equilibrated at 25°C. After 50 transfers, 1.0 ml. of sample was removed from every second tube and analyzed for peptide material by both the cyclohexane-Folin and ninhydrin methods giving the results reported in Fig. 19. A total of 13 ml. of diluent was necessary in the ninhydrin analysis. The pressor assay was conducted on safely diluted samples of lower phase from tubes 15, 18 and 21. Solvent from the tubes combined as indicated in Table 17 was evaporated and the remaining aqueous layer ether extracted several times and

Fig. 19. Distribution of peak 1, CCD 39, 2^{o} BuOH-0.5% TCA

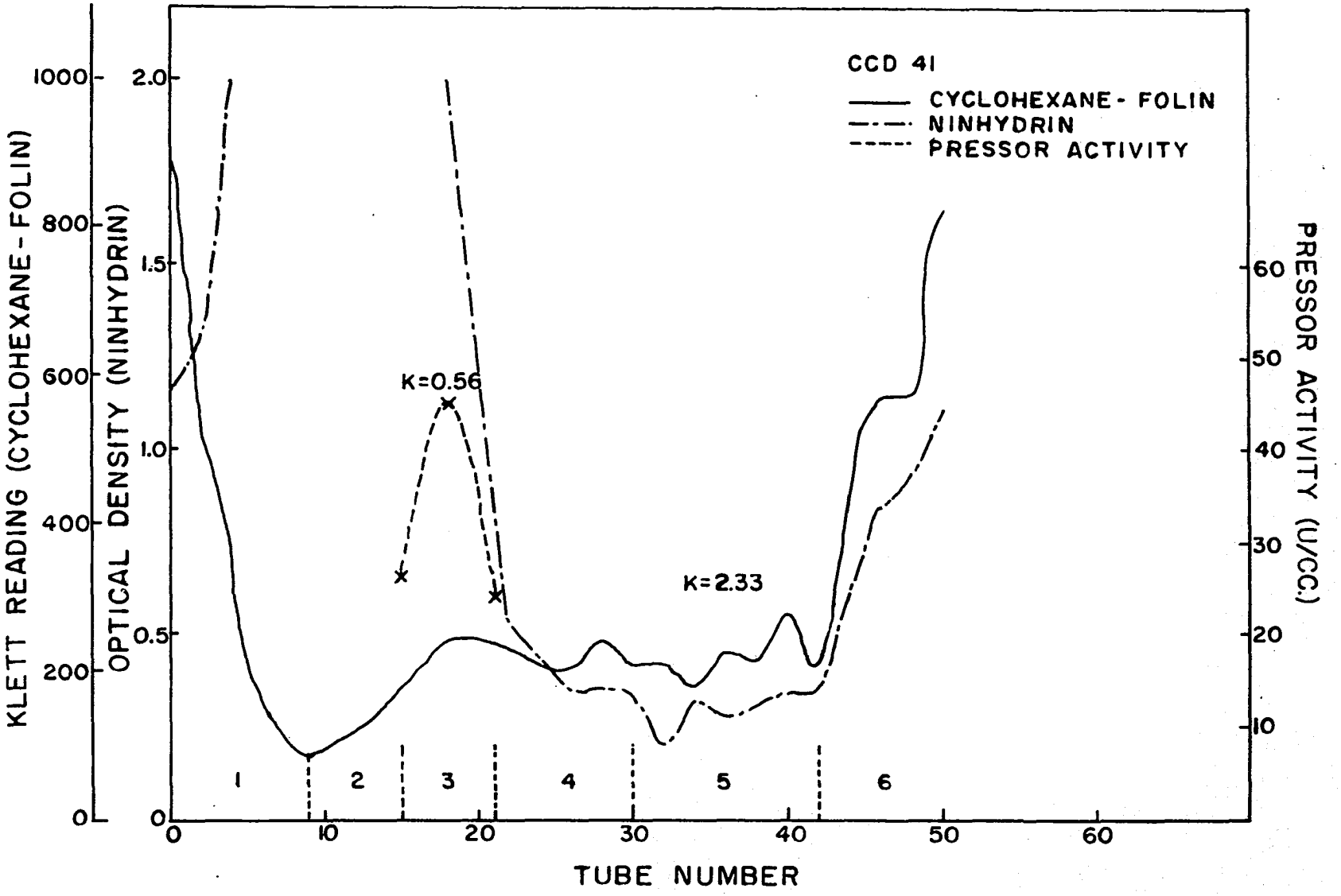

CCD 41
CYCLOHEXANE - FOLIN
NINHYDRIN
PRESSOR ACTIVITY
K=0.56
K=2.33
1
2
3
4
5
6
KLETT READING (CYCLOHEXANE - FOLIN)
OPTICAL DENSITY (NINHYDRIN)
PRESSOR ACTIVITY (U/CC.)
TUBE NUMBER
0
200
400
600
800
1000
0
0.5
1.0
1.5
2.0
10
20
30
40
50
60

Table 17. Weight, pressor and paper chromatography results of fractions obtained from CCD 41

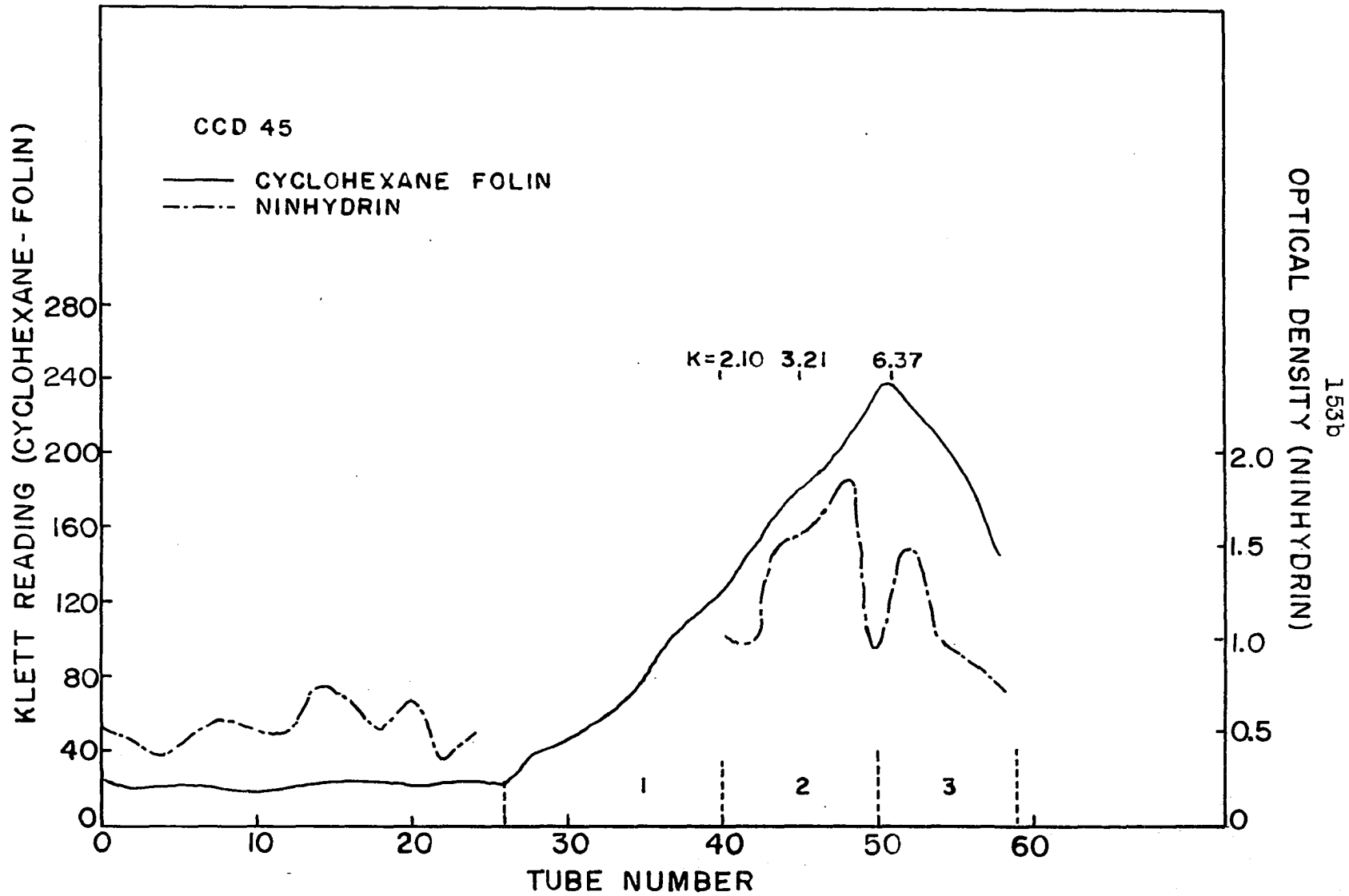
Peak	Tubes	LVP (U/mg.)	Wt. (g.)	R_F's (BAW 4:1:5)
1	0-9		0.2044	0^{+4} with some streaking upward
2	9-14	10.8	0.1496	0.07^{+}, 0.12^{+2}, 0.16^{+4}, 0.21^{+3} short streak above
3	15-21	61.3	0.1066	streak to 0.09^{+}, 0.15^{+4}, 0.19^{+2} streaking
4	22-30	5	0.1017	streak to 0.09^{+}, 0.15^{+}, 0.02^{+}
5	31-42		0.2054	streaking 0.12^{+3} streaking beyond
6	43-50		0.2211	streaking 0.18^{+3} streaking to 0.4^{+}

finally lyophilized to yield a TCA-free white powder in each case.

Weight recovery of 66% was obtained with a pressor recovery of 57%. Although the pressor recovery is low, this experiment afforded a rapid means of obtaining pressor activity in potency nearly equal that of commercial pitressin (192) and giving a five-fold purification.

To gain further knowledge of the area included in peak 5 of CCD 41, 30 mg. of this material was distributed (CCD 45) in the "microdistribution" apparatus using the same solvent system as in CCD 41. Fig. 20 shows the cyclohexane-Folin and ninhydrin analyses, each conducted on 0.25 ml. of each phase

Fig. 20. Distribution of peak 5, CCD 41, 2^{o} BuOH-0.5% TCA


CCD 45
CYCLOHEXANE FOLIN
NINHYDRIN
KLETT READING (CYCLOHEXANE-FOLIN)
OPTICAL DENSITY (NINHYDRIN)
K=2.10 3.21 6.37
1
2
3
TUBE NUMBER
0
40
80
120
160
200
240
280
0.5
1.0
1.5
2.0
0
10
20
30
40
50
60

of every second tube. Unfortunately the tubes designated as peak 1 were already pooled prior to the ninhydrin analysis and peaks 2 and 3 immediately afterward so that the ninhydrin results which appear spurious could not be repeated. The peak, from its shape, shows that the starting material was heterogeneous and the higher K value of the main peak appears greater than that of the original area; however this might be explained by overlapping of the faster moving material into peak 5 of CCD 41. All three peaks after isolation gave a similar pattern when subjected to paper chromatography using BAW (4:1:5) solvent for development. R_F's were 0.03^+, 0.05^+, 0.08^+, 0.12^{+2}, 0.15^{+3}, and a spot around 0.67^+, which may have been an artifact.

Column chromatography of CCD 41, peak 3 (IRC-50 18)

A glass chromatography column 2.1 cm. in diameter and 59.5 cm. long, topped with a 24/40 standard taper female joint was gravity-packed to a height of 42 cm. with IRC-50 ion exchange resin (200-400 mesh), buffered with 0.5 M ammonium acetate at a pH of 6.4. The column, equilibrated and run at room temperature (20-25°) was charged with 98.8 mg. of CCD 41 peak 3 (LVP = 61.3 U/mg.) at tube 13. The effluent fractions, collected in an automatic drop counter fraction collector, at a maximum rate of 1.5 ml./cm.^{2}/hr., had volumes of 2.5 to 3.0 ml. Fig. 21 shows the Folin analysis on a 1 ml. sample

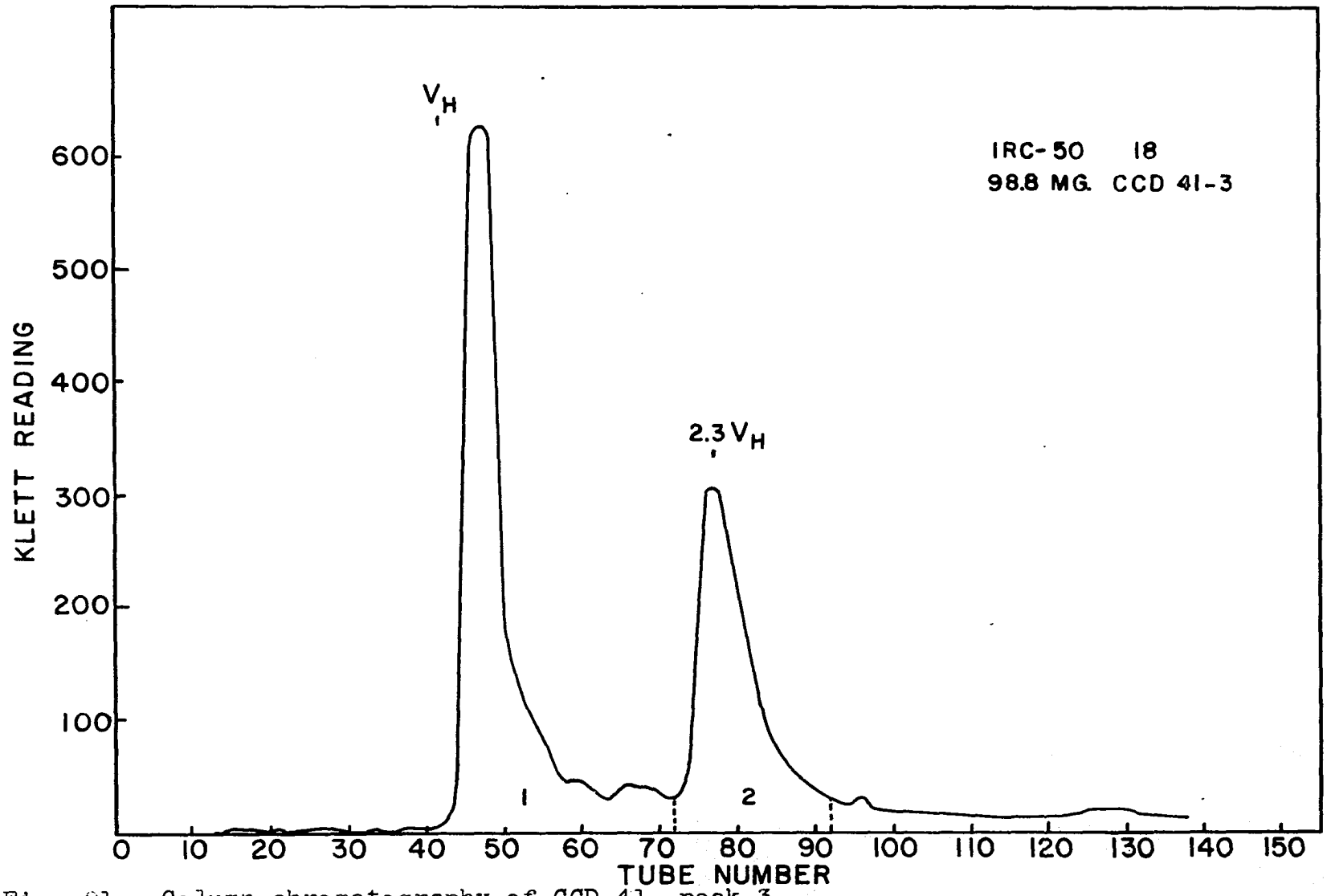


Fig. 21. Column chromatography of CCD 41, peak 3

taken from every second tube. At tube 398, the developing solvent was changed to 0.5 M ammonium hydroxide; however the effluent did not read pH 9 until tube 548. The column was stopped at tube 658. Table 18 shows the pooling scheme and peak number designation.

Table 18. Weight, pressor and paper chromatography results of fractions obtained from IRC-50 18

Peak	Tubes pooled	Wt. (mg.)	LVP (U/mg.)	R_F's (BAW 4:1:5)
1	42-72	12.8	0.52	0.02^{+}, 0.06^{+2}, 0.09^{+2}, 0.13^{+3}, 0.17^{+4}, 0.21^{+4}, 0.31^{+3}, 0.37^{+2}, 0.66^{+2}
2	73-92	4.9	220	slight streak to 0.09^{+}, 0.16^{+2}, streaking to 0.28^{+4}
3	93-196	4.7		0.02^{+}, 0.04^{+}, 0.11^{+}, 0.18^{+2}, 0.26^{+3}, 0.30^{+3} streaking higher
4	197-250	2	0.43	0.06^{+}, 0.11^{+2}, 0.14^{+}, 0.18^{+3}, 0.32^{+}, $0\ 40^{+}$, 0.05, 0.59^{+}
5	251-300	1.9		"
6	301-350	1.8		"
7	351-400	1.8		0.06^{+}, 0.12^{+2}, 0.19^{+2}, 0.42^{+}, 0.61^{+}
8	401-450	2	0.09	
9	451-500	1.1		
10	501-550	2.1		0.07^{+3}, 0.13^{+}, 0.20^{+}
11	551-600	2.9		0.07^{+}, 0.13^{+2}, 0.17^{+2}, 0.20^{+2} streaking 0.59^{+}
12	601-656	3.1		"

Assuming that 81.2 mg. of starting material was not used up for Folin analysis, only 50.6% of the recoverable weight was actually isolated. This poor recovery might be explained by: 1) column retention - it is difficult to envision peptide materials being retained on IRC-50 after treatment with 0.5 M ammonium hydroxide, however - 2) ammonium salts present in the starting material, which were subsequently sublimed when the peaks were lyophilized, 3) loss through manipulation or an incorrect starting weight. Moisture retention by these peptides during storage and weighing can cause large inaccuracies unless precautions are taken to prevent this error.

The pressor recovery amounts to only 24.9% of the starting material accounted for in the peaks assayed. The approximate weights reported for peaks 4 and 8 resulted when relyophilization of the peaks prior to weighing for pressor assays caused a loss of material.

Countercurrent distribution of Ce 208-2 (21-22) (CCD 44)

The lower phase of an equi-volume equilibrated sec-butanol-0.5% trichloroacetic acid (26°C) was distributed in the one milliliter "microdistribution machine". The first tube was charged with 32.2 mg. of Ce 208-2 (21-22) and the distribution was conducted for 59 transfers. Fig. 22 shows the curve which resulted from the cyclohexane-Folin analysis conducted on 0.25 ml. of each phase of every third tube. The

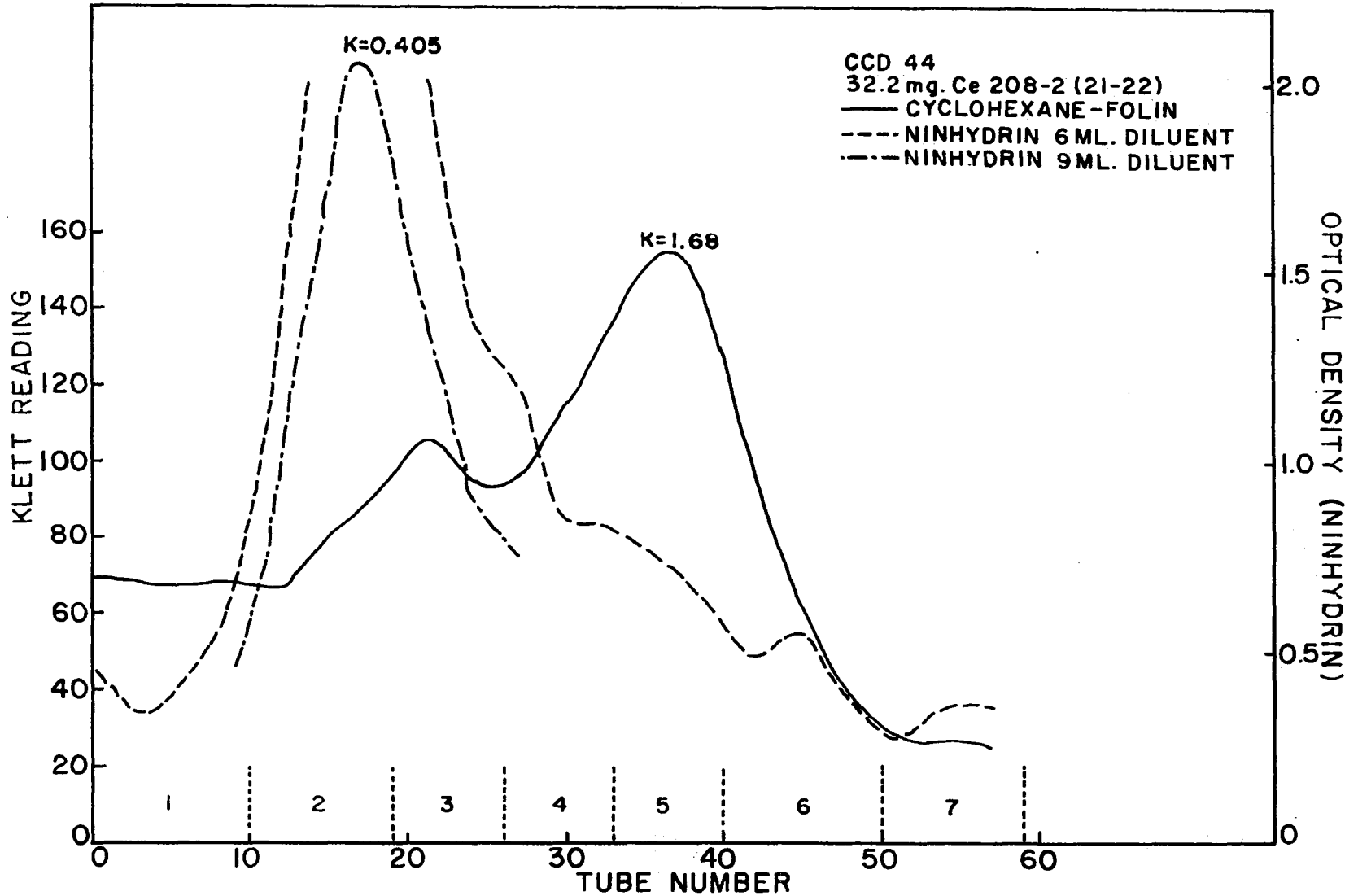


Fig. 22. Distribution of Ce 208-2 (21-22) in 2° BuOH-0.5% TCA

same sample size was employed in the ninhydrin analysis except that 6 ml. of diluent was used. The inset shows the curve resulting when 9 ml. of diluent was used to allow reading of the tubes in the peak ninhydrin area. Peaks indicated by the pooling scheme of Table 19 were obtained by repeated evaporations at temperatures below 40°C on the rotary evaporator, ether extractions and lyophilization.

The theoretical amount of material remaining after Folin and ninhydrin analysis was 26.8 mg.; actual recovery was 110.2% by weight and 114.3% by pressor activity.

Greatest interest was centered on peaks 3 and 5 as a result of the CRF assay. These areas were very near the K values reported for lysine vasopressin, 0.37,* and α-MSH, 2.1, (63) for this system. The CRF assay was repeated for these two fractions as well as an ACTH assay. An ACTH assay was performed on fraction 1. These results are reported in Table 20.

As reported in Ce 208-2, the starting material showed an ACTH activity present; however, no ACTH could be found in fraction 1, 3 or 5 in this experiment. Shepherd et al. (117) reported that oxycellulose treated "clinical" ACTH gave a slow and a fast moving peak having intermedin (MSH) and corticotropin (ACTH) activity. These components would correspond

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*See footnote of Experiment CCD 52.

Table 19. Weight and partition coefficients with pressor and CRF assay results of fractions obtained from CCD 44

Fraction	Tubes	Wt. mg.	LVP U/mg.	K	LVP mU/ inj.	γ/inj.	B control	B sample	ΔB	% inc.
1	0-10	7.7	0.04							
2	11-19	7.6	0.67	0.439	30.5	50	5.0±0.7(5)	16.45±2.8(5)		230
3	20-26	4.1	4.82	0.553	48.2	10	4.15±0.9(5)	18.25±3.9(3)	14.1	340
4	27-33	3.8	2.04		20.4	10	4.15±0.9(5)	10.6±1.8(5)		155
5	34-40	3.9	1.09	1.68	27.3	25	5.0±0.7(5)	21.1±4.2(4)	16.1	320
6	41-50	2.7	0.5		25	50	5.0±0.7(5)	16.95±1.6(5)	11.94	240
7	51-59	1.9	0.5		25	50	6.55±1.3(5)	8.95±2.0(5)		37

Table 20. CRF and ACTH assay results obtained from fractions 1, 3 and 5 of CCD 44

Fraction	ACTH mU	γ/inj.	LVP mU/inj.	B control	B sample	ΔB	% inc.
	1.0[a]			3.5±0.2(3)	26.9(?)(1)		
	0.25[a]				7.95±1.7(2)		
3[a]		20	96.4		4.3±0.9(2)		0
5[a]		50	54.5		2.5±0.3(4)		0
3[b]		5	24.1	7.0±0.5(2)	14.6±1.3(5)		110
5[b]		25	27.25		23.8±2.7(5)		240
	1.0[c]			3.2±0.4(5)	14.8(1)		362
	0.25[c]				5.75(1)		79.7
3[c]		10	48.2		1.45±0.2(2)		0
5[c]		25	27.25		1.75±0.0(2)		0

[a]Assayed 2/25/60.

[b]Assayed 3/17/60 using CRF assay.

[c]Assayed 3/25/60.

Table 20. (Continued)

Fraction	ACTH mU	γ/inj.	LVP mU/inj.	B control	B sample	ΔB	% inc.
	1.0[d]			3.0(1)	29.6±5.1(3)		886
	0.25[d]				9.1±1.9(4)		203
	1.0[e]			2.5±1.0(2)	16.75±2.5(3)		570
	0.25[b]				3.2±0.2(3)		28
1[e]		20	8		2.8±0.4(3)		0
		5	2		2.5±0.5(2)		0

[d]Assayed 5/4/60, ACTH repeat.

[e]Assayed 5/4/60.

to fractions 1 and 7 of this experiment. The possibility exists either that the ACTH activity was destroyed during the experiment or that it was present in the untested peaks. The results of this experiment showing the possible existence of two corticotropin-releasing factors was consistent with the work reported by Gros and DeGarihle in 1959 (42) and Guillemin et al. in 1960 (37, 109).

Table 21 shows R_F values of ninhydrin positive components developed in BAW (4:1:5) for the starting material Ce 208-2 (21-22) and the fractions of CCD 44.

Table 21. Paper chromatographic results of Ce 208-2 (21-22) and fractions obtained from CCD 44

Material	R_F's (BAW 4:1:5)
Ce 208-2 (21-22)	0^{+2}, 0.03^{+2}, 0.075^{+2}, 0.11^{+4}, 0.24^{+5}, 0.48^{+3}, 0.65^{+3}
1	0^{+3}, 0.07^{+}
2	0.02^{+}, 0.05^{+}, 0.08^{+}, 0.13^{+3} streaking, 0.22^{+4}, 0.26^{+4}, 0.33^{+4}, 0.33^{+3} streak 0.67^{+}
3	0.13^{+}, 0.17^{+3}, 0.24^{+4}, 0.34^{+}, 0.44^{+2}, 0.59^{+}
4	0.14^{+}, 0.18^{+}, 0.25^{+}, 0.46^{+}, 0.61^{+}, 0.69^{+3}
5	0.13^{+}, 0.19^{+}, 0.49, yellow spot below 0.73^{+4}
6	0.11^{+}, 0.18^{+}, yellow spot below 0.74^{+3}
7	0.06^{+}, 0.16^{+}, 0.64^{+}

An assay performed in the intact frog (61) showed no melanocyte stimulating activity in either peak 3 or 5 of this experiment or in Ce 208-2, 21-22, the starting material, at the 2.5 γ level. In a preliminary experiment, conditions identical to CCD 44 were used except that the microdistribution machine was charged with 10.1 mg. of Ce 208-2 (21-22). The Folin and ninhydrin analysis results are indicated by Fig. 23 with no additional dilutions necessary (CCD 42). Unfortunately the tubes 33-41 were pooled before the ninhydrin analysis could be performed. It can be seen that both curves are similar to CCD 44 although not identical. An explanation for this difference has not been found. Table 22 shows the result of a CRF assay on the isolated material corresponding to peaks 0, 1 and 2.

Table 22. CRF assay of fractions obtained from CCD 42

Sample	γ/inj.	LVP mU/inj.	B control	B sample	ΔB	% inc.
CCD 42 Peak 1	100	100[a]	4.1±0.7(4)	19.1±4.7(4)	15.0	365
	50	50[a]		11.9±3.4(4)	7.8	190
Peak 0	50		6.8±2.1(3)	10.9±1.8(3)	4.1	60
Peak 2	50			11.7±1.7(2)	4.9	70

[a]No pressor assays were performed; an estimate of this value is possible from consideration of the activity obtained in CCD 44 of material derived from a similar area.

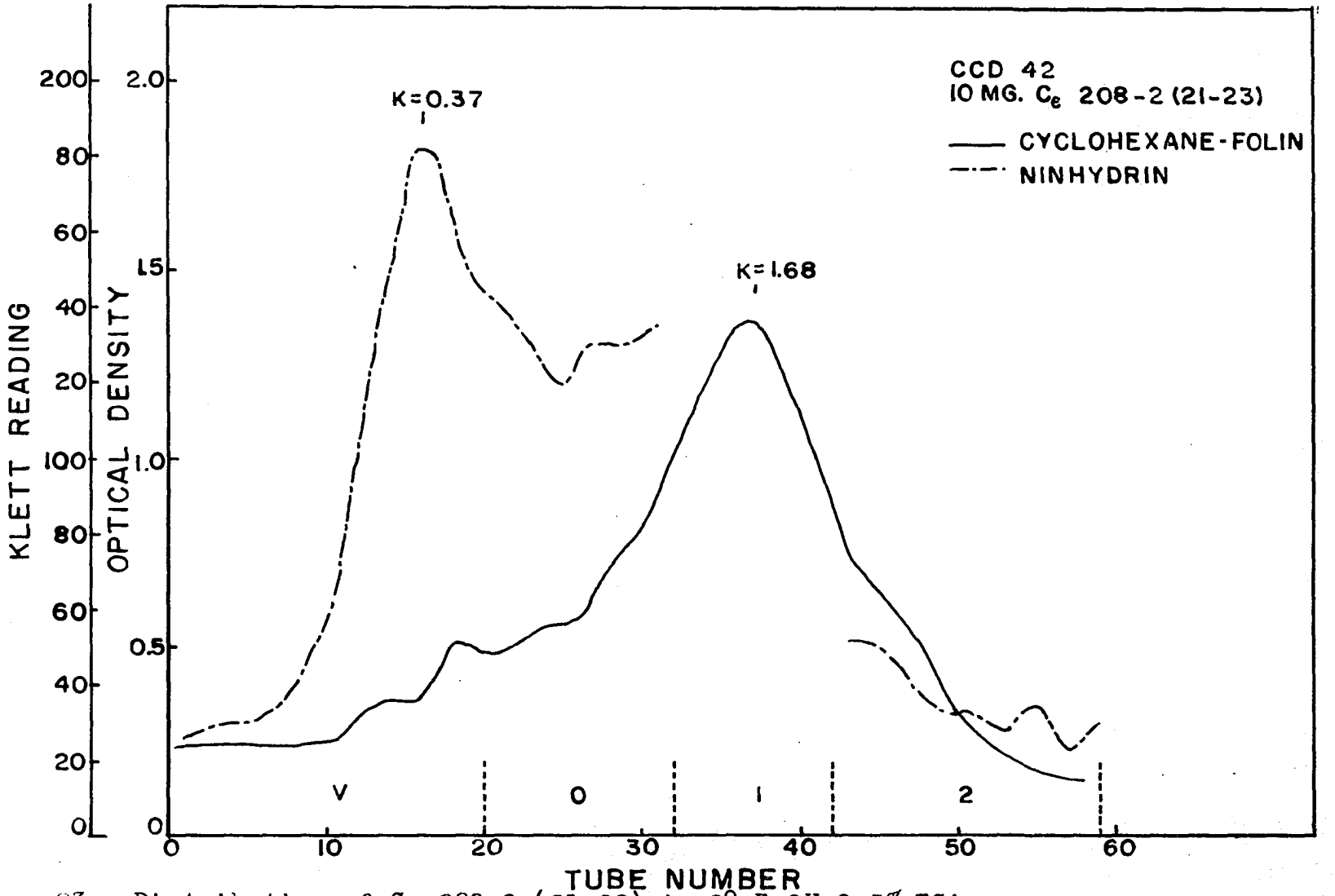


Fig. 23. Distribution of Ce 208-2 (21-22) in 2° BuOH-0.5% TCA.

A paper chromatogram of peak 1 developed in BAW (4:1:5) shows ninhydrin positive materials having R_F values of 0.18^{+}, 0.50^{+2} (yellow), 0.73^{+3} with a yellow spot directly below it.

Countercurrent distribution of pooled Kamm (4/20/60) (CCD 47)

Six hundred milliliters of lower phase of an equilibrated n-butanol:acetic acid:water (4:1:5) solvent system was placed in each of 15 commercial acid bottles. The n-butanol was taken from a drum, product of Commercial Solvents Corporation, Peoria, Illinois. The entire distribution was conducted at a room temperature of about 24°C. The first bottle was charged with 28.86 g. of "Pooled Kamm, April 20, 1960" which contained Kamm isolation yields of 5/23/58 (3.88 g.); 5/5/58 (5.43 g.); 6/26/59 (2.62 g.); 6/3/58 (5.67 g.); 6/17/58 (7.91 g.); 6/12/57 (3.97 g.) and which gave a pressor assay of 4.2 U/mg. This sample dissolved readily in the lower phase of the first bottle, giving a dark amber, translucent solution. The distribution was conducted with additions of 600 ml. of equilibrated upper phase with 75 oscillations and a settling out period of 1/2 to 3 hours. The actual transfers were performed by using a siphon connected to a water aspirator. Emulsions occurred in the first two bottles after several transfers and this lengthened the time necessary for each settling period. The Folin curve reported in Fig. 24 was obtained by using 0.5 ml. of each phase of sample neutralized with 0.3 ml. 6 N

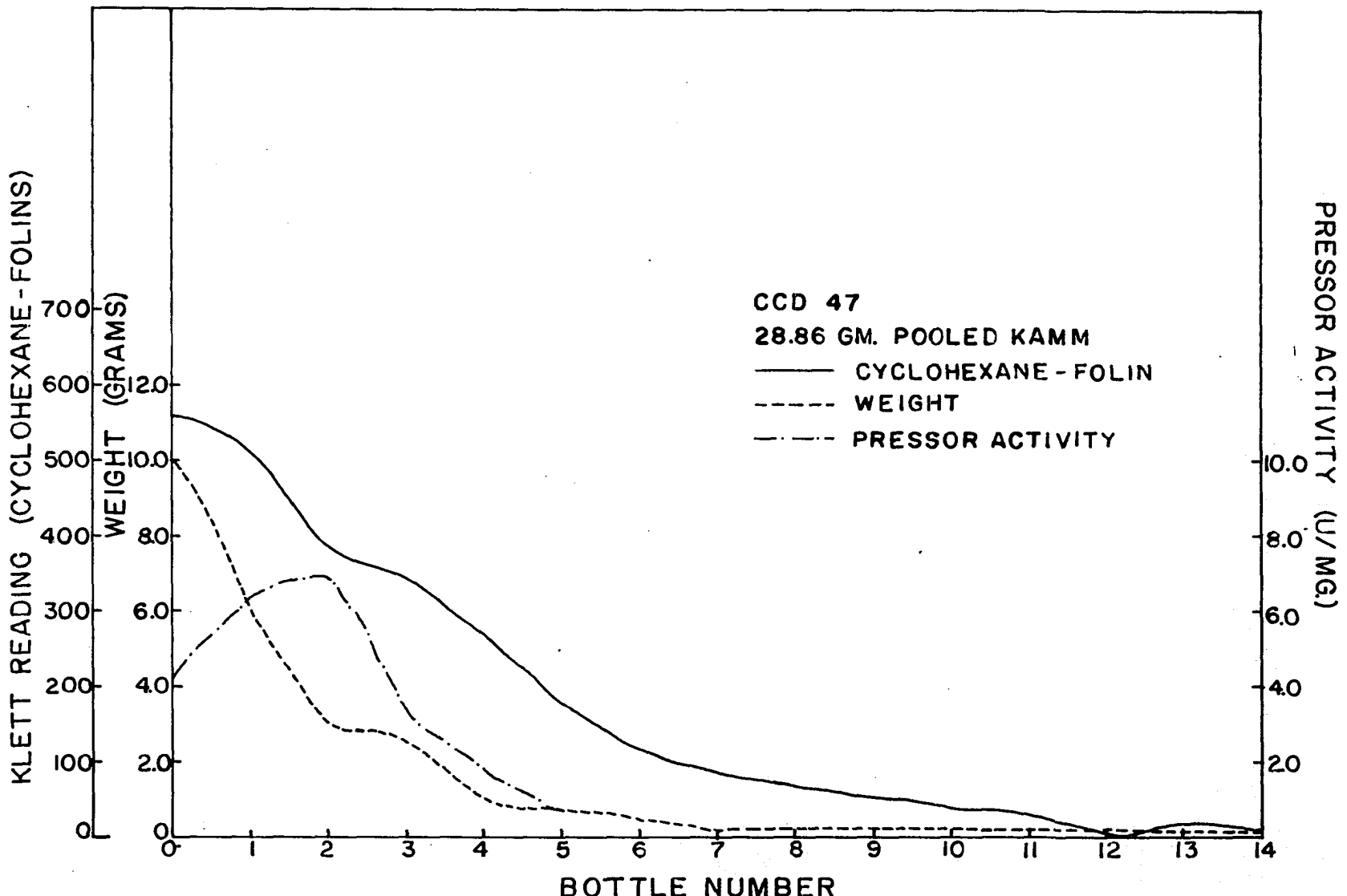


Fig. 24. Distribution of Kamm product in BAW (4:1:5)

Table 23. Weight, pressor and paper chromatography results of CCD 47

Tube	Wt. g.	LVP U/mg.	R_F's (BAW 4:1:5)
0	10.0347	4.1	0^{+4} lt. streaking to 0.15
1	6.1813	6.3	0^{+4}, 0.07^{+2}, 0.10^{+1}, 0.15^{+1} lt. streaking above to 0.34
2	2.9624	6.85	0.02^{+4}, 0.08^{+2}, 0.13^{+2}, streak to 0.27^{+3}, 0.31^{+3}, 0.37^{+} lt. streak beyond
3	2.5040	3.3	0.03^{+3}, 0.07^{+2}, 0.11^{+2}, 0.15^{+2}, 0.19^{+3}, 0.23^{+3}, 0.28^{+4}, 0.32^{+3} streak to 0.557^{+}
4	1.1800	1.8	streaking from 0 to 0.40, lt. streaking to 0.57
5	0.7306	0.65	lt. streaking to 0.11^{+2}, 0.15^{+2}, 0.19^{+2}, streaking to 0.29^{+2}, 0.33^{+2}, 0.37^{+2}, 0.45^{+2}, 0.54^{+4}
6	0.4622		
7	0.1997		
8	0.2014		0.12^{+} streaking to 0.25^{+}, 0.37^{+3} streaking to 0.71^{+3}
9	0.1527		0.2 streaking, lt. streaking from 0.4 to 0.65^{+}
10	0.1202		0.17^{+}, lt. streaking to 0.61^{+3}, streaking to 0.796^{+2}, streaking to 0.89
11	0.0918		0.17^{+}, v.lt. streaking to 0.61^{+}, 0.80^{+2}, 0.89^{+}
12	0.1145		0.17^{+}, v.lt. streaking to 0.09 with no distinct spots
13	0.0998		lt. ninhydrin pos. mat. 0.75 to 0.93
14	0.0895		"

sodium hydroxide and the cyclohexane procedure. The color was too intense for readings so further dilution with 5 ml. of water was necessary. The upper phase was removed on the rotary evaporator and the remaining solvent removed by lyophilization in 3 l. round-bottom flasks which were refrozen when melting occurred. Fig. 24 also shows the weight and pressor assay curve. The amber colored material moved with a high K value which was not reflected by the Folin or weight distribution curves.

The higher number tubes (9-14) contained very little ninhydrin positive material and, as also indicated by the Folin analysis, probably were not very rich in peptides. Weight recovery in this experiment was 87% and pressor recovery was 91%.

Countercurrent distribution of large scale CCD fractions (CCD 48, 49, 50 and 51)

The 54 tube, 30 ml. per phase, manually operated machine was employed, using equal volumes of freshly distilled sec-butanol and 0.5% trichloroacetic acid, equilibrated in the cold room, 3-5°C. Seventy-five oscillations were employed with a 15 min. settling time. Folin analysis was conducted on 0.5 ml. of each phase using the cyclohexane modification (10) and 0.25 ml. of each phase was used for the ninhydrin analysis. Further dilution of ninhydrin solution was made when necessary. Results are shown in Fig. 25, A, B, C and D.

Fig. 25. Distributions of peaks 2 and 3 of CCD 47, 2^{o}BuOH-0.5% TCA, 3-5^{o}C

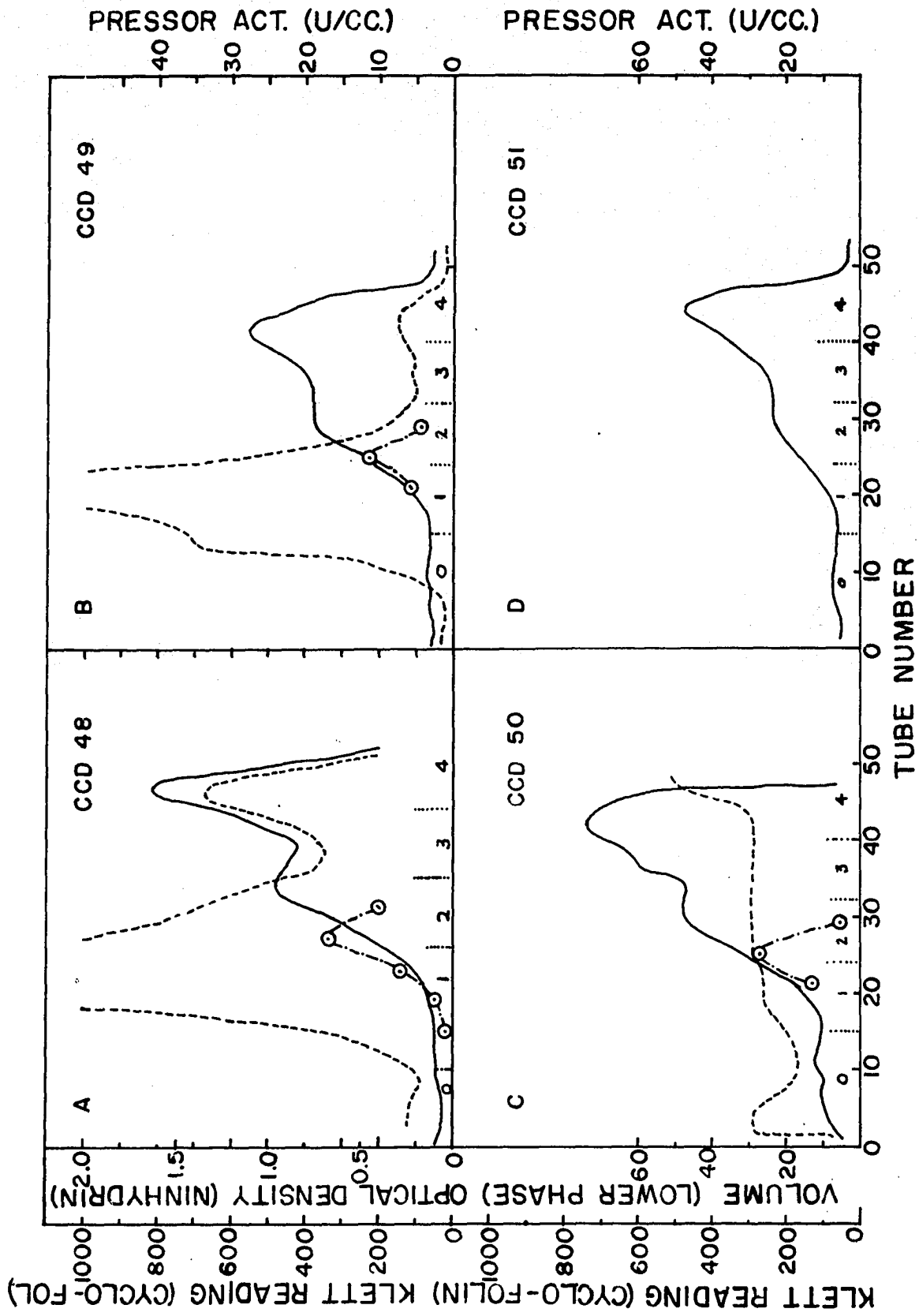

PRESSOR ACT. (U/CC.)
PRESSOR ACT. (U/CC.)
CCD 49
CCD 51
CCD 48
CCD 50
A
B
C
D
KLETT READING (CYCLO-FOLIN) KLETT READING (CYCLO-FOL)
VOLUME (LOWER PHASE) OPTICAL DENSITY (NINHYDRIN)
TUBE NUMBER

Table 24. Weight, paper chromatography and pressor assay results obtained from CCD 48, 49, 50 and 51

Peak	Tubes	Wt.(g)	R_F's (BAW 4:1:5)
	CCD 48 (Fig. 25A) 1.5 g. peak 3 of CCD 47, 56 transfers		
0	0.10	Lost	Large area at 0.0 to 0.1, weakly ninhydrin positive
1	11-26	0.2510	--
2	27-35	0.3143	0.05^{+1}, 0.11^{+2}, 0.15^{+3}, 0.19^{+2}, 0.28^{+1}, 0.4^{+1}, 0.67^{+2}
3	36-44	0.2972	streaking from 0 to 0.4, 0.65, 0.75
4	45-53	0.2957	--
	Pressor: tube 15 = 1, 19 = 2.2, 23 = 7, 27 = 16.7, 31 = 10 U/cc.		
	CCD 49 (Fig. 25B) 0.9473 g. peak 3 of CCD 47, 0.5559, peak 2 of CCD 47, 52 transfers		
0	0-15	0.0595	same as 48
1	16-23	0 2539	0.07^{+2}, 0.13^{+3}, 0.2^{+2} lt. streaking to 0.64^{+2}
2	24-32	0.3078	0.15^{+1}, 0.19^{+2}, 0 22^{+3}, 0.25^{+2}, 0.31^{+1}, 0 60^{+1}, 0.67^{+1}
3	33-40	0.2761	0.03^{+}, 0.08^{+2}, 0.13^{+3}, 0.15^{+3}, 0.19^{+3}, 0.22^{+3}, 0.25^{+2}, 0.28^{+1}, 0.31^{+1}, 0.57^{+}, 0.61^{+}
4	41-52	0.2571	streaking to 0.22.
	Pressor: tube 21 = 5.9, 25 = 13.4, 29 = 4.0 U/cc.		
	CCD 50 (Fig. 25C) 1.5 g. peak 2 of CCD 47, 49 transfers		
0	0-15	0.1743	same as 48
1	16-23	0 3377	same as 49
2	24-32	0.3479	--

Table 24. (Continued)

Peak	Tubes	Wt.(g.)	R_F's (BAW 4:1:5)
3	33-40	0.3207	0.03, 0.06, 0.10, 0.16, 0.21
4	41-53		streaking to 0.1^{+4}, 0.13^{+4}, 0.15^{+3}, 0.22^{+3}, 0.25^{+2}, 0.29^{+2} streaking to 0.43
	Pressor: tube 21 = 13.5, 25 = 27.4, 29 = 5.1 U/cc. CCD 51 (Fig. 25D) 1 g. peak 2 of CCD 47, 53 transfers		
0	0-15	0.0649	same as 48
1	16-23	0.2324	same as 49
2	24-32	0.1859	streaking from 0 to 0.7, predominant spot 0.22
3	33-40	0.1472	streaking from 0 to 0.26, 0.6
4	41-53	0.1900	--
	No pressor assays conducted		

The pressor area was located in CCD 48, 59 and 50 by assay of the indicated tubes.

A CRF assay of CCD 48, peak 2 gave a 320% increase in blood steroids at 3.0 γ (29 mU pressor act.) and 25% increase at 0.75 γ (7.3 mU pressor act.).

The K value for lysine vasopressin in this system is 0.37; the three distributions assayed gave values of 1.0 (CCD 48), 0.926 (CCD 49) and 0.89 (CCD 50). The fact that these distribution coefficients are higher than the reported

value may be due in part to the temperature difference. However, during the run, in each case, it was noted that there was a loss of lower phase in the second decade of tubes which appeared to have a symmetrical pattern. Fig. 25C shows a graph of this phenomena, which occurred in the area of heavy ninhydrin color. Peak O in every experiment gave a spot on paper chromatography similar to that obtained for ammonium sulfate in the BAW (4:1:5) solvent system. Peak O also gave a white precipitate on addition of barium chloride and gave off ammonia readily when heated with 6 N sodium hydroxide. This evidence hinted strongly at the presence of ammonium sulfate in the sample which was introduced in the Kamm procedure. Decrease in solubility of the organic phase by excess salt concentration could explain the curious displacement of lower phase in these experiments. This salt effect may have been largely responsible for the larger partition coefficient found for lysine vasopressin.

Continuous electrophoresis of countercurrent distribution fractions (Ce 225 and Ce 226)

Experimental conditions were identical to those reported for Ce 208-2, 3. Results are given in Tables 25 and 26 and shown in Fig. 26. Samples were material from fraction 2, CCD 48, 49, 50 and 51; Ce 225 was run on 0.6108 g. and Ce 226 on 0.5338 g., respectively.

Table 25. Weight, pressor, CRF and ACTH assay results of fractions from Ce 225

Fraction	Wt. g.	LVP U/mg.	γ/inj.	LVP mU/ inj.	B 100 ml.	B control	B inc.	CeU per inj.	CeU per	% inc.
11-12	36.4	2.0	15	30	26.6±2.8(4)	6.25±1.5(4)	20.35	5.5	0.367	330
13-14	63.3	17.4	1.8	31	15.3±1.8(5)	"	9.05	1.85	1.0	145
15-16	49.4	50.0	0.6	30	15.45±2.3(5)	"	9.20	1.87	3.12	150
17-18	39.3	12.4	2.4	30	22.2±2.3(5)	6.2±0.5(5)	16.0	3.6	1.5	260
19-20	40.0	1.6	18	29	17.6±0.7(5)	"	11.4	2.3	0.128	180
21-22	46.4	1.4	21	29	16.2±2.7(5)	"	10.0	2.03	0.097	160
23-24	31.5	1.1	27	31	19.6±2.2(4)	5.3±0.5(4)	14.3	3.08	0.114	270
25-26	22.8	0.8	36	29	18.2±1.4(5)	"	12.9	2.67	0.0741	240
27-28		1.2	24	29	13.3±1.7(5)	"	8.0	1.7	0.0709	150

Table 26. Weight and pressor assay results of Ce 226 fractions

Fraction	Wt. (mg.)	Pressor	Fraction	Wt. (mg.)	Pressor
11-12	57.7	4.8	1-2	--	
13-14	54.3	47.8	3-4	12.2	
15-16	43.1	76.0	5-6	21.2	
17-18	32.5	10.1	7-8	25.2	0.04
19-20	29.5	0.13	9-10	47.3	0.10
21-22	33.8	1.1	25-26	19.4	
23-24	27.6		27-28	16.8	Combined 1.2
			29-30	19.9	

In each case, the pressor peak occurred at fraction 15-16 coinciding very closely with the CRF activity. Using the assayed value of 9.7 U/mg. for CCD 48 peak 2 and the calculated value of 10.7 U/mg. for CCD 49 peak 2, the total amount of pressor activity in Ce 225 amounted to 6228 units. The recovery as assayed equaled 4274 units. The pressor activity recovery was thus 68% in comparison with a weight recovery of 771.9 mg. or 67% on the combined Ce 225 and Ce 226 experiments.

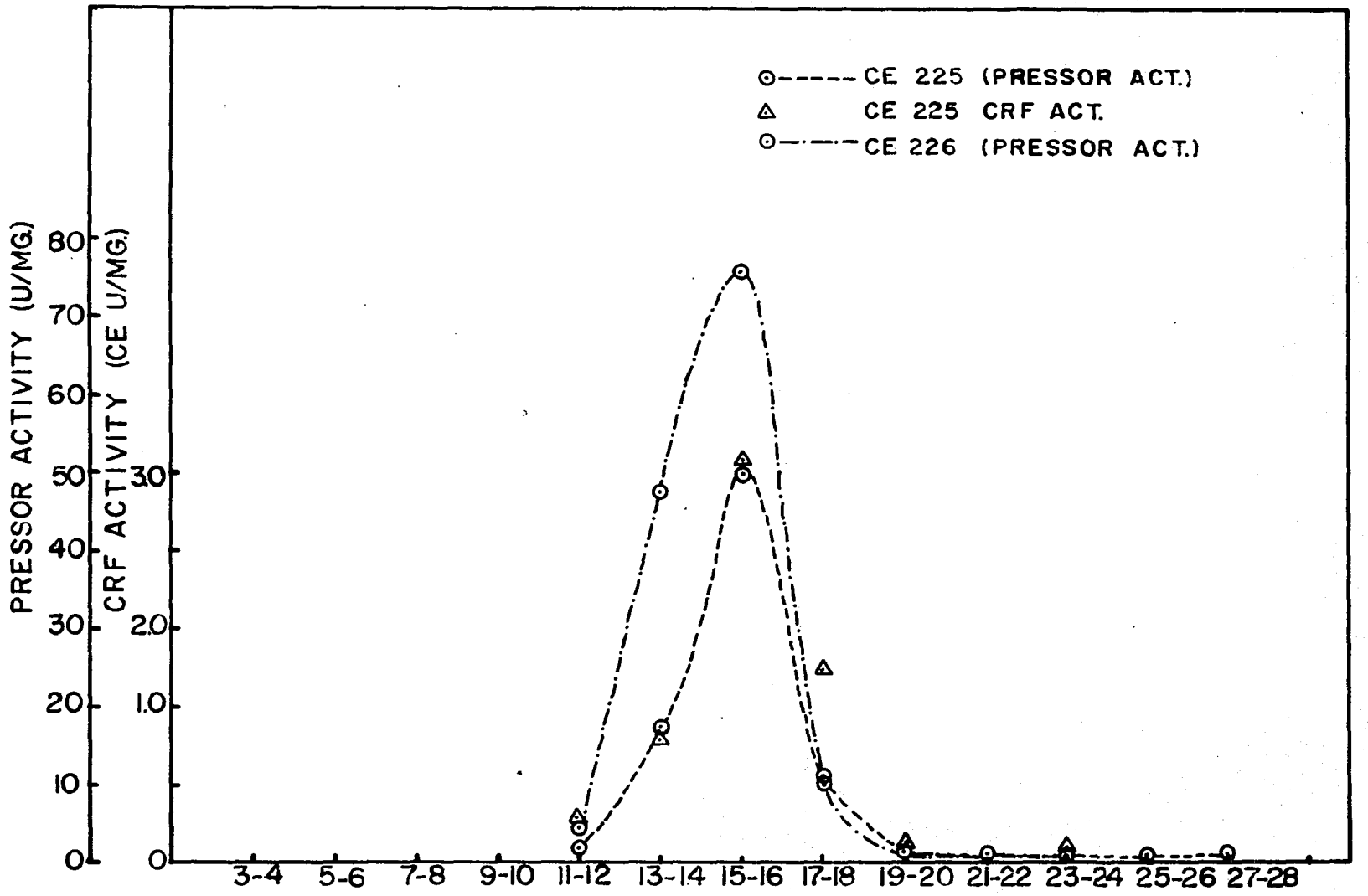


Fig. 26. Continuous electrophoresis of peaks 2 from CCD 48, 49, 50 and 51

Paper chromatograph of continuous electrophoresis sample (PC-120)

A 10 mg. sample of Ce 225, 17-18 (LVP 12.4 U/mg.) was streaked along a 19 cm. line of an EDTA-washed Whatman No. 1 paper measuring 22 cm. x 44 cm. The paper was developed in the cold room (3-5°C) for 36 hours in BAW (4:1:5) solvent which ascended to 31.8 cm. origin. After being allowed to dry in the cold room for 8 hours, a 2 cm. center strip and 1 inch strips from each edge were cut and sprayed with ninhydrin. Ninhydrin positive material had skewed upwards from left to right. The ninhydrin stained test strips were replaced and taped to the original chromatogram and a mark was placed 1 cm. from the colored area on all three strips. A line was drawn at 4 cm. connecting these points of equal R_F and segments 3 mm. in width were marked and cut crosswise to a height of 16 cm. These segments were cut into approximately 3 mm. squares and the resulting "confetti" placed into 4 inch lipless test tubes. The paper in each tube was extracted twice with 20 ml. of 1% cold acetic acid solution. It was found to be unnecessary to centrifuge the paper if the pipetting was conducted carefully. One milliliter aliquots were taken for the Folin analysis and 0.5 ml. samples for the ninhydrin analysis reported in Fig. 27. Table 27 gives a summary of the pressor and CRF assays conducted (6/10/60).

The ninhydrin and Folin curves appear to accurately

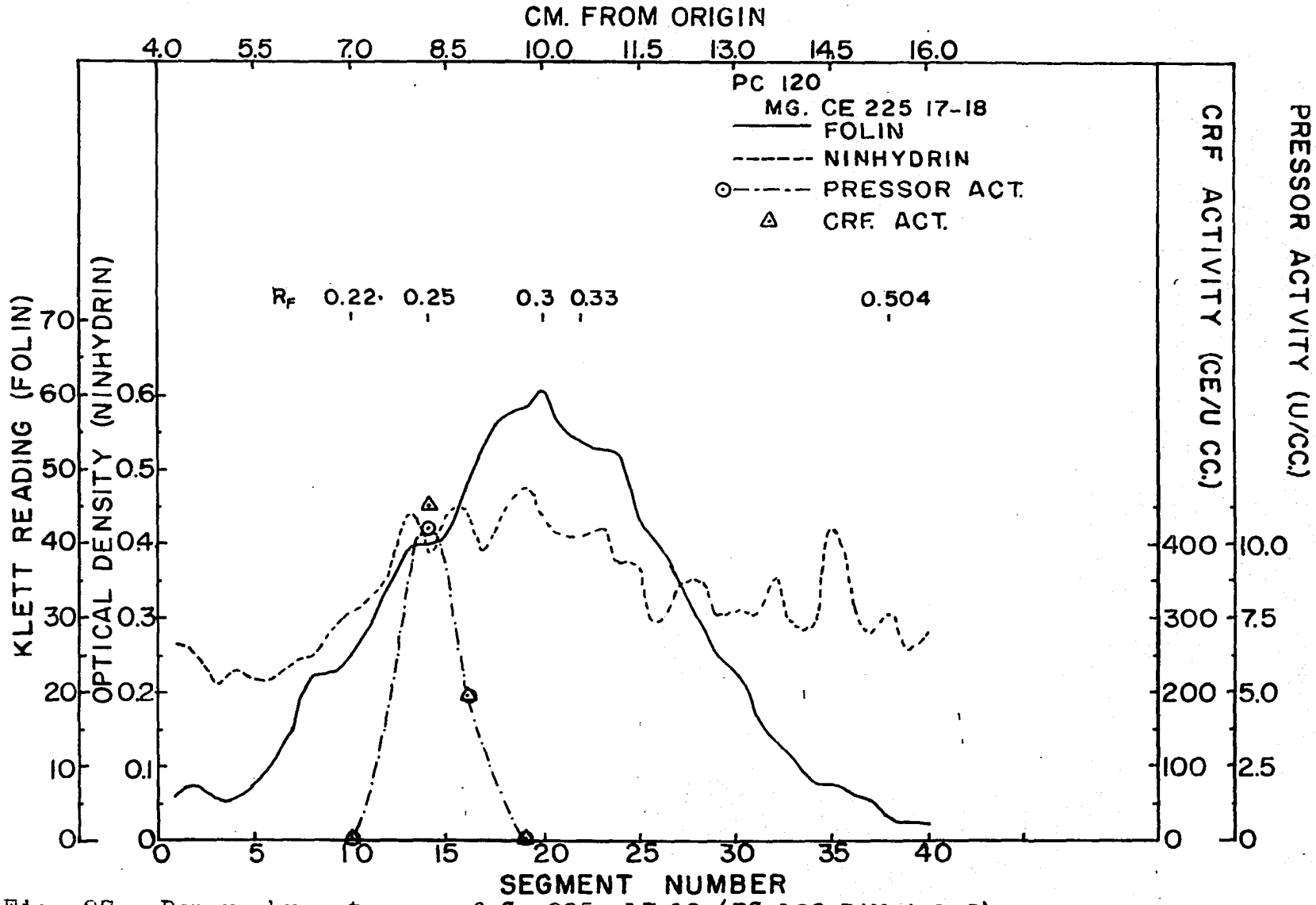


Fig. 27. Paper chromatogram of Ce 225, 17-18 (PC 120 BAW 4:1:5)

Table 27. Pressor and CRF assay results of selected tubes of PC 121

Tube	LVP U/cc.	LVP mU/ inj.	B control	B sample	ΔB	% inc.	CeU per ml.
10	0.082	16.4	7.3±0.5(5)	9.05±3.0(2)	1.75	25	4.6
14	10.5	25		10.8±0.4(5)	3.5	50	452
16	4.85	23		9.15±0.4(5)	1.85	25	195
19	0.056	14		7.5±0.7(3)	0.2	3	3.0

reflect the information contained on the ninhydrin stained center and side strips. There are at least eight ninhydrin positive components in the area depicted in Fig. 27 with another component at 22.5 cm. (R_F 0.708). The R_F value for lysine vasopressin in this experiment was 0.25. This is higher than the 0.12 reported by Rumsfield and Porter (99) and the 0.11 by Acher *et al*. (7) for the n-butanol:acetic:acid: water (4:1:5) solvent system. Schally *et al*. (115) gave an R_F of 0.05-0.20 for CRF in this sytem. Assays show the CRF activity in this experiment to have a slightly higher R_F, coinciding with the pressor peak at 0.25.

A comparison of effects of pretreatment of the paper and of solvent temperature was conducted during some preliminary experiments performed prior to PC 120. In the first two papers an attempt was made to equilize the concentration of

Table 28. Effects of temperature and washing of paper on the mobility and R_F of solvent and Ce 225 (17-18)

Paper	Time hrs.	Ht. of solvent	Temp.	R_F
Untreated	15.5	24.4	25	0.05^{+}, 0.08^{+}, 0.13^{+3}, 0.15^{+2}, 0.18^{+3}, 0.22^{+3}, 0.25^{+4}, 0.29^{+3}, 0.32^{+2}, 0.38^{+2}, 0.41^{+2}, 0.53^{+}, 0.57^{+}
EDTA washed	15.5	30.5	25	0.049^{+}, 0.08^{+}, 0.14^{+2}, 0.16^{+3}, (streak) 0.21^{+4}, 0.24^{+3}, 0.27^{+3}, 0.31^{+3}, 0.35^{+2}, 0.39^{+2}, 0.43^{+2}, 0.61^{+}, 0.69^{+}
EDTA washed[a]	36	33.7	3-5	0.16^{+2}(streak, 0.21^{+2}(streak), 0.25^{+3}, 0.29^{+3}, 0.35^{+4}, 0.37^{+3}(streak), 0.39^{+3}, 0.43^{+2}, 0.46^{+2}, 0.65^{+}

[a]0.6 mg. Ce 225 (17-18) streaked on 2 cm. line. Results essentially that found in PC 120.

Ce 225 (17-18) on the paper by alternating the spotting from the same capillary pipette.

Reduction of the temperature slows the solvent ascent considerably without improving the resolution noticeably. In fact, the lower moving components are undetectable on the papers developed at 3-5°C. Washing of the papers caused a more rapid movement of the solvent with no apparent effect on the resolution of the many components found in this continuous electrophoresis fraction.

Ion exchange chromatography of continuous electrophoresis sample (IRC-50 19)

A glass tube 2.16 cm. in diameter was filled to a height of 52 cm. with IRC-50 cation exchange resin buffered with 0.5 M ammonium acetate at pH 6.41 (68). After packing the column was moved to the cold room. The column was charged with 47.5 mg. of Ce 225 (15-16), LVP 50 U/mg.; and 26.9 mg. of Ce 226 (15-16) LVP 76 U/mg. A fraction collector with a drop counter was used to collect the effluent flowing at a rate of approximately 1.5 ml./cm.^{2}/hr. Effluent volume varied from 1.8 to 4.59 ml. because of erratic behavior of the collector after tube 59. Most tube volumes were in the 2-3 ml. range.

Addition of 0.5 M ammonium hydroxide to the column occurred at tube 186, and the column continued until tube 306. pHydrion paper revealed a pH of 9 at that point and the column was stopped. No detectable folin color was encountered after tube 126 until tube 286 when a slight rise was detected just prior to the pH change.

The Folin analysis curve shown in Fig. 28 was obtained from 0.5 ml. of sample. One-tenth milliliter of sample was removed from tubes 82, 86, 91, 96 and 100 for pressor and CRF assays. Before the assays were completed, the tubes were pooled into their various peaks as indicated in Table 29.

Weight recovery from this column amounted to 67%; recovery of both pressor and CRF activity approximated 30%. Some

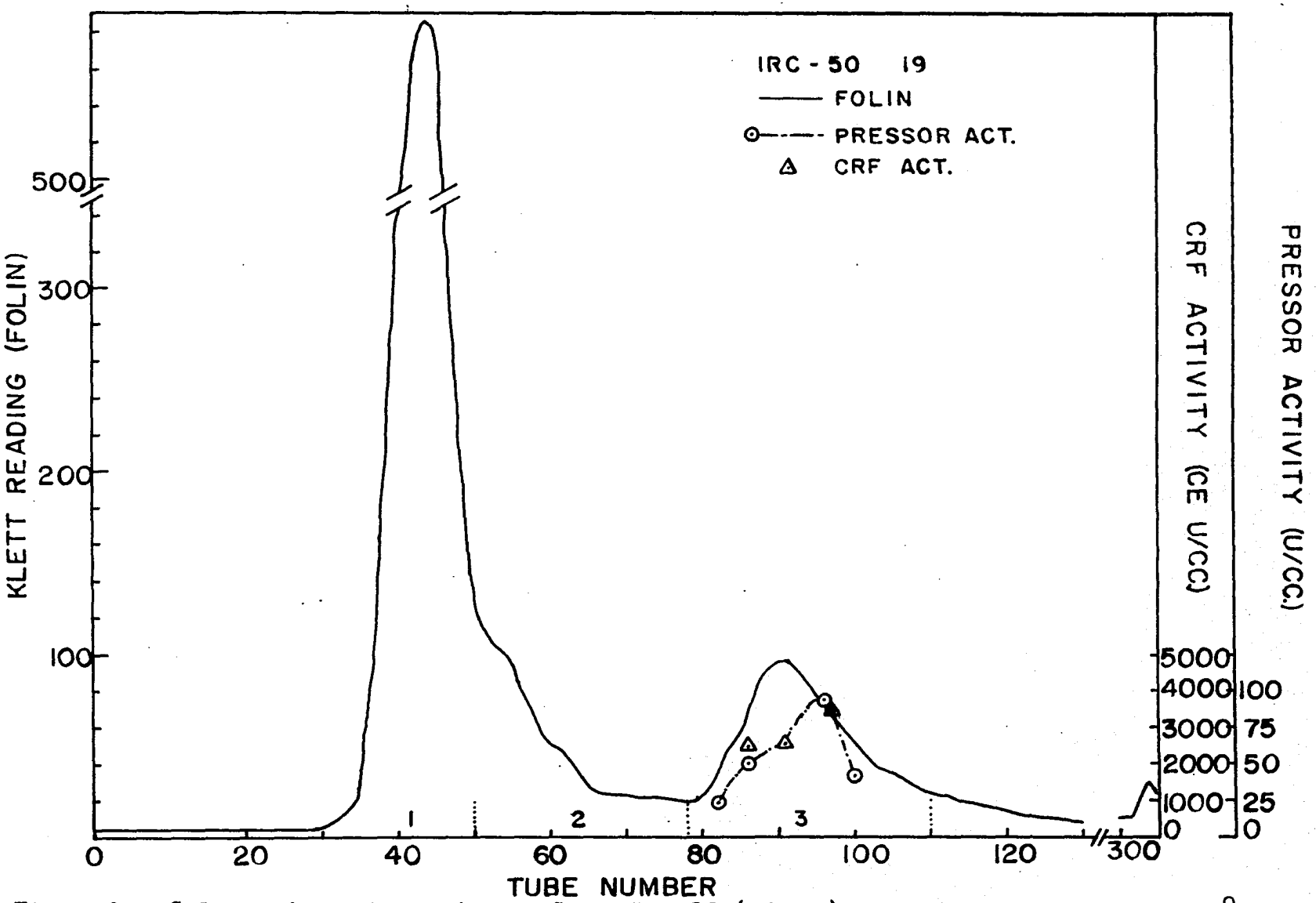


Fig. 28. Column chromatography of Ce 225, 226 (15-16) pH 6.41 0.5 M NH_4OAC 3-5°C

Table 29. Pressor and CRF assay results of tubes selected from IRC-50 19, peak 3; weight, pressor, MSH and CRF assay results of peaks from IRC-50 19

Tube or peak	LVP U/cc. or mg.	Wt.	MSH	LVP mU/ inj.	γ/ inj.	Control	B 100 ml.	ΔB	CeU per inj.	Dilu- tion factor	CeU/ cc. or γ	% inc.
82	24 U/cc.											
86	49 U/cc.			29		6.0± 0.9(4)	12.3± 2.0(4)	6.3	1.43	1650	2460	105
91	66 U/cc.			31.5		6.0± 0.9(4)	11.1± 1.2(4)	5.1	1.26	2060	2595	85
96	92 U/cc.			30		6.0± 0.9(4)	8.3± 2.8(4)	2.3	0.96	3003	2899	38
96	91 U/cc.			30		4.6± 0.4	9.0± 1.3(5)	4.4	1.18	3003	3543[a]	96
100	40											
1 32-50	.48 U/mg.	35.3	5+[b]	30	60	4.6± 0.6(5)	24.6± 1.3(5)	20.0	5.3		0.088	435
2 51-78	8 U/mg.	6.1		24	3	5.7± 0.8(4)	11.75± 0.3(3)	6.05	1.38		0.461	108
3 79-110	160 U/mg.	8.3	3-4[b]	27.2	.17	4.6± 0.6(5)	13.1± 2.5(5)	8.5	1.75		10.3	185

[a]Repeat experiments conducted giving better results.

[b]Inj. at 2.5γ.

inactivation may have occurred; it is interesting that both activities had the same percentage recovery within experimental error.

The vasopressin peak, peak 3, came off at 3.2 holdup volumes which is earlier than the 5 holdup volumes reported by Light et al. (68) on a column conducted at room temperature. They also reported pressor recovery yields of 75-100% of the starting activity using materials of much higher potency (210 U/mg.).

The R_F values shown in Table 30 were obtained for the three peaks in the BAW (4:1:5) solvent system. Calculation of the pressor recovery using the curve experimentally obtained by assay of the selected tubes would account for 85% of the starting activity. It is unknown why the actual recovery was only 30%.

Table 30. Paper chromatography results of peaks obtained from IRC-50 19

Peak	R_F's (BAW 4:1:5)
1	0.19, 0.23, 0.28 (trace amounts)
2	0.15^{+5}, 0.19^{+3}, 0.23^{+4}, 0.29^{+4}, 0.37^{+1}, 0.48^{+1}, 0.54^{+1}
3	0.12^{+1}, 0.19^{+3}, 0.25^{+4}, 0.31^{+2} (streaking)

Two dimensional "fingerprint" of IRC-50 19, peak 3

One-half milligram of IRC-50 19, peak 3 was spotted on a Whatman 3 mm. sheet marked as previously described. The solvent front reached 31 cm. Ninhydrin spray after electrophoresis at right angles revealed two positive areas. One faint spot measuring 1.5 cm. in diameter at an R_F of 0.155 and a distance of 6.9 cm. A double spot roughly five times as intense in ninhydrin color and measuring 4 cm. x 5 cm. was found at R_F 0.197 and 18.8 cm. This area was unusual in that the lower third in the electrophoresis direction gave a lighter somewhat yellowish spot.

Paper chromatography of IRC-50 19, peak 3 (PC 121)

A sample of 2 mg. of IRC-50 19, peak 3 was streaked on a 4 cm. line of an 8 cm. strip of Whatman 3 mm. paper. The developing solvent of acetone:water (3:2 v/v) was that of Schally et al. (115) with the modification that urea was excluded (108). At a temperature of 25-26°C in a period of 9 hours and 15 minutes, this solvent attained a height of 28.6 cm. The strip was dried in the cold room, then 1 cm. strips were cut crosswise for the entire length of the papergram after a 1 cm. test strip was cut lengthwise and sprayed with ninhydrin. The crosswise centimeter segments were cut into 1 cm. squares and extracted first with 2 ml. of 0.1%

acetic acid and then with 1 ml. of the same solvent. Fig. 29 shows the result of Folin analysis of 1 ml. samples and also two ninhydrin curves resulting from 0.5 ml. and 0.3 ml. samples. Also included are the results of the CRF and pressor assay reported in Table 31. The center strip shows a predominant ninhydrin-positive area at R_F 0.77 and a barely perceptible area at R_F 0.54. It will be noted that the biologically active areas coincide with the material analysis curves. A preliminary qualitative paper chromatogram run under the same conditions gave a large ninhydrin positive area at R_F 0.645 with some streaking beyond that and a faint spot at 0.47. Schally et al. (115) reported the R_F of lysine vasopressin in this system to be 0.69 and that of CRF at 0.35-0.46. Segment 14, corresponding to an R_F of 0.47, showed no CRF activity. Calculation of the pressor recovery from tubes 20 through 25 shows that 85% of the activity was lost. This loss was difficult to explain solely on the basis of mechanical loss of material. An experiment was conducted to investigate effects of acetone and temperature on pressor activity. A sample weighing 99γ was weighed into a vial and to it was added 1 ml. of acetone:water (3:2 v/v). This was allowed to remain at room temperature, 25°C, for 20 hours. Reassay of this material at dilutions where no solvent effects were encountered showed a loss of about 87% of the pressor activity. It is unknown at this time whether the urea employed by

Fig. 29. Paper chromatogram of IRC-50 19, peak 3 (PC 121 acetone:H_2O)

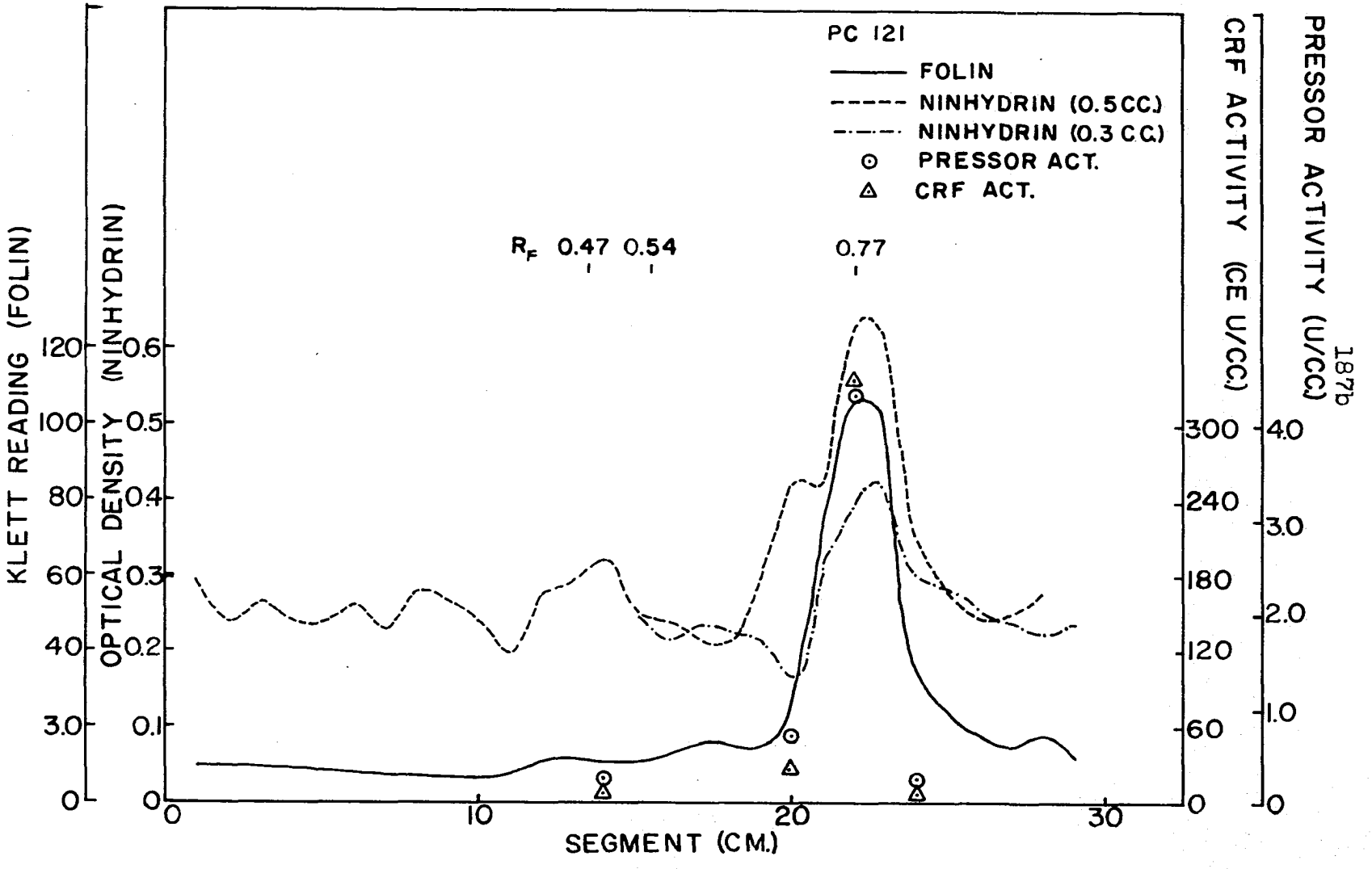

PC 121
FOLIN
NINHYDRIN (0.5CC.)
NINHYDRIN (0.3 C.C.)
PRESSOR ACT.
CRF ACT.
R_F 0.47 0.54
0.77
KLETT READING (FOLIN)
OPTICAL DENSITY (NINHYDRIN)
CRF ACTIVITY (CE U/CC.)
PRESSOR ACTIVITY (U/CC.)
SEGMENT (CM.)
0
10
20
30
120
100
80
60
40
30
0
0.6
0.5
0.4
0.3
0.2
0.1
0
300
240
180
120
60
0
4.0
3.0
2.0
1.0
0

Table 31. Pressor and CRF assay of tubes from PC 121

Tube	LVP U/cc.	LVP U/inj.	B 100 ml.	Control	ΔB	CeU/ inj.	Dilu- tion	CeU/ cc.	% inc.
14	0.2	17.1	8.4±0.8(5)	6.4±0.6(2)	2.0	0.96	11.55	11.1	31.3
20	0.7	30	12.25±0.9(4)	7.6±0.4(4)	4.65	1.2	23.1	27.8	61
22	4.3	30	18.0±2.5(4)	6.4±0.6(2)	11.6	2.35	142	333.0	181
24	0.2	30	11.8±1.6(3)	7.6±0.4(4)	4.2	1.15	6.6	7.6	55

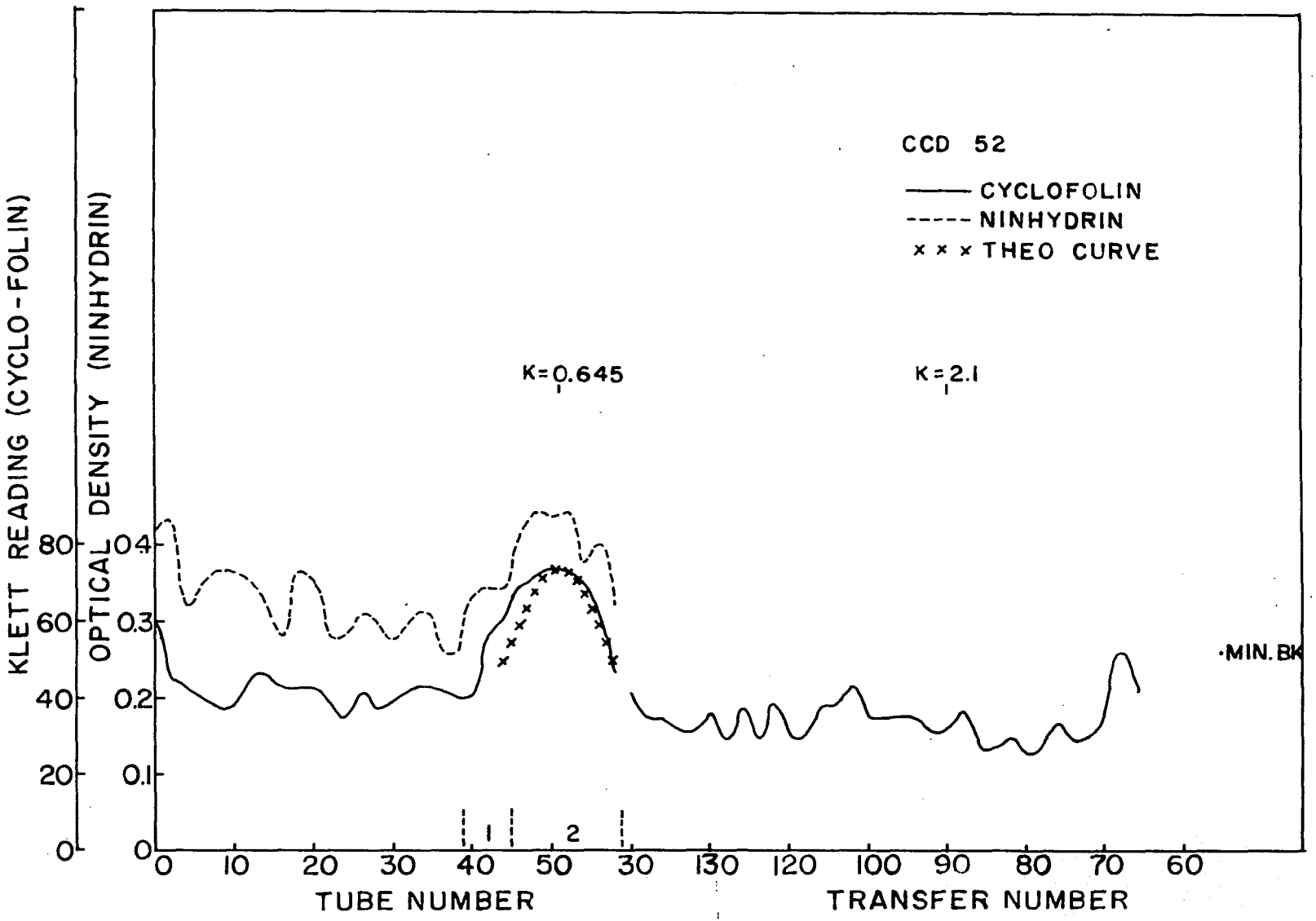
Schally (108) lends stability to this activity. Ressler (93) showed inactivation of oxytocin, a similar peptide to vasopressin, in 7 M urea and suggested this was due to hydrogen bond breaking in the molecule.

Calculation of the recovery of CRF activity showed a loss of approximately 86% resembling that of vasopressin very closely. The Folin color obtained is that to be expected from the amount of material placed on the paper.

Countercurrent distribution 52

A sample of 2.5 mg. of IRC-50 19, peak 3 was dissolved in the first tube of the 60 tube, 1 ml. all-glass "microdistribution machine" (10). The solvent system employed was 0.5% trichloroacetic acid equilibrated with an equal volume of freshly distilled sec-butanol at 3-5°C. The result of 130 transfers is given in Fig. 30. The Folin analysis reported was obtained from 0 5 cc. of each phase using the technique of Shepherd et al. (117). Addition of 0.5 cc. of lower phase to tubes 66 to 130 was used to give equivalent volumes. The ninhydrin curve was the result of using 0.6 cc. of the homogeneous contents of each tube obtained by rapid shaking and then applying the procedure of Stein and Moore (126) as modified by Hoften (131). Tubes 39, 43, 47, 51, 55 and 59 were set aside and 0.5 ml. of lower phase was taken for CRF and pressor assays. The results are shown in Table 32 and in

Fig. 30. Distribution of IRC-50 19, peak 3 in 2^{o}BuOH:0.5^{o}TCA ($3-5^{o}$C)


CCD 52
CYCLOFOLIN
NINHYDRIN
× × × THEO CURVE
K=0.645
K=2.1
KLETT READING (CYCLO-FOLIN)
OPTICAL DENSITY (NINHYDRIN)
0
20
40
60
80
0
0.1
0.2
0.3
0.4
1
2
0 10 20 30 40 50 30 130 120 100 90 80 70 60
TUBE NUMBER
TRANSFER NUMBER
·MIN. BK

1906

Table 32. CRF assays of CCD 52 (7/29/60 and 8/19/60)

Tube	B response	LVP mU/ inj.	LVP U/cc.	Control	ΔB	Dilu-tion factor	CeU/ inj.	CeU/ ml.	CRF/LVP
39			2.8						
43	12.0±1.65(3)	30	10.6	6.6[a]	5.4	347	1.3	451	.0434
47	12.7±1.3(4)	29	16	"	6.1	528	1.4	739	.0483
51	12.5±1.4(3)	28	22	"	5.7	777	1.36	1048	.0482
55	11.0±2.8(3)	30	15	"	4.4	508	1.18	600	.0393
59	11.6±0.6(4)	30	4.4	"	5.0	146.6	1.25	183	.0417
				Repeat 8/19/60					
43	14.1±2.4(3)	30		7.1±1.0(4)	7.0	347	1.52	528	.0507
47	19.5±2.3(3)	30		"	12.4	533	2.55	1360	.0851
51	17.9±2.4(4)	30		"	10.8	740	2.2	1626	.0734
55	17.6±4.7(4)	31		"	10.5	493	2.12	1045	.0684

[a]Not experimentally determined, calculated from three experiments preceding this one which had excellent precision.

Fig. 30 and Fig. 31. The calculated partition coefficient for vasopressin, taking tube 51 as the peak tube, is 0.646. This is higher than the 0.37 reported by Ward* at room temperature and obtained also in this laboratory using a sample supplied by him. Recovery of CRF calculated from the bioassay curve and correcting for the partition coefficient constant in assigning values for the upper phase (approximately 0.5) was 66% for the 7/29 data and 108% for the 8/19 data. Recovery of pressor activity using the same technique equalled 94%, showing little inactivation, if any, during the course of the experiment.

Tubes 39 to 45 were pooled and designated CCD 52, peak 1; tubes 46 to 59 were pooled and called CCD 52, peak 2. Material was recovered as previously described and lyophilized finally in glass vials. A pressor assay conducted on a weight of 91γ showed peak 2 to have an activity of 120-150 U/mg. This indicated that the maximum purity was equal to or greater than the original starting material, IRC-50 19, peak 3 (LVP = 160 U/mg.) since the weight of sample taken in the assay of peak 2 was so small and subject to possible error. The ninhydrin and Folin curves coincide fairly well with the CRF and pressor activity curves showing that negligible impurity could

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*Darrell Ward, M.D., Anderson Hospital of the University of Texas, Houston, Texas. Partition coefficient of lysine vasopressin in sec-butanol-0.5% trichloroacetic acid. Private communication. 1959.

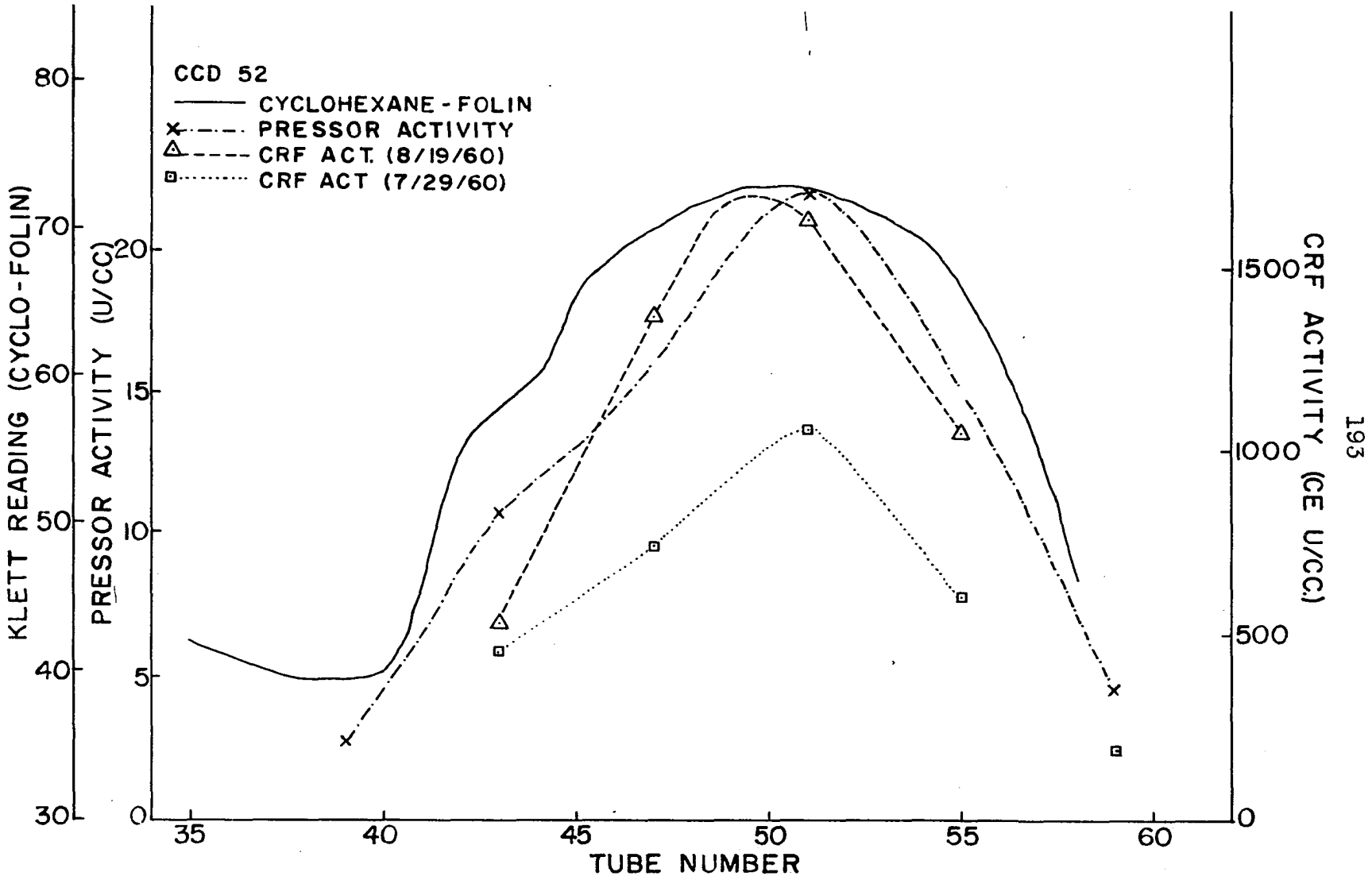


Fig. 31. CCD 52, peak 1 and 2 pressor and CRF assay results

have been present in this area and that the peak consisted almost entirely of CRF and LVP.

Amino acid analysis of CCD 52, peak 2

The hydrolysis technique was essentially that of Hirs et al. (51) with minor modifications. A sample weighing approximately 1 mg. was dissolved in 0.6 ml. of constant boiling hydrochloric acid contained in a 16 mm. x 150 mm. lipless Pyrex test tube. The sample was hygroscopic and so was weighed rapidly. The tube was immersed in a dry ice: acetone bath until the solution was frozen and then evacuated with an oil pump and sealed with an oxygen:gas hand torch. The tube was placed in a brass centrifuge cup in an oven at 100-110°C for 22 hours. When the cooled tube was opened an implosion occurred without loss of any of the light yellow solution, which was then evaporated to dryness over sodium hydroxide in a vacuum desiccator at room temperature over 43 hours. The hydrolysate was dissolved in 1.0 ml. of ion exchange water and the tube rinsed twice more with 0.5 ml. of ion exchange water. This solution of pH of 1.7 was titrated to a final pH of 6.21 with 6 N NaOH and permitted to stand unstoppered for 7 hours to oxidize the cysteine to cystine. Total volume was brought to 4 ml. and the pH to approximately 2 by addition of 2.0 ml. of 0.02 N HCL. The procedure used for analysis of the hydrolysate was that of Spackman et al.

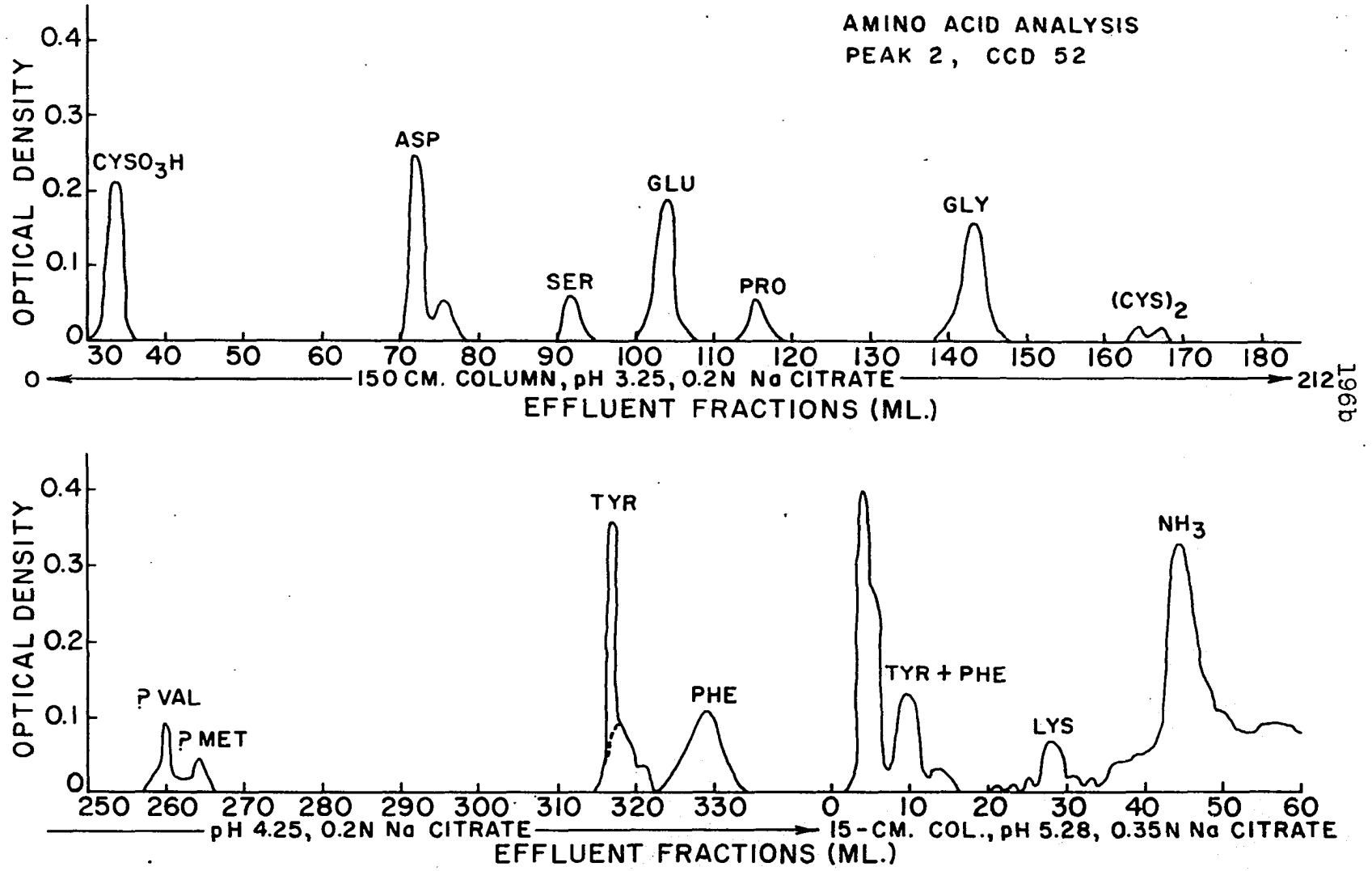
(123) using a fraction collector instead of the automatic recording apparatus. Results are shown in Table 33 and Fig. 33. The positive ninhydrin areas at tubes 190-200 and 257-261 cannot be explained since their effluent volumes do not agree with any usual hydrolytic products. It is to be noted that all of the amino acids identified are found in lysine vasopressin except serine (87). It is not known whether this is a free amino acid which came through the purification steps

Table 33. Amino acid content of peak 2, CCD 52

Amino acid	O.D.	μM leuc. equiv.	Color yield	μM A.A. sample	μM A.A. ml.
Cysteic acid	0.49	0.152	0.99	0.1535	0.1024
Aspartic acid	0.52	0.162	0.94	0.172	0.115
Serine	0.15	0.048	0.95	0.051	0.0406[a]
Glutamic acid	0.56	0.172	0.99	0.174	0.116
Proline	0.15	0.048	0.225	0.213	0.142
Glycine	0.62	0.192	0.95	0.202	0.135
Cystine	0.06	0.022	0.55	0.018	0.012
Tyrosine	0.32	0.100	1.00	0.100	0.0747[a]
Phenylalanine	0.59	0.184	1.00	0.184	0.1225
Lysine	0.21	0.066	1.10	0.060	0.12
Ammonia	1.096	0.34	0.97	0.35	0.70

[a]Corrected for hydrolytic decomposition (51).

Fig. 32. Spackman, Stein and Moore amino acid analysis of CCD 52, peak 2


AMINO ACID ANALYSIS
PEAK 2, CCD 52
OPTICAL DENSITY
0.4
0.3
0.2
0.1
0
CYSO3H
ASP
SER
GLU
PRO
GLY
(CYS)2
30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180
0
150 CM. COLUMN, pH 3.25, 0.2N Na CITRATE
212
EFFLUENT FRACTIONS (ML.)
1966
OPTICAL DENSITY
0.4
0.3
0.2
0.1
0
P VAL
P MET
TYR
PHE
TYR + PHE
LYS
NH3
250 260 270 280 290 300 310 320 330 0 10 20 30 40 50 60
pH 4.25, 0.2N Na CITRATE
15-CM. COL., pH 5.28, 0.35N Na CITRATE
EFFLUENT FRACTIONS (ML.)

or whether it is an integral part of another peptide present in amounts roughly equal to one-half the amount of the starting material on a molar basis. This is consistent with the pressor activity of the starting material, 160 U/mg., which show it to be approximately 57% vasopressin, assuming a value of 280 U/mg. for pure lysine vasopressin.

If this peptide is assigned a concentration of 0.04 μM per milliliter, then subtraction of this amount from the other amino acids will permit insufficient molar quantities of tyrosine and cystine to permit these amino acids to appear in the non-vasopressin peptide. The appearance of cysteic acid instead of cystine may be the result of oxidation by elemental chlorine in the constant boiling hydrochloric acid used in the hydrolysis which had been prepared almost a year prior to its use.* Disagreeable amounts of ammonia are ordinarily found in acids exposed to the laboratory air and it is extremely difficult to obtain ammonia-free hydrochloric acid. A correction for ammonia contamination is customarily applied.

Paper chromatograph of IRC-50 19, peak 3 (PC 122)

A sample of 0.25 mg. of IRC-50 19, peak 3 was pipetted

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*C. H. W. Hirs. Brookhaven National Laboratory, Long Island, N.Y. Effect of commercial reagent grade hydrochloric acid on amino acid analysis. Personal communication. 1960.

onto an 8 in. x 8 in. sheet of Whatman No. 1 chromatography paper at the intersection of two lines 1 inch from the left edge of the paper and 1.5 inches from the lower edge. The edge with the larger margin was stapled together so that the rust which develops after irrigation in BAW (4:1:5) can be cut off and still allow sufficient edge for irrigation of the chromatogram with m-cresol:water (95:5) in the other direction. Ninhydrin spray gave three spots at R_F's of 0.0^{+2}, 0.303, 0^{+3} and 0.35, solvent front (with tailing)$^{+4}$, for BAW (4:1:5) and m-cresol:water (95:5) respectively. A second papergram gave a similar result.

Since Schally et al. (115) reported that lysine vasopressin and corticotropin-releasing factor have R_F's of 0.85 and 0.46-0.64 in the m-cresol:water system, this gave a method for separating lysine vasopressin from the other components known to be present in IRC-50 19, peak 3. The remaining material in the vial containing IRC-50 19, peak 3 was dissolved in 50 λ of ion-exchange water and 40λ of this solution was streaked over a 1.5 cm. center area of an 8 in. x 8 in. Whatman No. 1 chromatogram. The chromatogram was developed in a solvent using freshly distilled m-cresol and water (95:5) in the cold room, 3-5°C. The remaining 10 λ which was unspotted was used for a pressor assay and gave an activity of 57.5U, thus the total units placed on the paper amounted to 230U. The chromatogram was removed after 13.5

hours with the solvent front at 10.7 cm. and allowed to remain hanging in the cold room for 3 additional hours. A 1 inch strip centered on the middle of the streaked area was cut out and divided into three areas corresponding to R_F's of 1 cm. below the origin to 0.33, 0.33 to 0.66, and 0.66 to 1 cm. above the solvent front. Each area was cut into segments of 1 square centimeter and extracted three times with 5 ml. portions of cold 0.25% acetic acid solution. The combined extracts were lyophilized and the flasks rinsed three times with 0.5 cc. portions of ion-exchange water. The combined rinses were centrifuged to remove the paper and lint and pipetted into vials and relyophilized. Results of the pressor and CRF assay are reported in Table 34.

Since 126 units of pressor activity were recovered, this amounted to 54.8% of the original activity. Inactivation, mechanical loss, or incomplete extraction are all possible explanations for this low recovery. The curious splitting

Table 34. Pressor and CRF assay results of fractions obtained from PC 122

Fraction	R_F	LVP U/cc.	LVP mU/inj.	B control	B sample	B	% inc.
1	0-0.33	83.8	10	4±0.6(3)	5.9±1.0(5)	1.86	46.5
2	0.33-0.66	12.2	10.2		8.3±1.0(4)	4.29	107
3	0.66-1.00	30	9.9		5.2±0.6(4)	1.26	30

of the pressor activity might be explained by the possibility of multiple spots occurring due to the lysine ϵ-amino and α-amino group of cysteine; lysine is known to form two spots under certain conditions (8). Another possibility might be overloading of the chromatogram, but a subsequent experiment showed that 1.0 mg. of a similar peptide fraction (LVP = 167 U/mg.) gave no indications of overloading under similar conditions.

This CRF assay may be inaccurate because of the unusually low controls encountered. If the controls were raised to 5.0, then the value for peak 2 would become a 66% increase; this is still considerably higher than the known CRF activity of 10 mU of lysine vasopressin in this assay (7% increase). The lowered values for peaks 1 and 3 would not be unreasonable with a control value of 5.

SUMMARY

1. A Kamm product (Pitressin Intermediate B) was shown to contain at least 30 ninhydrin positive components, presumably peptidic in nature.
2. An acetone extract of hypothalamic tissue was shown to contain at least eight ninhydrin positive components and an aqueous extract to contain 16 ninhydrin-staining components.
3. Chromatographic "Fraction D" was shown to contain pressor, MSH and CRF activity. Analysis of this mixture revealed the presence of 10 to 14 peptides ranging in size from 5 to 13 amino acids.
4. No CRF or MSH activity was obtained from cold or hot 0.25% acetic acid extracts of hypothalamic tissue at the levels tested.
5. Experiments (FD (12/14/59), CCD 44 and IRC-50 19, peak 1) indicated the presence of a CRF containing no pressor activity but possessing either inherent or contaminating MSH activity. This mixture of peptides gave a CRF activity at relatively higher doses.
6. A peptide fraction containing lysine vasopressin (160 U/mg.) showed strong CRF activity at doses of 0.17 μg.
7. The CRF activity contained in the purified pressor active fraction could not be separated from lysine vasopressin by column chromatography, paper chromatography or

countercurrent distribution.

8. The CRF activity of the purified pressor active fraction could be accounted for by the known CRF activity of synthetic lysine vasopressin.
9. Vasopressin is shown to fit the anatomical and physiological criteria necessary to the role of the neurohumoral mediator in neurotropic stress.
10. Therefore, vasopressin, because of its high ACTH-releasing activity and concentration, is concluded to be the most probable "physiological corticotropin-releasing factor".

BIBLIOGRAPHY

1. Abel, J. J. and M. C. Pincoffs, Proc. Natl. Acad. Sci. U.S. 3, 507 (1917).

2. Acher, Roger, Ann. Revs. Biochem. 21, 547 (1960).

3. Acher, Roger and Jacqueline Chauvet, Biochim. et Biophys. Acta. 12, 487 (1953).

4. Acher, Roger, Jacqueline Chauvet and Marie-Therese Lenci, Bull. Soc. Chim. Biol. 40, 2005 (1958).

5. Acher, Roger, Jacqueline Chauvet and Marie-Therese Lenci, Biochim. et Biophys. Acta 31, 545 (1959).

6. Acher, Roger, Jacqueline Chauvet and Marie-Therese Lenci, Compt. Rend. 248, 1435 (1959).

7. Acher, Roger, A. Light, V. du Vigneaud, J. Biol. Chem. 233, 116 (1958).

8. Aronoff, S., Science 110, 590 (1949).

9. Bargmann, W. and E. Scharrer, Am. Scientist 39, 255 (1951).

10. Bell, P. H., K. S. Howard, R. G. Shepherd, B. M. Finn and J. H. Meisenhelder, J. Am. Chem. Soc. 78, 5059 (1956).

11. Block, R. J., E. L. Durrum and G. Zweig. Manual of paper chromatography and paper electrophoresis. New York, N.Y., Academic Press, Inc. 1955.

12. Brown, R. A., M. Davies, M. Englert and H. R. Cox, J. Am. Chem. Soc. 78, 5077 (1956).

13. Casentini, S., A. De Poli, S. Hukovic and L. Martini, Endocrinology 64, 483 (1959).

14. Condliffe, P. G., J. Biol. Chem. 216, 455 (1955).

15. Craig, L. C. and O. Post, Anal. Chem. 21, 500 (1949).

16. Dale, Henry H. Evidence concerning the endocrine function of the neurohypophysis and its nervous control. In Heller, H., ed. The neurohypophysis. pp. 1-9. London, England, Butterworths Scientif Publications. 1957.

17. Daniels, P. M. and M. M. L. Pritchard, Quart. J. Exp. Physiol. 41, 215 (1956).

18. de Garihle, Privat, Claude Gros, Jacqueline Chauvet, Cl. Fromageot, C. Mialhe-Voloss and J. Benoit, Biochim. et Biophys. Acta. 29, 603 (1958).

19. de Garihle, Privat, Claude Gros, J. Porath and Eva-Brita Lindner, Experientia 16, 414 (1960).

20. Dekanski, J., Brit. J. Pharmacol. 7, 567 (1952).

21. de Wied, D. and I. Arthur Mirsky, Endocrinology 64, 955 (1959).

22. du Vigneaud, V., Inter. Cong. Biochem., Brussels, 1955. Proc. 3, 49 (1955).

23. du Vigneaud, V., D. T. Gish and P. G. Katsoyannis, J. Am. Chem. Soc. 76, 4751 (1954).

24. du Vigneaud, V., C. Ressler, J. M. Swan, C. W. Roberts, Panayotis G. Katsoyannis and S. Gordon, J. Am. Chem. Soc. 75, 4879 (1953).

25. du Vigneaud, V., R. R. Sealock and R. H. Sifferd with O. Kamm and I. W. Grote, J. Biol. Chem. 100, xciv (1933).

26. Fortier, Claude and Darrell N. Ward, Can. J. Biochem. and Physiol. 36, 111 (1958).

27. Geschwind, Irving I. Species variation in protein and polypeptide hormones. In Gorbman, Aubrey, ed. Comparative endocrinology. pp. 421-435. New York, N.Y., John Wiley and Sons, Inc. 1959.

28. Guillemin, R., G. W. Clayton, H. S. Lipscomb and J. D. Smith, J. Lab. Clin. Med. 53, 830 (1959).

29. Guillemin, R., G. W. Clayton, J. D. Smith and H. S. Lipscomb, Endocrinology 63, 349 (1958).

30. Guillemin, R., W. E. Dear, Buford Nichols, Jr. and H. S. Lipscomb, Proc. Soc. Exptl. Biol. Med. 101, 107 (1959).

31. Guillemin, R. and Claude Fortier, Trans. N. Y. Acad. Sci., 15, 138 (1953).

32. Guillemin, R. and W. R. Hearn, Proc. Soc. Exptl. Biol. Med. 89, 365 (1955).

33. Guillemin, R., W. R. Hearn, W. R. Cheek and D. E. Householder, Federation Proc. 15, 84 (1956).

34. Guillemin, R., W. R. Hearn, W. R. Cheek and D. E. Householder, Endocrinology 60, 488 (1957).

35. Guillemin, R., B. Nichols and H. Lipscomb, Compt. Rend. 247, 1662 (1958).

36. Guillemin, R. and A. V. Schally, Endocrinology 65, 555 (1959).

37. Guillemin, R., A. Schally, R. Anderson, H. Lipscomb and J. Long, Compt. Rend. 250, 4462 (1960).

38. Guillemin, R., A. V. Schally, R. N. Anderson and J. L. Long, Federation Proc. 19, 239 (1960).

39. Guillemin, Roger, Federation Proc. 14, 65 (1955).

40. Guillemin, Roger, Endocrinology 56, 248 (1955).

41. Guillemin, Roger, Endocrinology 63, 349 (1958).

42. Gros, C. and M. P. de Garihle, Compt. Rend. 249, 2234 (1959).

43. Hardy, T. L., D. O. Holland and J. H. C. Nayler, Anal. Chem. 27, 971 (1955).

44. Harris, G. W., Brit. Med. J. 1951, Part 2, 559 (1951).

45. Harris, G. W., Brit. Med. J. 1951, Part 2, 627 (1951).

46. Harris, J. I. and P. Roos, Biochem. J. 71, 434 (1959).

47. Hays, Edwin E. and Wilfrid F. White, Recent Progr. in Hormone Research 10, 265 (1954).

48. Hilton, J. G., L. F. Scian, Con D. Westermann, J. Nakano and O. R. Kruesi, Endocrinology 67, 298 (1960).

49. Hirs, C. H. W., S. Moore and W. H. Stein, J. Biol. Chem. 195, 677 (1952).

50. Hirs, C. H. W., S. Moore and W. H. Stein, J. Biol. Chem. 200, 493 (1953).

51. Hirs, C. H. W., W. H. Stein and S. Moore, J. Biol. Chem. 211, 941 (1954).

52. Holmes, R. L. and S. Zuckermann, J. of Anat. 93, 1 (1959).

53. Hume, David M., Annals of Surgery 138, 548 (1953).

54. Irving, G. W., Jr. and V. du Vigneadu, J. Biol. Chem. 123, 485 (1938).

55. Irving, G. W., Jr., H. M. Dyer and V. du Vigneaud, J. Am. Chem. Soc. 63, 503 (1941).

56. Ito, Shinji and Kazuko Machida, Seitai No Kagaku 5, 181 (1954). (Original not available; abstracted in Chemical Abstracts 51, 4572d (1957).)

57. Kamm, O., T. B. Aldrich, I. W. Grote, L. W. Rowe, E. P. Bugbee, J. Am. Chem. Soc. 50, 573 (1928).

58. Kappeler, H. and R. Schwyzer, Experientia 16, 415 (1960).

59. Katz, A. M., W. J. Dreyer and C. B. Anfinsen, J. Biol. Chem. 234, 2896 (1959).

60. Kwaan, H. C. and H. J. Bartlestone, Endocrinology 65, 982 (1959).

61. Landgrebe, F. W. and H. Waring. Biological assay of the melanocyte expanding hormone from the pituitary. In Emmens, C. W., ed. Hormone assay. pp. 141-171. New York, N.Y., Academic Press, Inc. 1950.

62. Lee, T. H., J. Biochem. 233, 917 (1958).

63. Lee, T. H. and A. B. Lerner, J. Biol. Chem. 221, 943 (1956).

64. Le Gros Clark, W. E. Morphological aspects of hypothalamus. In Le Gros Clark, W. E., J. Beattie, G. Riddoch and N. M. Dott, eds. The hypothalamus. pp. 1-68. Edinburgh, Oliver and Boyd Publishers. 1938.

65. Lerner, A. B. and T. H. Lee, J. Am. Chem. Soc. 77, 1066 (1955).

66. Li, C. H., Advances in Protein Chem. 11, 138 (1956).

67. Li, C. H. and H. M. Evans, The Hormones 1, 665 (1948).

68. Light, A., R. Acher and V. du Vigneaud, J. Biol. Chem. 228, 633 (1957).

69. Long, C. N. H., Ann. Rev. Physiol. 18, 409 (1956).

70. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall, J. Biól. Chem. 193, 265 (1951).

71. Martini, L. and C. Morpurgo, Nature 175, 1127 (1955).

72. McCann, S. M. and J. R. Brobeck, Proc. Soc. Exptl. Biol. Med. 87, 318 (1954).

73. McCann, S. M. and A. Fruit, Proc. Soc. Exptl. Biol. Med. 96, 566 (1957).

74. McCann, S. M. and P. Haberland, Proc. Soc. Exptl. Biol. Med. 102, 319 (1959).

75. McDonald, R. K., H. W. Wagner and V. K. Weise, Proc. Soc. Exptl. Biol. Med. 96, 652 (1957).

76. McDonald, R. K. and V. K. Weise, Proc. Soc. Exptl. Biol. Med. 92, 481 (1956).

77. McDonald, R. K., V. K. Weise and R. W. Patrick, Proc. Soc. Exptl. Biol. Med. 93, 348 (1956).

78. Metuzals, J., J. Comp. Neurol. 113, 103 (1959).

79. Miller, J. H. and M. D. Bogdonoff, J. Appl. Physiol. 6, 509 (1954).

80. Mirsky, I. A., M. Stein and G. Paulisch, Endocrinology 55, 28 (1954).

81. Moore, Stanford and William H. Stein, Advances in Protein Chem. 11, 191 (1956).

82. Mote, John R., The physiological basis for the action of ACTH in human beings. Chicago 11, Illinois, The Armour Laboratories. 1951.

83. Nagareda, C. Susan and R. Gaunt, Endocrinology 48, 560 (1951).

84. Nichols, Buford, Jr. and R. Guillemin, Endocrinology 64, 914 (1959).

85. Peterson, E. A. and H. A. Sober, J. Am. Chem. Soc. 78, 751 (1956).

86. Pfuderer, A. P. Structural studies on the β-lactoglobulins. Unpublished Ph.D. Thesis. Ames, Iowa, Library, Iowa State University of Science and Technology. 1961.

87. Popenoe, E. A., H. Claire Lawler and V. du Vigneaud, J. Am. Chem. Soc. 74, 3713 (1952).

88. Popenoe, E. A. and V. du Vigneaud, J. Biol. Chem. 205, 133 (1953).

89. Porter, J. C. and J. C. Jones, Endocrinology 58, 62 (1956).

90. Porter, J. C. and H. W. Rumsfield, Jr., Endocrinology 58, 359 (1956).

91. Porter, J. C. and H. W. Rumsfield, Jr., Endocrinology 64, 948 (1959).

92. Ramachandran, L. K. and T. Winnick, Biochim. et Biophys. Acta. 23, 533 (1957).

93. Ressler, C., Science 128, 1281 (1958).

94. Roberts, S., Ciba Colloquia on Endocrinology 11, 167 (1957).

95. Royce, P. C. and G. Sayers, Proc. Soc. Exptl. Biol. Med. 98, 70 (1958).

96. Royce, D. C. and G. Sayers, Proc. Soc. Exptl. Biol. Med. 98, 676 (1958).

97. Royce, P. C. and G. Sayers, Federation Proc. 18, 132 (1959).

98. Royce, P. C. and G. Sayers, Proc. Soc. Exptl. Biol. Med. 103, 447 (1960).

99. Rumsfield, H. W., Jr. and J. C. Porter, Arch. Biochem. Biophys. 82, 475 (1959).

100. Rumsfield, H. W., Jr. and J. C. Porter, Endocrinology 64, 942 (1959).

101. Sachs, Howard, Biochim. et Biophys. Acta. 34, 572 (1959).

102. Sachs, Howard, J. of Neurochem. 5, 297 (1960).

103. Saffran, M. and A. V. Schally, Can. J. Biochem. and Physiol. 33, 408 (1955).

104. Saffran, M. and A. V. Schally, Endocrinology 56, 523 (1955).

105. Saffran, M., A. V. Schally and B. G. Benfey, Endocrinology 57, 439 (1955).

106. Sayers, G., Physiol. Revs. 30, 241 (1950).

107. Sayers, M. A., G. Sayers and L. Woodbury, Endocrinology 42, 379 (1948).

108. Schally, A. V. In vitro studies on the control of the release of ACTH. Unpublished Ph.D. Thesis. Montreal, Quebec, Library, Allan Memorial Institute of Psychiatry. 1957.

109. Schally, A. V., R. N. Anderson, H. S. Lipscomb, J. M. Long and R. Guillemin, Nature 188, 1192 (1960).

110. Schally, A. V., R. N. Anderson, J. M. Long and R. Guillemin, Proc. Soc. Exptl. Biol. Med. 104, 290 (1960).

111. Schally, A. V. and R. Guillemin, Proc. Soc. Exptl. Biol. Med. 100, 138 (1959).

112. Schally, A. V. and R. Guillemin, Texas Repts. Biol. Med. 18, 133 (1960).

113. Schally, A. V., W. Nelkin, H. S. Lipscomb, J. M. Long and R. Guillemin, Federation Proc. 20, 185 (1961).

114. Schally, A. V. and M. Saffran, Proc. Soc. Exptl. Biol. Med. 92, 636 (1956).

115. Schally, A. V., M. Saffran and B. Zimmermann, Biochem. J. 70, 97 (1958).

116. Scharrer, E. and B. Scharrer, Recent Progr. in Hormone Research 10, 183 (1954).

117. Shepherd, R. G., K. S. Howard, P. H. Bell, A. R. Cacciola, R. S. Child, M. C. Davies, J. P. English, B. M. Finn, J. H. Meisenhelder, A. W. Moyer and J. van der Scheer, J. Am. Chem. Soc. 78, 5051 (1956).

118. Shepherd, R. G., S. D. Wilson, K. S. Howard, P. H. Bell, D. S. Davies, S. B. Davis, E. A. Eigner and N. E. Shakespeare, J. Am. Chem. Soc. 78, 5067 (1956).

119. Shizume, K., A. B. Lerner and T. B. Fitzpatrick, Endocrinology 54, 553 (1954).

120. Sideman, M. and H. Sobel, Proc. Soc. Exptl. Biol. Med. 103, 274 (1960).

121. Slusher, M. A. and S. Roberts, Endocrinology 55, 245 (1954).

122. Smith, P. E., J. Am. Med. Assoc. 88, 158 (1927).

123. Spackman, D. H., W. H. Stein and S. Moore, Anal. Chem. 30, 1190 (1958).

124. Steelman, S. L. and R. Guillemin, Proc. Soc. Exptl. Biol. Med. 101, 600 (1959).

125. Stein, W. and S. Moore, J. Biol. Chem. 176, 367 (1948).

126. Stein, W. H. and S. Moore, J. Biol. Chem. 211, 907 (1954).

127. (Stormont, R. T.), J. Am. Med. Assoc. 147, 326 (1951).

128. Swingle, W. W., L. J. Brannick, A. F. Parlow and W. Barrett, Proc. Soc. Exptl. Biol. Med. 92, 107 (1956).

129. Thoma, J. A., H. B. Wright and D. French, Arch. Biochem. Biophys. 85, 452 (1959).

130. Tuppy, H., Biochim. et Biophys. Acta. 11, 449 (1953).

131. v. Horsten, B., Acta. Chem. Scand. 10, 1432 (1956).

132. Waring, H. and F. W. Landgrebe, The Hormones 2, 458 (1950).

133. Weber, E. J. and W. R. Hearn, Federation Proc. 20, 164 (1961).

134. Winnick, T., R. E. Winnick, R. Acher and C. Fromageot, Biochim. et Biophys. Acta. 18, 488 (1955).

135. Wirz, H. The location of antidiuretic action in the mammalian kidney. In Heller, H., ed. The neurohypophysis. pp. 157-169. London, England, Butterworths Scientific Publications. 1957.

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Very frequently during the course of this investigation, the problem took on the aspects of a rapidly revolving windmill. When we carefully plotted a stratagem to attack and subdue one arm, it would swiftly and silently move away leaving our flank exposed to the buffeting action of the second arm. Over the years, this quixotic tale repeated itself until, inevitably and inexorably, we yelled "enow" and laid down our broken lance and battered hides and wrote this chronicle in warning to others who pass this way.

Indeed if we are to liken this problem to that famous tale then the role of that slightly mad man, Don Quixote de La Mancha must be reluctantly assumed, although this author perhaps insults that fine gentleman by doing so. Presuming this then, to that Cervantes, Dr. W. R. Hearn, must go the blame for assembling the characters in this tragic comedy for it was he who created, inspired and supplied the materials necessary to the battle. To these characters, he also showed great love in their greatest folly and the understanding necessary of a creator toward his creation. For giving us our birth we give him our gratitude but in the true tradition of obstinancy reserved for offspring we reserve the right to mock him now and then although no sign of ammonium chloride shall make itself seen upon these pages.

In our tale, Rozinante becomes the plodding drudge-horse

of the imagination and the lance becomes the blunted probings of the mind while the rusted armor must be the corrosion of indolence.

Fortunately the author's faithful Sancho Panzo in the person of Frank Spellman far surpasses his fictional counterpart in that he possesses all of the virtues and very few of the deficiencies. The beloved Squire will never know the depth of gratitude and although the author never promised a governorship, he only wishes it were his to give.

Again, my Dulcinea del Toboso far surpassed the fictional attributes enumerated by the Don. Miss Evelyn J. Weber was all of those in reality and yet tempered her wisdom and exasperation with great kindness. For this the author is most thankful and can only quote gratefully for "her whole person without parallel". To Miss Weber's attendants, Mrs. Peggy Randolph and Mrs. Nancy Borks, the author wishes to add his heartfelt thanks.

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APPENDIX

Abbreviations

AAAD	Adrenal ascorbic acid depletion
ACTH	Adrenocorticotropic hormone
ADH	Antidiuretic hormone (vasopressin)
AVP	Arginine vasopressin
B	Corticosterone
BAW	Butanol:acetic acid:water
CCD	Countercurrent distribution
CeU	Continuous electrophoresis units for CRF (Ce 225, 13-14)
CMC	Carboxymethyl cellulose
CRF	Corticotropin (ACTH)-releasing factor
DOC or DOCA	Desoxycorticosterone or desoxycorticosterone acetate
EDTA	Ethylenediaminetetraacetic acid
F	17-Hydroxycorticosterone
γ	Gamma (microgram)
IRC-50	Amberlite carboxylic acid resin
λ	Lambda (microliter)
LVP	Lysine vasopressin
mγ	Milligamma (millimicrogram)
MSH	Melanocyte stimulating hormone
17-OHC	17-Hydroxycorticosterone

R_F	Ratio of the component mobility to the solvent front mobility
TCA	Trichloroacetic acid
U	Units of biological activity