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# Corticotropin-releasing activity of lysine vasopressin

Evelyn Joyce Weber
*Iowa State University*

# CORTICOTROPIN-RELEASING ACTIVITY OF LYSINE VASOPRESSIN

by

Evelyn Joyce Weber

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Head of Major Department

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Dean of Graduate College

Iowa State University
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# TABLE OF CONTENTS

# HISTORICAL

Some of the biological activities of the posterior pituitary hormone, vasopressin, have been known for many years. In 1859 Oliver and Schäfer (109) found that extracts of the whole pituitary gland were capable of increasing the blood pressure of a dog when injected intravenously. Three years later Howell (66) demonstrated that the substance responsible for this pressor action was obtainable only from the posterior lobe of the pituitary.

While studying the pressor action of posterior lobe extracts, Dale (23) in 1909 noted that these crude extracts also had a uterine contracting or oxytocic effect. The next year Ott and Scott (110) recognized a milk-ejecting action on the mammary gland by these extracts.

In 1912 Frank (45) postulated a relationship between the neurohypophysis and diabetes insipidus, a disease characterized by a large increase in urinary secretion. He believed that the disease was due to an excessive outpouring of a diuretic hormone from the pituitary. von den Velden (163) and Farmi (41) tried injections of posterior lobe extracts on cases of diabetes insipidus and were surprised to learn that these preparations had an antidiuretic effect and could be used successfully to treat the disease.

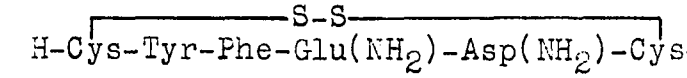
All of these biological activities were attributed to a single substance until 1928 when Kamm et al. (73) worked out

a separation scheme which gave two active fractions. These crude fractions were given the commercial names Pitressin and Pitocin. The vasopressin fraction exhibited most of the pressor and antidiuretic activities, and the oxytocin the uterine contracting and galactogogic effects.

Kamm and his coworkers achieved their separation by a hot dilute acetic acid extraction of acetone-dried posterior pituitary tissue and a series of solvent and salt precipitations. Little was known about the chemical structure of the active principles at that time except that they were basic and probably amines.

After further purification of the Kamm extract from beef pituitaries by electrophoresis (70) and countercurrent distribution (159), hydrolysis of the highly active vasopressin fraction revealed that it contained cystine, tyrosine, phenylalanine, glutamic acid, aspartic acid, proline, arginine and glycine in approximately equimolar ratios. Three moles of ammonia were also present.

Performic acid oxidation indicated that the disulfide linkage of cystine formed a cyclic structure (111). Amino acid sequence studies, utilizing the dinitrofluorobenzene method of Sanger (132), the stepwise degradation scheme of Edman (40) and trypsin cleavage, enabled Acher and Chauvet (1) and du Vigneaud *et al.* (38) in 1953 to propose a structure for arginine vasopressin:




The final proof of this structure came when du Vigneaud and his coworkers (36) synthesized arginine-vasopressin in 1954 and demonstrated that it possessed the same biological and chemical properties as the hormone isolated from beef pituitaries.

An interesting species difference was discovered when a purified vasopressin fraction from hog pituitaries was hydrolyzed. Lysine was found to have replaced arginine (112). du Vigneaud's group also synthesized the porcine hormone (38).

The syntheses of arginine and lysine vasopressins have made available hormones which are known to be free of pituitary contaminants having other biological activities. With synthetic vasopressin the biological activities intrinsic to this hormone can be investigated.

## Biological Activities of Vasopressin

### Pressor activity

The increase in blood pressure produced by injection of vasopressin is due mainly to the constriction of the arterioles or proximal parts of the capillaries (60). Immediately after the injection the heart beat becomes slightly slower, but the force of the beats of both the auricle and ventricle are augmented (109). Vasopressin seems to affect the

musculature of the heart and blood vessels directly because severing the spinal cord does not abolish the pressor action.

For many years vasopressin has been considered unimportant physiologically in the control of cardiovascular function, because it was necessary to inject large pharmacologic doses to demonstrate a blood pressure increase. In the dog 200 mU. of vasopressin had to be injected to produce a satisfactory pressor response although the antidiuretic action of as little as 0.25 mU. could be detected (160). Recently, by measuring both blood flow and pressure in the femoral artery, Potter and Sutfin (116) have shown that vasopressin injected intra-arterially in doses of 0.1 to 5 mU. induced vasoconstriction without significant elevation of blood pressure.

Kitchin (80) also observed a vasoconstriction after infusion of Pitressin when he studied blood flow in the human hand and forearm. An increase in capillary resistance was noted by Kramár et al. (81) in man after an intramuscular injection of 30 mU. of vasopressin although no antidiuretic activity could be demonstrated. At a higher dose of 1 U. both effects were observed. Vasopressin appears to be vasoactive as well as antidiuretic at low levels.

Pressor activity is usually assayed by directly cannulating the carotid artery and then measuring the rise in blood pressure following the intravenous injection of a sample. The assay animal frequently chosen is the anesthe-

tized rat. The method of Dekanski (29) utilizing the dibenamine-blocked rat will be discussed in detail later.

Antidiuretic activity

The antidiuretic effect of this hormone is illustrated by the decrease in urine volume which occurs after injection of vasopressin into a hydrated animal. Vasopressin increases the rate of water reabsorption in the kidney so that the osmotic pressure of the urine is raised above that of the plasma.

The role of the antidiuretic hormone in urinary electrolyte excretion has been very difficult to assess. Thorn (157) has reviewed the conflicting literature and discussed possible sources of variation in results.

The location of antidiuretic action in the nephron of the kidney has been studied by two different approaches. Direct information about the composition of the fluid in various parts of the nephron has been obtained by micropuncture of the tubules. Wirz (164) has perfected this technique. The indirect method is based on renal clearance studies, which measure the rates at which the kidney excretes various substances relative to their concentration in the plasma.

From the results of clearance experiments Smith (152) has proposed a widely accepted theory that there must be three separate sites and three different mechanisms of water

reabsorption in the mammalian kidney. In the proximal tubule there is an active reabsorption of the solutes and, to maintain an isoosmotic state, a passive reabsorption of the water. Eighty to 90 per cent of the filtered water is reabsorbed here. In the distal tubule there is a further active reabsorption of sodium, and if antidiuretic hormone is present, water also is reabsorbed. The maximum dose of vasopressin will promote water reabsorption until the distal tubule fluid is isotonic. Dilution of the urine will occur with lower doses. At the third site, water must be actively abstracted to concentrate the isotonic tubule fluid above the osmotic pressure of plasma. Smith believes that this process occurs in the collecting ducts.

Wirz has suggested another theory for concentration of the urine. He has proposed that there is a hair-pin countercurrent system in the loops of Henle. This countercurrent concentrating mechanism is initiated by the antidiuretic hormone when it opens the pores in the descending limb and allows water to pass out of the tubule.

Although the exact location of antidiuretic action is not clear, increasing membrane permeability appears to be the primary function of the hormone. In 1960 Schwartz and his coworkers in an interesting series of papers (43, 118, 146) suggested a scheme for a mechanism of action of vasopressin on membrane permeability at the molecular level. Their in-

vestigations of the interactions of tritiated vasopressin with rat kidney or toad bladder indicate that a covalent bond is formed between the hormone and the membrane proteins. A disulfide-thiol exchange reaction results in a hormone-receptor disulfide bond. This group proposed that following this initial reaction a series of disulfide-sulfhydryl reactions may alter the tertiary structure of the membrane proteins to such an extent that water and certain solutes can now easily pass through.

Evidence for the disulfide bond was substantiated by the release of 46-58 per cent of the bound vasopressin after treatment with cysteine, mercaptoethylamine or thioglycolate. Complete inhibition of vasopressin action occurred at high concentrations of the sulfhydryl reagents, N-ethyl-maleimide or methylmercuric bromide. This is the first identification of a physiologically significant chemical bond formed between a hormone and a target organ.

These workers also suggested that the very low activities of acetylated and iodinated vasopressins may be due to specific stereochemical and electrostatic requirements between the hormone and its receptor for the formation of the disulfide bond.

The substitution of lysine in porcine vasopressin for arginine has shown an interesting species difference in antidiuretic potency. In dogs van Dyke et al. (162) found that

1 U. of lysine vasopressin had only one-sixth the antidiuretic activity of 1 U. of arginine vasopressin. When lysine vasopressin was injected into rats, Sawyer (133) noted that the antidiuresis was as intense as with arginine vasopressin but the duration of the antidiuresis was less. In the pig, the porcine hormone lysine vasopressin had the same or perhaps even greater antidiuretic activity than arginine vasopressin (104).

Thorn (157) has written a critical evaluation of the procedures which have been used to measure antidiuretic activity. He concluded that intravenous injection of the sample into an anesthetized hydrated rat and measurement of the osmolality of the urine as the index of antidiuretic response gave the most reliable results.

Recently de Wied (30) devised an automatic feedback system that maintained the hydration of the rat at a rate identical to the rate of urine excretion. With this procedure there was little variation in baseline urine output and urine flow could be used as the index of antidiuretic activity. van Dyke et al. (160) preferred heavily hydrated, unanesthetized dogs.

Oxytocic activity

The structure of oxytocin is identical to that of arginine-vasopressin except that isoleucine and leucine have

replaced phenylalanine and arginine respectively. With this close chemical relationship it is not surprising that vasopressin has some oxytocic activity.

Fitzpatrick (42) has listed two effects of oxytocin on the uterus. In the non-pregnant female at the time of mating the uterine contractions propel the seminal fluid toward the Fallopian tubes. At parturition the contractions help to expel the foetus. In the mammary gland oxytocin exerts a milk ejecting effect by causing a contraction of the myoepithelia of the alveoli (22).

There are many oxytocin assay systems, such as the isolated rat uterus, chicken blood pressure, milk ejection pressure in the rabbit mammary gland, the cat uterus in situ and others (10). The relative oxytocic activity of vasopressin seems to depend on the assay procedure used. The following table compares the values obtained with highly purified arginine-vasopressin and oxytocin.

Table 1. Potencies of purified oxytocin and vasopressin (160)

| Assay | Oxytocin (U./mg.) | Vasopressin (U./mg.) |
|---|---|---|
| Isolated rat uterus | 500 | 30 |
| Avian depressor | 500 | 85 |
| Milk ejection in the rabbit | 500 | 100 |
| Rat pressor | 7 | 600 |

Corticotropin releasing activity

The existence of a hypothalamic neurohumoral mediator that regulates adrenocorticotropin (ACTH) release under conditions of stress was postulated by de Groot and Harris (27, 28) and independently by Hume (68). The experimental evidence from which de Groot and Harris formulated this theory was that electrical stimulation of the hypothalamus of a rabbit gave a reduction in the number of circulating lymphocytes similar in magnitude and time relation to that following an emotional stress stimulus or an injection of ACTH (27, 28). Hume (68) obtained similar results using unanesthetized dogs and eosinopenia as an index of release of ACTH from the anterior pituitary. These investigators and others (46, 97, 115) demonstrated that lesions in the hypothalamus inhibited stress-induced release of ACTH.

The posterior pituitary hormones, vasopressin and oxytocin, are elaborated by neurosecretory cells in the hypothalamus (7, 145). By suitable staining techniques (122) the neurosecretory material containing the hormones can be traced from its origin in the supraoptic and paraventricular nuclei of the hypothalamus down the axons of the hypothalamohypophysial tracts to the posterior pituitary. Here the hormones are stored until released. Recent evidence indicates that there are direct vascular connections from the posterior to the anterior lobe of the pituitary (24).

Lauber (84) demonstrated that there is a correlation after bilateral adrenalectomy in the rat between the release of the antidiuretic hormone (ADH) and the loss of stainable secretory material. She did this by comparing bioassays of plasma ADH levels with densitometric estimations of the neurosecretory material.

Mirsky et al. (103) also have presented evidence that vasopressin is formed in the hypothalamus rather than in the posterior pituitary. They found that hypophysectomized, adrenalectomized rats responded to noxious stimuli by a marked increase in plasma antidiuretic activity. Because both ADH release from the hypothalamus and ACTH release from the anterior pituitary occurred in response to adrenalectomy or noxious stimuli, these workers proposed that the antidiuretic hormone might serve as the hypothalamic neurohormone which activated the anterior pituitary.

Hypothalamic lesions which destroyed the supraoptico-hypophyseal tract blocked ACTH release, but McCann and Brobeck (94) were able to induce ACTH discharge with large doses of Pitressin. The minimal effective dose of Pitressin was approximately 600 mU. in these rats, which were tested three or more weeks after lesioning. ACTH activity was measured by adrenal ascorbic acid depletion. Later McCann and Fruit (95) discovered that rats which had been lesioned only 48 hours prior to the assay were more sensitive to Pitressin. The

minimum effective dose for the acute hypothalamic-lesioned rats was 100 mU. Linear log dose response was demonstrated from 100 to 1000 mU. of pressor activity. Synthetic lysine vasopressin evoked an almost identical response in these animals. It is interesting to note that Pitressin, which is usually a mixture of beef and pork vasopressins, had only 83 per cent of the potency of synthetic lysine vasopressin at the same pressor dosage.

McDonald and his coworkers (99) tried intravenous infusions of Pitressin and highly purified arginine and lysine vasopressins in man. Adrenocorticotropin release was measured by the increase in concentration of free 17-hydroxy-corticosteroids in the plasma. Two units of highly purified lysine vasopressin gave an increase more than twice that observed for two units of arginine vasopressin. The response to Pitressin was only slightly lower than that given by lysine vasopressin, and the authors concluded that all the ACTH-releasing activity of Pitressin could be accounted for by its pressor content. To investigate the possibility that Pitressin and the vasopressins isolated from natural sources might contain contaminants having ACTH-releasing ability these workers (100) also compared Pitressin and synthetic lysine vasopressin. They ruled out the existence of an active contaminant after finding no significant difference between the 17-hydroxy-corticosteroid levels given by 2 U. of synthetic

lysine vasopressin and 2 U. of Pitressin.

Steroid-blocked rats have been used as assay animals. Smelik and de Wied (151) administered prednisolone. Twenty-four hours later the steroid-inhibited rats showed no adrenal ascorbic acid depletion with adrenaline or serotonin. Only Pitressin was effective.

Casentini et al. (19) blocked nonspecific stimuli in the rat with 9-alpha-fluoro-hydrocortisone. Intravenous injections of 300 mU. of Pitressin and lysine vasopressin gave significant adrenal ascorbic acid depletion. With intracarotid injections of Pitressin much smaller doses, 6 to 24 mU., were effective. Pitressin did not exert a direct effect on the adrenals nor contain ACTH, because 300 mU. produced no fall in adrenal ascorbic acid in hypophysectomized rats.

Another interesting experiment performed by this group was with hypophysectomized rats bearing functional pituitary grafts in the anterior chambers of their eyes. Acetylcholine, histamine, serotonin, Pitocin and synthetic oxytocin did not release ACTH when injected directly into the graft. Pitressin did at the low level of 10 mU.

In 1955 an in vitro assay system was devised by Saffran and Schally (130). Corticotropin-releasing factors were detected by their ability to release ACTH from rat anterior pituitaries incubated in vitro. In this assay hypothalamic tissue alone was inactive but the combination of hypothalamic

tissue with arterenol or epinephrine increased ACTH release threefold. Posterior pituitary tissue plus arterenol was even more active and increased ACTH release six to eight times.

Guillemin and Hearn (52) also independently developed an in vitro assay. Pitressin released ACTH in their system when cultured with anterior pituitaries for four days, but purified vasopressin did not. These results led the investigators to suggest that the ACTH-releasing activity of Pitressin might be due to an active contaminant of this commercial material rather than the vasopressin.

Saffran and his group (131) also reached the same conclusion after they had separated a corticotropin-releasing factor (CRF) from Pitressin by paper chromatography in two systems, butanol:acetic acid:water (4:1:5) and water-saturated phenol.

Guillemin and his coworkers (55) prepared a more active ACTH-releasing fraction (Fraction D) by paper chromatography in acetone:diethylene glycol:0.5% aqueous urea (6:1:3). In this system Fraction D ran close to the front with a mean Rf value of 0.9. The pressor-containing fraction (Fraction B) was found at a mean Rf of 0.4. Only these two fractions showed ACTH-releasing ability with Fraction D almost twice as active as the pressor fraction.

Fraction D obtained from posterior pituitary tissue was active in the Saffran and Schally in vitro assay at 100 $\mu$g.

It had a low pressor activity (4 to 50 mU./mg.) and some melanocyte stimulating hormone (MSH) activity. Hypothalamic Fraction D had 30 to 100 times greater CRF activity, no vasopressin effect and more MSH activity.

Electrophoresis of the posterior pituitary Fraction D in acetic acid concentrated the activity in a D-delta fraction which stimulated ACTH release at 0.5 to 2.5 μg. in the in vitro assay. The D-delta fraction upon hydrolysis showed 17 amino acids and therefore was believed to be a mixture of peptides.

Schally et al. (144) obtained a highly active CRF by using a series of four chromatographic systems, butanol: acetic acid:water (4:1:5), water-saturated m-cresol, acetone: water (3:2), and propanol:water (4:1). This CRF fraction was active in releasing ACTH at doses as low as 1 mμg. Hydrolysis of this fraction consistently showed the amino acids of lysine vasopressin plus major amounts of serine and histidine and smaller variable proportions of alanine and arginine.

Schally (139, 143) also tested various vasopressin preparations in the in vitro assay. Lysine vasopressin purified to 200 U./mg. showed a 40 per cent increase in ACTH release at a level of 0.5 μg., but this result was not statistically significant. Synthetic lysine vasopressin with an activity of 300 U./mg. was active at a dose of 0.067 μg. but not active at 0.5 μg. or 0.002 μg. However, only one

assay at the high level was reported.

Commercial Pitressin was active in the slightly modified *in vitro* system of Swingle *et al.* (156). An ACTH-releasing fraction was separated from the pressor fraction by countercurrent distribution or by destruction of the pressor activity b. hydrolysis. Later in an addendum to their reprints this group admitted that their *in vitro* assay gave non-specific and irreproducible results (55).

In 1959 Nichols and Guillemin (108) published the results of a study of the effects of endogenous and exogenous vasopressin on ACTH release in unanesthetized dogs. They found no correlation between antidiuresis and ACTH release measured by increase in the 17-hydroxy-corticosteroids. When lysine vasopressin was injected into the carotid artery the dose required to stimulate ACTH secretion was 3000 to 7000 times the amount of vasopressin needed to produce maximal antidiuresis. These workers felt that vasopressin could not be the mediator of both responses unless there was a large sensitivity difference between the two reactions.

McDonald and his group (98) found a variation in the responses of normal human subjects to stresses of fluid deprivation, hypertonic saline, nicotine, hand immersion in ice water and insulin-induced glycemia. Endogenous ADH release without an increase in ACTH, or a rise in ACTH without a detectable release of vasopressin, occurred in these sub-

jects even under the same stress. These authors concluded that there was no correlation in the release of the two hormones.

de Wied (32) noted that ACTH release in response to neurogenic stress such as strange environment, sound, and pain, required vasopressin. Compared with controls, rats which had been neurohypophysectomized had low levels of plasma free corticosterone after neurogenic stress but treatment with Pitressin tannate restored pituitary response. However, when the neurohypophysectomized rats were exposed to systemic stress such as hemorrhage, histamine injection, or nicotine injection, the ACTH-response was similar to that of the sham-operated controls. This is an interesting difference in response to two types of stress.

In 1958 Guillemin et al. (54) introduced an in vivo CRF assay utilizing rats blocked with nembutal plus morphine. The release of ACTH was determined by measuring the concentration of free corticosterone in the plasma by the fluorometric method of Silber et al. (149) as modified by Guillemin et al. (49). In these pharmacologically blocked rats a linear log dose relationship was observed between ACTH release and Fraction D in the range of 10 to 1000 μg. Almost identical results were obtained when Fraction D was injected into rats with lesions of the median eminence of the hypothalamus.

When highly purified lysine vasopressin (287 U./mg.) was

tested in the in vivo assay, Guillemin et al. (51) concluded that there was a threshold for ACTH release at 80-100 mU. of pressor activity. At 80 mU. of pressor activity there was a large ACTH release (150% increase in plasma corticoids over controls) and from this point ACTH release increased linearly with the log dose of vasopressin. Below 80 mU. no statistically significant discharge occurred. To eliminate this ACTH-releasing activity of high pressor dosages, it was suggested that all samples should be diluted to 30 mU. of pressor activity before being assayed for CRF.

Work on purification of CRF continued. In 1959 for larger scale preparation Schally and Guillemin (141) concentrated CRF by chromatography on carboxymethylcellulose (CMC). Column fractions were assayed at 30 pressor milliunits in nembutal-morphine-blocked rats. The pressor and CRF activities had very similar mobilities on the CMC column, but the maximum CRF activity was found in the early tubes of the vasopressin peak.

The chromatographic behavior on CMC and IRC-50 cation exchange resin of highly purified arginine and lysine vasopressins, $\alpha$-MSH and ACTH were studied by Schally and Guillemin (142), in order that their locations would be known when crude pituitary extracts were chromatographed. CRF activity appeared to follow oxytocin and precede lysine vasopressin, $\alpha$-MSH, arginine vasopressin and ACTH on CMC columns,

but lysine vasopressin, ∝-MSH and CRF moved very close together. On IRC-50 oxytocin and lysine vasopressin moved ahead of the CRF, but ∝-MSH, arginine vasopressin and ACTH came off the column near the peak of ACTH-releasing activity.

Porter and Rumsfeld (113, 114, 129) obtained blood from the hypothalamico-hypophyseal portal vessels of dogs in order to test for the presence of the neurohumoral agent in the blood draining the hypothalamus. After low-temperature alcohol fractionation of the blood plasma proteins by the method of Cohn et al. (21) the ACTH-releasing activity was found only in fraction $III_0$. The injection of a fraction $III_0$ prepared from carotid artery blood did not cause reduction in the adrenal ascorbic acid of the hydrocortisone-inhibited rat. The CRF activity of portal vessel plasma fraction $III_0$ was labile to trypsin and pepsin. It also was destroyed by refluxing 1 hour in 0.1 N sodium hydroxide but not by 2 hours of refluxing in 0.1 N hydrochloric acid. Dialysis data indicated that the active factor was associated with a large carrier protein.

An ACTH-releasing fraction also was prepared by Rumsfeld and Porter (128) from an acetone extract of beef hypothalami. After chromatography on Dowex 50x2 resin with volatile formate buffers, the active material was fractionated by countercurrent distribution (CCD) in sec-butanol and 0.05 per cent acetic acid. Paper chromatography in butanol:acetic acid:

water (4:1:5) of the fast and slow-moving CCD components gave single spots with Rf's of 0.64 and 0.41 respectively. Arginine and lysine vasopressins are usually found at 0.15 and 0.11 in this system.

Additional evidence for a CRF distinct from vasopressin and ACTH was found by Royce and Sayers (126). Acetic acid extracts of calf hypothalamus gave adrenal ascorbic acid depletion in median eminence lesioned rats even after absorption of 85-90 per cent of the pressor activity on oxycellulose. This CRF was pepsin-labile, whereas the pressor activity and the activity of added ACTH were unaffected by pepsin.

McCann and Haberland (96) corroborated these results by obtaining acid extracts of beef or rat stalk-median eminence area in which vasopressin accounted for only 10 to 20 per cent of the ACTH-releasing activity of the fraction. However, in contrast to the oxycellulose purification of the CRF by Royce and Sayers, McCann and Haberland found that the oxycellulose adsorbed almost all of the ACTH-releasing material. In acetic acid extracts of posterior pituitaries vasopressin alone accounted for all the corticotropin-releasing activity.

Further purification of the hypothalamic ACTH-releasing material was achieved in 1960 by Royce and Sayers (127) by chromatography on CMC columns. Discontinuous gradient elution with 0.05, 0.1, 0.2 and 0.4 M ammonium acetate buffers gave two active fractions. These CRF fractions emerged with the

0.01 M and 0.1 M eluents, whereas the 0.2 M buffer contained most of the pressor activity, and ACTH was found in the 0.4 M eluate. These results suggested that there might be two chemically distinct CRFs.

In March of 1960 Guillemin et al. (57) reported that in three different CCD systems CRF activity corresponded to the α-MSH peak. de Garilhe and his coworkers (26) also obtained a CRF fraction that appeared to be closely related to α-MSH. All the amino acids of α-MSH were present in this fraction plus threonine and leucine. The CRF material and α-MSH did not appear to be identical because of the two additional amino acids, the low MSH activity of the CRF fraction and the previously observed low ACTH-releasing activity of highly purified α-MSH and synthetic formyl α-MSH.

In June of 1960 Guillemin and his group (56) proposed that there was evidence for two chemically distinct CRFs in porcine posterior pituitary. One was closely related to α-MSH and was named α-CRF, while the other, β-CRF, was associated with vasopressin. They had separated α-CRF from α-MSH by a CMC column and paper chromatography in butanol: acetic acid:water (4:1:5) and m-cresol:water (95:5). The α-CRF contained all the amino acids of α-MSH plus threonine, leucine and alanine. It was active at relatively high dosages, 2-3 μg. in the in vivo CRF assay and 0.5 μg in vitro. Its MSH activity was 0.1 to 0.3 x $10^7$ U./mg. and inherent ACTH

activity 0.1 U./mg. The pressor activity of $\alpha$-CRF was very low, 0.1-0.2 U./mg.

The $\beta$-CRF was detected on the descending edge of the vasopressin peak after CCD in n-butanol:0.3 M p-toluene-sulphonic acid (140). In this system the vasopressin peak was well separated from the $\alpha$-MSH and $\alpha$-CRF area. A partial separation of $\beta$-CRF from vasopressin was obtained by the same series of paper chromatographic systems originally developed by Schally et al. (144). $\beta$-CRF was much more active at 0.05 $\mu$g. in vitro and 0.1 $\mu$g. in vivo than $\alpha$-CRF. Guillemin and his coworkers (140) proposed that $\alpha$-CRF may be only a precursor in the biosynthesis of ACTH and that $\beta$-CRF is the true chemical mediator of ACTH release.

The discovery of $\alpha$-CRF has helped to explain the ACTH-releasing activity of most of the fractions which were carefully characterized as different from vasopressin. Lazzari (86) has re-evaluated the published data on corticotropin-releasing factors and was able to classify almost all as either $\alpha$-CRF or $\beta$-CRF. The question still remains, however, whether $\beta$-CRF is a separate entity or a manifestation of the inherent ACTH-releasing ability of vasopressin. This problem will only be resolved by either isolation of a chemically distinct $\beta$-CRF or proof that vasopressin itself is the most active corticotropin-releasing factor.

Sideman and Sobel (148) have concluded that lysine vaso-

pressin is the CRF of the guinea pig. By electrophoresis in acetate buffer a fraction was isolated from Pitressin that was active in releasing corticoids and contained only the eight amino acids of vasopressin.

Kwaan and Bartelstone (82) injected a very small dose, 2 mU., of arginine vasopressin directly into the third ventricle of the brain of a dog and found a significant rise in the adrenal corticoids. Intraventricular injections of 2 mU. of oxytocin or intravenous injections of 20 mU. of arginine vasopressin did not increase the 17-hydroxy-corticosteroid levels. These results illustrate that smaller doses of vasopressin may be effective if they can reach the pituitary directly via the portal vessels.

A direct vasopressin stimulation of the isolated adrenal gland was shown by Hilton et al. (63). With direct arterial perfusion minimal activity was detected with doses as low as 7 mU. of arginine vasopressin. Royce and Sayers (125) also noted an extrapituitary action of vasopressin after intravenous injection of large doses (2.5-5.0 U.) into hypophysectomized or decapitated rats. Other investigators (48, 96, 148) were unable to demonstrate a direct effect on the adrenals by intravenous injections.

One of the greatest problems in assessing corticotropin activity has been the development of a sensitive and reliable assay. According to McCann (93, p. 673):

> . . . the ideal assay animal to test for possible neurohumoral activators of ACTH release would be one in which the ubiquitous pituitary-adrenal response to stress was completely blocked while leaving unimpaired the sensitivity of the pituitary and adrenal to the neurohumor and ACTH, respectively.

Sayers et al. (137) in their 1958 review stated that none of the current CRF bioassays completely met all of these requirements.

Saffran et al. (131) developed the in vitro pituitary assay to avoid some of the complexities of in vivo assays. However, three groups (8, 44, 124) suggested that the assay was not specific but that the increase in ACTH was due to inhibition of the degradation of ACTH or to leakage from the pituitary cells rather than secretion of the hormone. Guillemin and Schally (55) re-evaluated the technique and concluded there was no destruction of ACTH in the system under the conditions used and, therefore, there could be no inhibition. In their hands the in vitro assay was specific for corticotropin-releasing factors, because there was a correlation in the data obtained with the in vitro and in vivo assays. The severe limitation of this method is that it does not show a linear log dose response with increasing doses of CRF. Consequently, it remains a qualitative rather than a quantitative test.

Rats with hypothalamic lesions have been used as assay animals (54, 94, 126), but there has been so much disagreement

over the sensitivity of these animals that it is difficult to interpret the assay data. Royce and Sayers (126) observed that median eminence lesioned rats gave the same response to ACTH as did 24-hour hypophysectomized rats. McCann and Haberland (96) stated that rats with lesions were three times less sensitive to ACTH than hypophysectomized rats. Dear and Guillemin (25) found lowered adrenal response to ACTH but a compensatory increase in pituitary sensitivity.

The presence of diabetes insipidus was not considered as an adequate guide to animals with "effective" hypothalamic lesions by Guillemin and his group (51). Eighteen to 19 hours after lesioning they stressed the rats with ether. Only those animals with low plasma corticosteroid levels were selected. No correlation was observed between the inhibition of stress-induced ACTH release and diabetes insipidus.

de Wied (31) used a slightly different method to judge the effectiveness of hypothalamic lesions in rats. Corticoid production by the left adrenal in vitro after ether anesthesia was the index of the effectiveness of the lesion. Immediately after the removal of the left adrenal, the assay sample was injected. Fifteen minutes later the right adrenal was removed. Steroidogenesis by the right adrenal in vitro measured the CRF activity of the sample. The log dose response curve obtained was linear between 5 and 180 mU. of Pitressin. Purified lysine vasopressin gave similar results

in this assay.

Hydrocortisone and morphine are used as blocking agents in CRF assay animals with the assumption that these agents act on the hypothalamus to inhibit the release of the neurohormone in response to nonspecific stimuli. The exact site or mode of action of these agents is not known.

Morphine does not interfere with the response of the adrenal to ACTH (18) but in steroid-blocked rats the adrenal sensitivity is less than that of hypophysectomized rats (19). Morphine plus nembutal anesthesia is also relatively more effective than corticosteroids in the inhibition of reaction to intense stimuli such as laparotomy and unilateral adrenalectomy (107). Perhaps the greatest advantage of the nembutal-morphine-blocked rat is that there is no destruction of the vascular and nervous tissue as there is in electrolytic lesioning.

Leeman et al. (87) and Munson (106) have suggested that CRF assays must be performed in both morphine-nembutal-blocked animals and in animals anesthetized with nembutal alone. They have proposed that the true CRF will give the same response in both, but a non-specific stress would be inhibited in the morphine-blocked animal. A crude hypothalamic extract prepared by this group (88) gave essentially the same effect in both assays while the response to 80 mU. of Pitressin was reduced in the morphine-blocked animals. However, the crude

extract contained some ACTH and vasopressin and perhaps MSH and ∝-CRF since no measurements of MSH activity were made on the extract. Further purification of this crude hypothalamic material must be carried out to reveal the nature of its CRF activity.

For many years ACTH release has been assessed by the Sayers and Sayers (138) adrenal ascorbic acid depletion method. In 1958 Slusher (150) noted that lesions in the posterior and midcentral portions of the hypothalamus specifically inhibited corticosterone release without altering the ascorbic acid depletion response to stress. Lesions in the basal tuberal area inhibited adrenal ascorbic acid depletion but had no effect on steroid release.

When Guillemin et al. (51) measured both adrenal ascorbic acid depletion and plasma corticosteroid levels in the same animals, variations in corticoid levels were found with no change in the adrenal ascorbic acid concentration. The fluorometric determination of corticosterone in rats appears to be a more direct and sensitive measurement of adrenal response to ACTH release.

Other activities of vasopressin

In 1955 Ingle and Li (69) observed another interesting property of vasopressin. This was its ability to enhance work performance in adrenalectomized, hypophysectomized rats.

Sreter and Friedman (154) suggest this effect is due to a direct action by vasopressin on cationic exchanges across muscle membranes.

Vasopressin and oxytocin stimulate the oxidation of glucose in mammary gland slices (47). Vasopressin has one-sixth the activity of oxytocin, a ratio similar to the ratio of their milk-ejection activities. A glycogenolytic effect of vasopressin in the canine liver has also been observed (11).

## Biological Activities after Inactivation of Vasopressin

Inactivation studies of a hormone are of great interest because they may offer some clues as to its mode of action. These studies are also practical because of isolation and storage problems.

Adamsons et al. (5) studied the spontaneous decomposition at room temperature of synthetic and natural neurohypophysial hormones in aqueous buffers over a pH range of 3.0-8.5. Partially purified synthetic lysine vasopressin (38 U./mg.) showed maximal stability at pH 3 and 5 with 87 to 90 per cent of its activity still remaining at the end of 28 days. Arginine vasopressin also was most stable at the same acidic pHs. A highly purified arginine vasopressin (400 U./mg.) decomposed faster than a crude sample (175 U./mg.). There was a parallel loss of both antidiuretic and pressor activities during the destruction of the vasopressins.

The enzymes pepsin and pancreatic carboxypeptidase do not affect vasopressin (158). Trypsin cleaves between the carboxyl group of arginine or lysine and the adjoining amino group of glycine. Trypsin has been used to destroy the pressor activity of a crude sample without harming the oxytocin (105).

Hooper (65) has prepared a soluble vasopressinase, probably a peptidase, from human placenta. Slices of mammalian kidney have also been shown to inactivate vasopressin (153). This system may contain several enzymic activities directed toward either the disulfide or amide bonds.

Chemical reduction of the disulfide bond of vasopressin leads to loss of pressor and antidiuretic activity. Ames et al. (6) incubated Pitressin in a 0.01 M solution of thioglycolate at pH 7.5. After 2.5 hours at room temperature only 2 per cent remained of the original antidiuretic activity of 100 mU./ml. Schally (139) used the same conditions to study CRF activity and found that all pressor activity of a crude pituitary extract was destroyed but the results on CRF were inconclusive. When the thioglycolate concentration was increased to 0.05 M and the time of the reaction to 24 hours, both activities were abolished.

Oxidative cleavage of the disulfide bond with performic acid also destroyed the pressor (111) and CRF activities (139).

Sideman and Sobel (148) used inactivation studies to support their contention that lysine vasopressin is the corticotropin-releasing factor in the guinea pig. After mild acid and alkaline hydrolysis, iodination and incubation with placental extract both the CRF and pressor activities of Pitressin were reduced to the same extent.

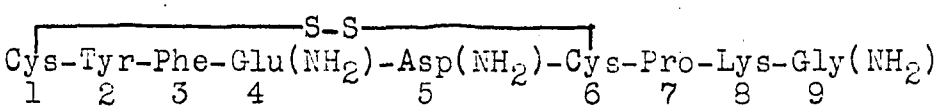
In a footnote to his paper on the direct adrenal action of vasopressin Hilton et al. (63) stated that the ACTH-like activity of vasopressin was lost after 2 hours at room temperature in a saline solution although there was no loss of pressor activity under these conditions.

## Biological Activities of Vasopressin Analogues

The relation of chemical structure to hormonal activity also has been studied by means of analogues. The two vasopressins, arginine vasopressin and lysine vasopressin, have been found in the neurohypophyses of many mammals. Arginine vasopressin, originally isolated from beef, has also been identified as the pressor hormone of man (91), horse (2), sheep (3), cat, dog, rat, rabbit, camel, monkey (161), opossum and spiny anteater (135). Lysine vasopressin, the porcine hormone, has been found in only one other animal, the hippopotamus (61).

Two other naturally occurring analogues of vasopressin are known. Oxytocin, the other posterior pituitary hormone

of mammals, could be called isoleucine$^{3}$-leucine$^{8}$-vasopressin. In order to designate various analogues of lysine vasopressin the following numbering system has been used.




Recently vasotocin, Ileu$^{3}$-Arg$^{8}$-vasopressin has been identified from the pituitaries of chickens (20, 105), fish (62, 117, 136), frogs (4), alligators (135), turtles and lamphreys (134).

This new hormone, containing the ring of oxytocin and the side chain of arginine vasopressin, was synthesized in 1958 by Katsoyannis and du Vigneaud (78) before it was discovered as a natural product. The presence of arginine in the side chain increases the pressor activity above that of oxytocin, while the oxytocin ring structure gives higher avian depressor response than that of the vasopressins.

The chemical synthesis of arginine vasopressin and lysine vasopressin opened the way for the synthesis of many analogues of the natural hormones. These analogues afford an excellent opportunity to study the relationship between structure and biological activities.

Table 2 lists the biological activities of lysine vasopressin and closely related synthetic polypeptides. Ileu$^{3}$-vasopressin is the lysine analogue of vasotocin. Arginine vasotocin and lysine vasotocin have very similar biological

Table 2. Lysine vasopressin analogues

| Analogue | Rat pressor U./mg. | Avian depressor U./mg. | Rat uterine contracting U./mg. | References |
|---|---|---|---|---|
| Lys-vasop. | 280 | 30 | 4 | (34) |
| $Arg^8$-vasop. | 400 | 85 | 30 | (37, 76) |
| $Ileu^3$-$Arg^8$-vasop. (Vasotocin) | 125 | 150 | 75 | (78) |
| $Ileu^3$-vasop. (Lys-vasotocin) | 130 | 190 | -- | (17, 79) |
| $Leu^8$-vasop. (Oxypressin) | 3 | 45 | 20 | (10, 16, 76) |
| $His^8$-vasop. | 1.5 | 4.6 | 1.5 | (77) |
| $Phe^2$-vasop. | 55 | 0.5 | <0.1 | (15, 101) |

activities with lysine vasotocin showing slightly higher values. $Leu^8$-vasopressin in contradistinction to vasotocin contains the ring portion of vasopressin linked to the tripeptide amide side chain of oxytocin. It is commonly called oxypressin. Substitution of leucine for the basic amino acids lysine or arginine decreased the pressor activity greatly. In $His^8$-vasopressin a weakly basic amino acid was substituted for the strongly basic acids. The pressor activity of this polypeptide was very low. One of the requirements for high pressor activity appears to be a strongly basic amino acid in the side chain.

Another functional group in the lysine vasopressin molecule is the hydroxyl group of tyrosine. The replacement of the phenol group by a phenyl to give $Phe^2$-vasopressin diminished the pressor activity but did not abolish it. The hydroxyl group is thus not essential. The replacement of phenylalanine in the third position by tyrosine, however, gave very low pressor activity. $Tyr^3$-vasopressin had 1.6 U./mg. of pressor activity and $Phe^2$-$Tyr^3$-vasopressin had only 0.14 U./mg. (15).

du Vigneaud and his group (39) have obtained further evidence that basicity may be very important for high pressor function. When the free amino group on the cystine residue was eliminated, the desamino-lysine vasopressin had only half the activity of the natural hormone. $\beta$-mercaptopropionic acid was used in the place of cysteine to prepare this analogue.

The addition of a methyl group to the nitrogen of the C-terminal peptide bond caused a marked loss of biological activity. This $sarcosine^9$-lysine vasopressin had only 0.45 U./mg. of pressor activity and no activity in the avian depressor and rat uterine contracting assays (102). The substitution of a methyl for a hydrogen on the imino group of the terminal peptide bond of the side chain may interfere with hydrogen bonding.

The oxytocin analogues with non-basic leucine in the

side chain generally have very low pressor activity. The synthetic compounds have usually been compared in only three assays, the rat pressor assay and the two recommended for oxytocic activity, avian depressor and rat uterine contracting. Berde et al. (10) have suggested that all analogues should be tested in a battery of assays. The varying ratios of activities obtained with oxytocin analogues in five different assays can be seen in Table 3. The substitution of valine or leucine for the third amino acid of oxytocin decreased the avian depressor and the rat uterine contracting activities much more than the milk ejection potency, whereas $Ileu^{8}$-oxytocin showed much higher activity in the avian depressor assay than in any other test. A variety of biological assays are needed to characterize structural analogues and also may be helpful in developing synthetic compounds with highly desirable biological properties.

Desamino-oxytocin (39) is an example of a synthetic polypeptide with selective biological action. It had a higher avian depressor activity (578 U./mg.) than oxytocin but only one-third of its low pressor activity. This analogue again illustrates that amino groups are not necessary for oxytocic activity. A desamino-desoxy-oxytocin in which phenylalanine had replaced tyrosine was relatively low in avian depressor activity (140 U./mg.). The presence of the phenolic group in oxytocin enhances its activity. The synthesis of (O-methyl-

Table 3. Oxytocin analogues

| Analogue | Rat pressor U./mg. | Rat anti-diuretic U./mg. | Avian depressor U./mg. | Rat uterine contracting U./mg. | Milk ejection, rabbit U./mg. | References |
|---|---|---|---|---|---|---|
| Oxytocin | 5.0 | 5.0 | 450 | 450 | 450 | (72) |
| $Ileu^{8}$-oxytocin | 6.0 | 1.1 | 498 | 289 | 328 | (72) |
| $Val^{8}$-oxytocin | 9.0 | 0.8 | 280 | 200 | 310 | (72) |
| $Val^{3}$-oxytocin | 0.2 | 0.8 | 57 | 59 | 207 | (10, 16, 72) |
| $Leu^{3}$-oxytocin | ~5.0 | ~5.0 | 42 | 45 | 101 | (10, 16, 72) |
| $Phe^{2}$-oxytocin | ~1.0 | ~1.3 | 63 | 31 | 141 | (9, 14, 71) |
| $Glu^{5}$-oxytocin | 0.0 | 0.0 | < 1 | < 0.1 | ~ 1 | (10, 16) |

Tyr)$^2$-oxytocin (85) and (N-methyl-Tyr)$^2$-oxytocin (67) resulted in compounds with less than 1 per cent oxytocic activity.

du Vigneaud and his group (35) have prepared glycyl-oxytocin. This compound with glycine attached to the amino group of the cystine gave such unusual assay responses that no estimates of its potencies could be made. The responses were very slow and prolonged as compared with the natural hormones.

Ressler (119) discovered that the cyclic disulfide ring alone of oxytocin has low but significant rat uterine contracting (3.3 U./mg.) and milk ejecting (1.1 U./mg.) activities. Changes in the size of the ring produce inactive compounds. If tyrosine is eliminated from the ring, the resulting heptapeptide amide disulfide is inactive. The enlargement of the ring by an additional tyrosine (59) results in a nonapeptide with negligible oxytocic activity. Beyerman and Bontekoe (12, 13) have prepared a tyrosine homologue of vasopressin but did not report biological activities in their papers.

The disulfide ring was expanded by one and two methylene units in isoasparagine-oxytocin (92) and isoglutamine-oxytocin (120). Both of these analogues were inactive. Isoglutamine-oxytocin even inhibited the pressor activity of arginine vasopressin (121).

Guttman and Boissonnas (58) have prepared ten analogues

Table 4. Analogues of oxytocin and vasopressin

| | |
|---|---|
| $Ser^2$- oxytocin | $Ser^2$-$Ileu^3$-vasopressin |
| $Ser^2$-$His^3$-oxytocin | $Ser^2$-$His^3$- vasopressin |
| $His^2$-$Phe^3$-oxytocin | $His^2$-$Ser^3$- vasopressin |
| $Try^3$-oxytocin | $His^2$- vasopressin |
| | $Ser^3$- vasopressin |
| | $Try^3$- vasopressin |

of oxytocin and lysine vasopressin containing serine, histidine or tryptophan in the second or third position. The oxytocic and vasopressic activities of all these analogues were lower than 1/1000 to 1/10000 of the corresponding activities of the parent hormones.

Although many synthetic peptides related to $\alpha$-MSH have been tested and found to have corticotropin-releasing activity (26, 74, 75, 90), only a few analogues of vasopressin have been tried in CRF assays. Rinne et al. (123) found that oxytocin did not deplete ascorbic acid in the cortisone-blocked rat. Neither $Arg^8$-oxytocin nor $Leu^8$-vasopressin produced any significant increase in adrenal corticosteroid output when injected directly into the third ventricle of the dog by Kwaan and Bartelstone (82) at levels of 5 $\mu\mu$g.

# MATERIALS AND METHODS

## Materials

### Princeton hog posterior pituitary powder

Princeton Laboratories Inc. of Princeton, New Jersey (courtesy of Dr. William Kleinberg) generously donated several kilograms of hog posterior pituitary lobe powder. This powder had been prepared by acetone desiccation, homogenization and lyophilization of the posterior pituitaries and had an oxytocin and pressor activity of approximately 1500 U./g. This powder was the starting material for most of the isolation work.

### Continuous electrophoresis starting material

The Princeton posterior pituitary powder was extracted according to the procedure developed by Kamm *et al.* (73). This extract, usually designated as Kamm product, had 4-5 U./mg. of pressor activity.

A large scale countercurrent distribution of Kamm product was carried out in *n*-butanol:acetic acid:water (4:1:5). After 14 transfers most of the weight and pressor activity remained in the first four tubes. Material from the leading edge of the pressor peak (Tubes 2 and 3) was submitted to continuous electrophoresis. The Kamm extractions and the CCD were performed by Eugene Lazzari and his assist-

ant; details are given in his Ph. D. thesis (86).

Synthetic lysine vasopressin

du Vigneaud lysine vasopressin Synthetic lysine vasopressin was kindly provided by Dr. V. du Vigneaud, Department of Biochemistry, Cornell University Medical College, New York, N. Y. The sample (JM V 68/16) was reported to contain 280 International Units per mg. In our laboratory two assays of this sample gave 268 U./mg. (95% fiducial limits 248-291) and 267 U./mg. (95% fiducial limits 256-279). The mean of these two assays, 282 U./mg., was taken as the pressor activity of the sample.

Sandoz vasopressin Synthetic lysine vasopressin (Batch No. 067 01) also was generously donated by Sandoz Pharmaceuticals Co., Hanover, N. J. (courtesy of Dr. H. Schwarz). This material was received in solution. Each sealed vial held 1 cc. of saline containing 10,000 mU. of pressor activity.

Drugs

Nembutal Powder was provided by Abbott Laboratories, North Chicago, Ill. (courtesy of Dr. J. D. Taylor). The heparin-sodium (Nutritional Biochemicals Corp., Cleveland, Ohio) was obtained in a powdered form having 100 U./mg. activity. Crystalline urethan (ethyl carbamate-Fisher Scientific

Company, Fairlawn, N. J.) and USP-Powder morphine sulfate (Merck and Co., Inc., Rahway, N. J.) were used. Fresh solutions of all drugs were prepared before each bioassay.

Animals

Rats Male albino rats were obtained from the Holtzman Company, Madison, Wisconsin. The rats were shipped by Air Express and then kept in the laboratory at least a week before they were used for bioassays.

Frogs Jumbo size frogs (Rana pipiens) with light skins were purchased from E. G. Steinhilber and Co., Oshkosh, Wisconsin, for the MSH assays.

## Methods

### Continuous flow electrophoresis

A Spinco Model CP - Continuous Flow Paper Electrophoresis Cell was used with a Spinco Con-stat constant current supply. Prior to each run the system was equilibrated with the electrolyte, 0.5 N acetic acid, for four to six hours. The flow rates of the electrolyte to the side wicks and onto the hanging curtain were adjusted until a trace of dye moved straight down the paper curtain.

A 1% solution of the sample in the electrolyte was prepared. The sample was continuously fed at a rate of 0.9 ml. per hour onto a tab of the hanging curtain near the anode.

During the run the current was maintained at 30 milliamps which provided 530-540 volts. The system was cooled by running cold tap water through the cooling plate behind the hanging curtain and by recirculating the electrolyte from a six liter bottle immersed in an ice bath. Every 24 hours the electrolyte was changed.

The electrophoresis fractions were numbered 1 to 32 from the cathode to the anode. All fractions were lyophilized.

Bioassays

Pressor assay The pressor activities of the various samples were determined by the method of Dekanski (29) with slight modifications. Rats weighing 200 to 300 g. were injected subcutaneously with urethan (190 mg./100 g. B. W.). After approximately two hours when the rat was completely anesthetized, the trachea was cannulated with a short glass tube, and one carotid artery was dissected ready for cannulation. The femoral vein next to the inguinal ligament was cannulated with a needle, because the vein collapses easily with manipulation. Heparin (200 U./100 g. B. W.) was injected into the rat by way of the femoral vein. All the samples were administered in 0.2 ml. of physiological saline and washed into the femoral vein by an equal amount of saline.

A polyethylene cannula inserted into the carotid artery led to a Statham pressure transducer (Model P23Db) which in

turn was connected to a Sanborn amplifier and recorder (Model 141). The blood pressure of the rat was blocked by intravenous injections of dibenamine (200-300 μg./100 g. B. W.) to a base level of about 50 mm. Hg. Unknown samples were compared with two levels (4 mU. and 8 mU.) of USP Posterior Pituitary Reference Standard.

Corticotropin-releasing factor assay The in vivo assay procedure of Guillemin et al. (51) was used to measure CRF activity. All assays were performed in the morning, because Guillemin and his group (50) found that the resting level of plasma corticoids was the lowest during this period.

Male rats weighing 150-170 g. were used. Each rat was placed on the following time schedule: Nembutal (4.9 mg./100 g. B. W.) was injected intraperitoneally at zero time. After the rat was asleep, it was placed on an electric blanket in a box through which 95% oxygen-5% carbon dioxide was passed at a flow rate of 7.5 l./min. Thirty-five minutes after the nembutal, morphine (2.0 mg./100 g. B. W.) was injected intraperitoneally. This was a modification of Guillemin's assay in which morphine was given 25 minutes after nembutal and was done to assure that the rats were completely anesthetized. A small opening was made in the neck of the rat 15 min. after the morphine injection, and the sample to be tested was injected into the exposed external jugular vein. All samples were administered in 0.3 ml. of physiological saline.

Exactly 15 minutes after the sample had been given, the animal was opened at the midline of the abdomen. The viscera were lifted out, and 5 ml. of blood were withdrawn into a heparinized syringe from the dorsal aorta. This operation was completed within 40 seconds. The blood was centrifuged immediately to obtain the clear plasma. The plasma samples were frozen until the fluorometric determination of the corticosteroids was done. The interval of 15 minutes after sample injection was selected because the maximal concentration of plasma corticosteroids has been found 15 minutes after stress or injection of ACTH (48).

During the assay a careful record was kept of the condition of each rat. If an animal appeared to be awake or moribund, his corticosteroid value was not included in the final assay results.

The determination of the plasma free corticosteroids was carried out on 2 ml. aliquots of plasma according to the method of Silber (149) as modified by Guillemin (49). A Farrand fluorometer was used with the filter system suggested by Sweat (155).

Chloroform was freshly distilled over sodium carbonate before each determination. A deionized water sample was used as a reagent blank. Galvanometer readings of the water blank were of the order of 1.0-2.5.

For sulfuric acid induced fluorescence of corticoids the

11-hydroxy group is essential and the $\alpha,\beta$ unsaturated ketone in ring A also exerts a strong influence. A 17-hydroxy group has little effect. Corticosterone (cpd. B) was chosen as the standard for the fluorometric determinations, because it is the principal steroid found in the rat.

Two cpd. B standards and an internal plasma pool standard were used in every fluorometric determination. The internal standard was prepared by pooling plasma from stressed rats. The animals were stressed with ether anesthesia, and the blood was withdrawn from the abdominal aorta 15 minutes later. The pooled plasma was then divided into aliquots and frozen. The value for the corticosteroid concentration of a plasma pool does not vary even after the samples have been stored for many months (48).

The cpd. B level of the plasma pool showed good reproducibility from one fluorometric determination to another. The variations for three different plasma pools are listed in Table 5.

Uninjected rats or rats injected with physiological saline were introduced randomly throughout each assay as controls. The CRF activity of a sample was calculated as the per cent increase in corticosteroids over the level of the combined controls.

The sensitivity of the rat preparations was tested by injecting USP-ACTH in the nembutal-morphine-blocked animals.

Table 5. Concentrations of cpd. B in the internal plasma pool standards

| Pool | Number of determinations | Plasma - cpd. B μg./100 ml. |
|---|---|---|
| A | 21 | 32.1 ± 0.7[a] |
| B | 24 | 49.4 ± 0.9 |
| C | 10 | 33.4 ± 0.4 |

[a]Standard error of the mean.

Table 6. Concentrations of plasma free corticosteroids in nembutal-morphine-blocked rats after injection of ACTH

| Number of rats | ACTH mU. | Plasma - cpd. B μg./100 ml. |
|---|---|---|
| 5 | 0.205 | 13.8 ± 2.8[a] |
| 3 | 0.410 | 27.8 ± 2.5 |
| 4 | 0.820 | 33.8 ± 0.95 |

[a]Standard error of the mean.

With increasing doses of ACTH a graded response was obtained. These data indicate that the morphine-nembutal-blocked rat is as sensitive to ACTH as the hypophysectomized rat, for Guillemin et al. (48) obtained very similar values after injecting USP-ACTH into hypophysectomized rats.

Table 7. Concentrations of plasma free corticosteroids in the hypophysectomized rat after injection of ACTH (48)

| Number of rats | ACTH mU. | Plasma - cpd. B μg./100 ml. |
|---|---|---|
| 6 | 0.2 | 12.4 ± 1.6[a] |
| 6 | 0.4 | 27.8 ± 1.8 |
| 6 | 0.8 | 38.2 ± 2.3 |

[a]Standard error of the mean.

ACTH assay Male rats in the same weight range as those used for the CRF assays were hypophysectomized by the parapharyngeal approach by Mrs. Peggy Randolph. Twenty-four hours after the operation the rats were anesthetized with ether. The sample was injected into the external jugular vein, and a blood sample was withdrawn 15 minutes later from the abdominal aorta exactly as was done in the CRF assays. The concentrations of corticoids in the plasma were determined fluorometrically as previously. Two dose levels (1.0 mU. and 0.25 mU.) of USP Corticotropin Reference Standard (1.41 U./mg.) were injected randomly throughout the assay, so that the activity of the sample could be calculated in ACTH units/mg.

MSH assay Melanocyte stimulating activity was assayed in both intact and hypophysectomized frogs by the semi-quantitative method of Landgrebe and Waring (83). Hypophysec-

tomized frogs are more reliable assay animals, because their melanophores do not respond to nonspecific agents.

The frogs were placed under a light several hours before the assay. Then the melanophores in the web of the foot were checked under a low power microscope. If the melanophores were contracted, the frog was selected as an assay animal and injected with the sample. After 1 1/2 hours the size of the melanophores was observed again and compared to the Hogben Melanophore Index (64). On this scale increasing sizes of expanded melanocytes were given numbers from 1 through 5. The approximate MSH activity of a sample was judged by comparing the size of the expanded melanophores with that obtained with a standard of known MSH activity.

The MSH standard was prepared according to the water extraction method of Shizume et al. (147) except that pork posterior pituitary powder was used instead of beef. From 2 g. of porcine pituitary powder 1.0157 g. of standard was prepared. This was slightly more than twice the weight yield extracted from beef. Only 490 mg. were obtained from 2 g. of beef posterior pituitary powder. The activity of porcine glutamyl-$\beta$-MSH, however, is $2 \times 10^7$ U./mg. but that of beef seryl-$\beta$-MSH only $0.2\text{-}0.4 \times 10^7$ U./mg. (89). The species difference in activities canceled out the difference in weight yield, and the two standards showed almost the same activity. Injections of 1.25 $\mu$g. and 2.5 $\mu$g. of porcine standard gave

melanophore index readings of 2-3 and 3-4. One unit of MSH activity was defined as 0.04 μg. of the standard.

Inactivation studies

Temperature inactivation The loss of pressor activity and CRF activity of synthetic lysine vasopressin was followed after storage for varying lengths of time at -15°C., 25°C. and 37°C.

pH inactivation The stability of the activities in acidic and basic solution at room temperature and 100°C. was examined. All solutions were adjusted to pH 7 before bioassay.

Thioglycolate inactivation Thioglycolate reduction of crude and synthetic vasopressin preparations was studied by varying the concentration of thioglycolate from 1 to 600 moles per mole of vasopressin.

Pepsin inactivation The conditions for the pepsin inactivation experiments were essentially the same as those used by Royce and Sayers (126). The ratio of pepsin to milliunits of pressor activity was 4 μg./cc. to 160 mU./cc. After the solutions had been incubated at 37°C. for 2 hours, the action of the enzyme was stopped by adjusting the pH to 7.

# RESULTS AND DISCUSSION

## Continuous Electrophoresis

### Continuous electrophoresis of methanol extract (CE 73)

In 1957 CRF active fractions had been prepared from crude posterior pituitary materials in two ways. Schally (139) used paper chromatography in four different solvent systems; Guillemin et al. (53) obtained their D-delta fraction by a combination of paper chromatography and electrophoresis. Both of these procedures were so laborious and time-consuming that they were poorly suited to large scale preparation work. In an attempt to find a more efficient large scale isolation procedure continuous electrophoresis (CE) was investigated.

The starting material was a methanol extract (86) of Kamm product from Princeton hog posterior pituitary powder. A 1% solution was prepared by dissolving 1.0107 g. of methanol extract in 100 ml. of the electrolyte, 0.5 N acetic acid. Instead of keeping the electrolyte in ice the entire apparatus was moved into the cold room for this run. Under these conditions a voltage of 600-610 V. was attained at a constant current of 28 ma. The electrophoretic fractions were collected and lyophilized during the 94 hr. run. The weight recovery, shown in Table 8, was 71%.

In this experiment the pressor peak was found at fraction

Table 8. Weight, pressor and CRF results of CE 73

| Fraction | Migration from anode cm. | Weight mg. | Pressor activity U./mg. | In vitro CRF potency |
|---|---|---|---|---|
| 1-4 | 30.2 | 33 | 0.5 | -- |
| 5-6 | 26.4 | 25 | 2.2 | -- |
| 7-8 | 24.6 | 56 | 22.0 | -- |
| 9-10 | 22.7 | 55 | 31.25 | -- |
| 11-12 | 20.8 | 63 | 3.7 | -- |
| 13-14 | 18.9 | 54 | 0.0 | 1.9 |
| 15-16 | 17.0 | 70 | 0.6 | 2.0 |
| 17-18 | 15.1 | 86 | 0.8 | 1.7 |
| 19-20 | 13.2 | 111 | 0.45 | 1.6 |
| 21-24 | 11.3 | 74 | 0.45 | -- |
| 25-32 | 7.5 | 100 | 0.5 | -- |

9-10. The in vitro CRF assays were performed by Dr. Roger Guillemin at Baylor University College of Medicine, Houston, Texas. In the expression of the in vitro results a potency of 1.0 meant that there was no increase in ACTH secretion over the control pituitary flask and therefore no CRF activity. A potency of 2.0 was equivalent to the activity of Fraction D of Guillemin et al. (58). The four CE fractions on the anode side of the vasopressin peak (F13-14, F15-16, F17-18 and F19-20) appeared to be almost as potent as Fraction D. No CRF

assays were carried out on the electrophoretic fractions which had pressor activity, because this was an attempt to show separation of the two activities.

Paper chromatography in butanol:acetic acid:water (4:1:1) showed that all the electrophoretic fractions were gross mixtures of ninhydrin-positive materials.

Continuous electrophoresis of countercurrent distribution fractions (CE 208-2,3)

The results from the previous continuous electrophoresis experiment indicated that low pressor, CRF active fractions could be obtained with this procedure. In 1959 after an *in vivo* CRF assay was set up in our own laboratory, the continuous electrophoresis separation was reinvestigated in order to test the fractions in our assay.

A cleaner starting material was obtained by using samples from a countercurrent distribution (CCD 39) of Kamm product in butanol:acetic acid:water (4:1:5) (86). Because Schally *et al.* (144) had reported that CRF had an Rf value slightly higher than lysine vasopressin on a paper chromatogram in the butanol:acetic acid:water system, the leading edge (Tubes 2 and 3) of the CCD 39 pressor peak was used. The material from Tube 2 had 9.62 U./mg. pressor activity and Tube 3 had 4.22 U./mg.

For CE 208-3 a sample of 0.4464 g. of CCD 39-Tube 3 was dissolved in 0.5 N acetic acid. The weight and pressor

results for this electrophoresis run are shown in Table 9. The weight recovery was 66% and the pressor 62%.

In this electrophoresis the pressor peak had shifted to F15-16. However, the starting material was different from

Table 9. Weight and pressor results of CE 208-3

| Fraction | Weight mg. | Pressor activity U./mg. |
|---|---|---|
| 7-8 | 16.5 | |
| 9-10 | 65.9 | 0.065 |
| 11-12 | 84.8 | 1.03 |
| 13-14 | 35.8 | 19.3 |
| 15-16 | 12.9 | 28.2 |
| 17-18 | 12.9 | 0.53 |
| 19-20 | 17.9 | 0.44 |
| 21-22 | 19.5 | 0.64 |
| 23-24 | 21.1 | 0.03 |
| 25-26 | 0.4 | |
| 27-28 | 8.2 | |

that used in the previous run (CE 73), and the electrophoresis was carried out at room temperature with the electrolyte in an ice bath. Under these conditions 30 ma. of current gave only 530-540 V.

Identical conditions were used for CE 208-2 which was run on 1.0688 g. of CCD 39-Tube 2. Table 10 shows that the pressor peak again occurred at F15-16. The greater pressor activity of this fraction reflects the higher activity of the starting material. The weight recovery was 89% for CE 208-2.

Table 10. Weight and pressor results of CE 208-2

| Fraction | Weight mg. | Pressor activity U./mg. |
|---|---|---|
| 1-2 | 1.7 | |
| 3-4 | 5.3 | |
| 5-6 | 6.1 | |
| 7-8 | 38.1 | <0.02 |
| 9-10 | 124.6 | <0.02 |
| 11-12 | 169.6 | 0.5 |
| 13-14 | 92.0 | 17.4 |
| 15-16 | 52.3 | 50.0 |
| 17-18 | 46.3 | 6.7 |
| 19-20 | 42.8 | 1.1 |
| 21-22 | 42.9 | 1.25 |
| 23-24 | 43.3 | 0.6 |
| 25-26 | 17.1 | 1.1 |
| 27-28 | 30.4 | 0.8 |
| 29-30 | 13.3 | <0.02 |
| 31-32 | 7.9 | |

but only 46.5% for the pressor activity. Since 208-3 and 208-2 were run under identical conditions, the reason for the lower recovery in CE 208-2 is unknown. Perhaps there was some inactivation during the lyophilization of the samples.

The CRF activities of fractions from CE 208-2 were measured in nembutal-morphine-blocked rats. The results are given in Table 11. All the samples were assayed at pressor dosages below 80 mU., the threshold value at which Guillemin et al. (54) had stated that lysine vasopressin began to show inherent CRF activity. Although the pressor dosages were low, the peak of the CRF potencies appeared to coincide with that of the pressor activities. Fraction 15-16 gave a 140% increase in plasma corticosteroids over controls at 0.6 µg. per injection.

As in the previous electrophoresis run the fractions on the anode side of the pressor peak were low in pressor activity but showed CRF activity as if there had been some separation of vasopressin and CRF. Because ACTH also will release corticoids in the CRF assay, the ACTH content of Fz1-22 was examined. The results are shown in Table 12. Although these assays were only roughly quantitative, Fz1-22 appeared to have approximately 0.02 U. of ACTH per mg. In the CRF assay 45 µg. of Fz1-22 were injected. This would be equivalent to 0.9 mU. of ACTH which would account for almost all of the CRF activity of this fraction.

Table 11. CRF assays of fractions from CE 208-2

| Fraction | Pressor activity U./mg. | μg./inj. | Pressor dose mU./inj. | Plasma - cpd. B μg./100 ml. Control | Sample | Cpd. B incr. | % incr. |
|---|---|---|---|---|---|---|---|
| 13-14 | 17.4 | 0.6 | 10.4 | (5)[a] 8.1 ± 0.5[c] | (3) 9.8 ± 2.8 | 1.7 | 20 |
| 15-16 | 30.0 | 0.6 | 30.0 | (5) 8.1 ± 0.5 | (5) 19.4 ± 2.1 | 11.3 | 140 |
| 17-18 | 6.7 | 2.4 | 16.1 | (4) 7.4 ± 2.1 | (3) 25.1 ± 4.8 | 17.7 | 240 |
| 19-20 | 1.1 | 2.4 | 2.6 | (4) 5.8 ± 1.5 | (4) 4.8 ± 0.9 | 0.0 | 0 |
| | " | 45.0 | 49.5 | (4) 7.4 ± 2.1 | (5) 35.4 ± 2.8 | 28.0 | 380 |
| 21-22 | 1.25 | 45.0 | 56.2 | (3) 6.7 ± 1.7 | (3) 31.8 ± 1.3 | 25.1 | 375 |
| 23-24 | 0.4 | 45.0 | 18.0 | (4) 5.8 ± 1.5 | (5) 13.2 ± 2.4 | 12.4 | 215 |
| 25-26 | 1.1 | 45.0 | 49.5 | (4) 5.8 ± 1.5 | (4) 27.9 ± 4.0 | 22.1 | 380 |
| 27-28 | 0.8 | 45.0 | 36.0 | (5) 8.0 ± 0.5 | (5) 12.7 ± 1.4 | 4.7 | 60 |

[a]Number of rats.

[c]Standard error of the mean.

Table 12. ACTH assay of CE 208-2 (Fr1-22)

| Date of assay and fraction | Pressor activity U./mg. | µg./inj. | Pressor dose mU./inj. | Plasma - cpd. B µg./100 ml. Control | Sample |
|---|---|---|---|---|---|
| (2/12/60) | | | | | |
| ACTH | | 1.0 mU. | | (5)[a] 3.45 ± 0.3[b] | (3) 18.1 ± 0.5 |
| | | 0.25 mU. | | " | (3) 5.1 ± 1.0 |
| Fr1-22 | 1.25 | 90.0 | 112.5 | " | (4) 29.4 ± 1.8 |
| | " | 22.5 | 28.1 | " | (3) 8.7 ± 0.9 |
| (3/10/60) | | | | | |
| ACTH | | 1.0 mU. | | (4) 2.4 ± 0.1 | (4) 26.7 ± 2.6 |
| | | 0.25 mU. | | " | (4) 8.1 ± 2.4 |
| Fr1-22 | 1.25 | 112.5 | 140.5 | " | (3) 23.6 ± 5.8 |
| | " | 28.1 | 35.1 | " | (4) 13.1 ± 3.2 |

[a]Number of rats.

[b]Standard error of the mean.

No melanophoretic activity was observed in F21-22 after injection of 2.5 µg./dose in the in vivo frog assay of Landgrebe and Waring (83).

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

The starting material for these electrophoresis runs had been fractionated previously by countercurrent distribution in two solvent systems. Kamm product was distributed in butanol:acetic acid:water (4:1:5) (CCD 47). Then tubes 2 and 3 of CCD 47 were submitted to countercurrent distribution in sec-butanol:0.5% trichloroacetic acid (1:1). Four separate distributions (CCD 48, 49, 50 and 51) were made to provide enough material for electrophoresis. The pressor peak of each CCD was located by assay, and the tubes from the descending part of the pressor curves were pooled (86).

CE 225 was run on 0.6108 g. from CCD 48 and CCD 49. The pressor activity of the material from CCD 48 was 9.7 U./mg. A pooled sample from CCD 50 and CCD 51 of 0.5338 g. was used for CE 226. The weight and pressor results for CE 225 and CE 226 are listed in Table 13. The weight recovery was 67% for the combined experiments. The highest pressor activity was found in F15-16 in both runs.

In order to compare the CRF activities of the continuous electrophoresis samples, F13-14 of CE 225 was arbitrarily chosen as a standard. One milliunit (CE mU.) of CRF activity

Table 13. Weight and pressor results for CE 225 and CE 226

| Fraction | Weight mg. | Pressor activity U./mg. |
|---|---|---|
| CE 225 | | |
| 11-12 | 36.4 | 2.0 |
| 13-14 | 63.3 | 17.7 |
| 15-16 | 49.4 | 50.0 |
| 17-18 | 39.3 | 13.4 |
| 19-20 | 40.0 | 1.6 |
| 21-22 | 46.4 | 1.4 |
| 23-24 | 31.5 | 1.1 |
| 25-26 | 22.8 | 0.8 |
| CE 226 | | |
| 3-4[a] | 12.2 | |
| 5-6[a] | 21.2 | |
| 7-8[a] | 25.5 | 0.04 |
| 9-10[a] | 47.3 | 0.10 |
| 11-12 | 37.7 | 4.8 |
| 13-14 | 54.3 | 47.8 |
| 15-16 | 43.1 | 76.0 |
| 17-18 | 32.5 | 10.1 |
| 19-20 | 29.5 | 0.13 |
| 21-22 | 33.8 | 1.1 |
| 23-24 | 27.6 | |
| 25-26 | 19.4 | |
| 27-28[a] | 16.8 | 1.2 |
| 29-30[a] | 19.9 | 1.9 |

[a]These fractions were combined from CE 225 and CE 226.

was defined as the increase in cpd. B/100 ml. plasma over the controls given by 1 μg. of F13-14. The results of an *in vivo* CRF assay of F13-14 at dose levels of 0.9, 1.8, 3.6 and 7.2 μg. are listed in Table 14a. The log dose response curve obtained from the CRF data is drawn in Figure 1.

An ACTH assay of CE 225, F13-14 in 24 hour hypophysec-

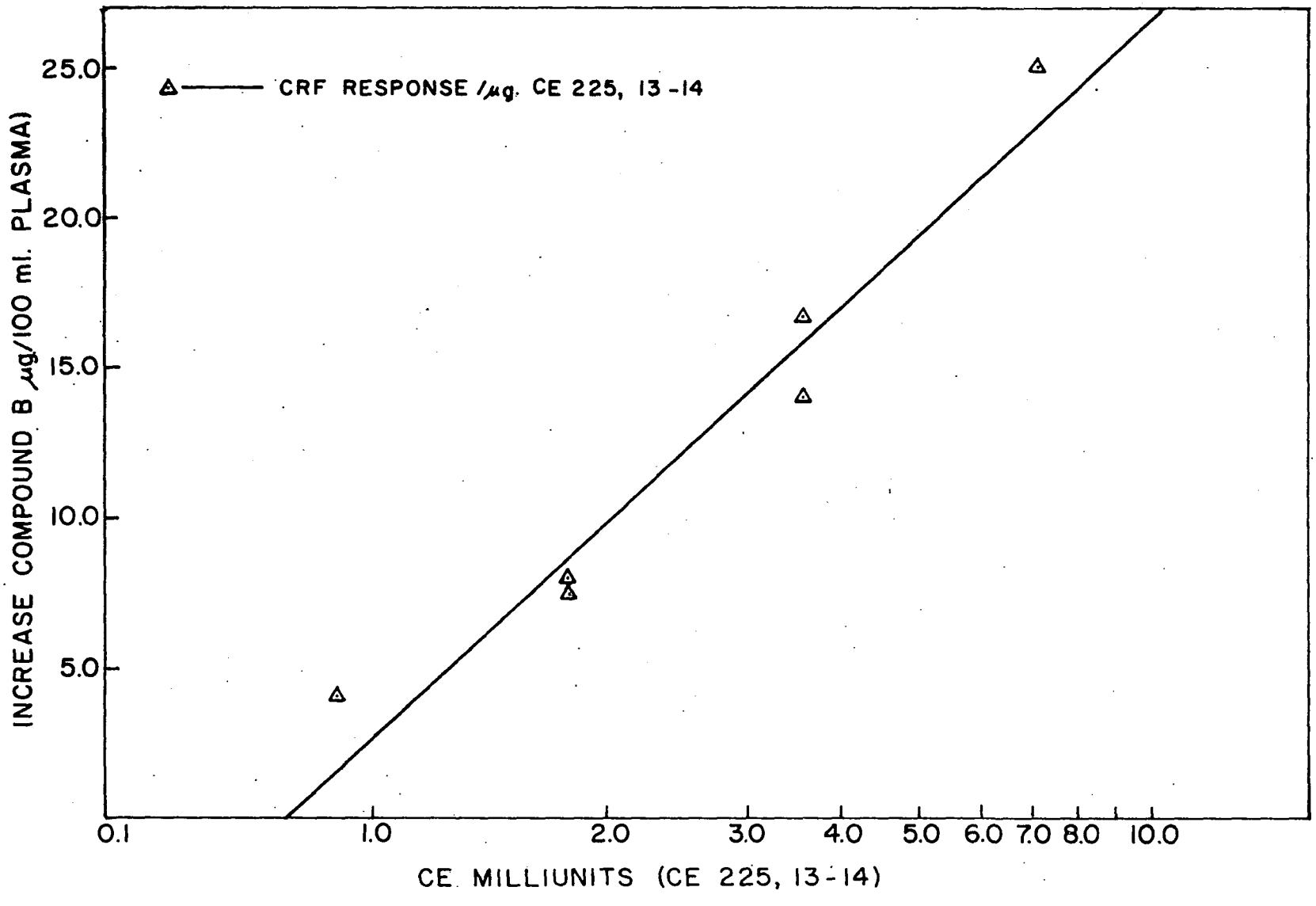
Table 14a. Assays of CE 225 (F13-14) for CRF activity and ACTH activity

| Sample | µg./inj. | Pressor activity mU./mg. | Pressor dosage mU./inj. | Plasma - cpd. B µg./100 ml. Control | Sample | Cpd. B incr. |
|---|---|---|---|---|---|---|
| 13-14 | 0.9 | 17.7 | 15.95 | (4)[a] 4.25 ± 1.2[b] | (4) 8.3 ± 1.7 | 4.05 |
| | 1.8 | " | 31.9 | " | (4) 11.6 ± 1.3 | 7.35 |
| | 3.6 | " | 63.7 | (4) 5.25 ± 1.1 | (4) 21.8 ± 1.3 | 15.55 |
| | 7.2 | " | 127.3 | " | (4) 30.4 ± 1.6 | 25.15 |
| ACTH | 0.25 mU. | | | (3) 2.85 ± 0.3 | (5) 13.6 ± 3.1 | |
| | 1.0 mU. | | | " | (5) 23.4 ± 1.6 | |
| 13-14 | 0.9 | 17.7 | 15.95 | " | (5) 2.9 ± 0.2 | 0.0 |
| | 3.6 | " | 63.7 | " | (8) 4.0 ± 0.4 | 1.15 |

[a] Number of rats.

[b] Standard error of the mean.

Figure 1. Increase in plasma corticosterone in nembutal-morphine blocked rats due to CE 225, F13-14


CRF RESPONSE /μg. CE 225, 13-14
INCREASE COMPOUND B μg/100 ml. PLASMA)
25.0
20.0
15.0
10.0
5.0
0.1
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
10.0
CE. MILLIUNITS (CE 225, 13-14)

tomized rats also is shown in Table 14a. This fraction had negligible ACTH activity.

The CRF assays of the fractions of CE 225 were all performed at pressor levels of 30 mU. per injection. This was the level recommended by Schally and Guillemin (141) as free from CRF activity due to vasopressin. The results of these assays are shown in Table 14b.

The CE units for CRF activity were calculated using the provisional CE 225, F13-14 standard. A graph (Figure 2) of the pressor activities and the CRF activities of the fractions from CE 225 demonstrates that the peaks of both activities occur at F15-16. Continuous electrophoresis did not separate the two activities. It was also noted that many of the fractions gave similar per cent increases in corticoids at the constant pressor dosages of 30 mU.

Continuous electrophoresis of a countercurrent distribution fraction (CE 236)

Another continuous electrophoresis run (CE 236) was made on material from tube 1 of the countercurrent distribution (CCD 47) in butanol:acetic acid:water (4:1:5) of Kamm product. This starting material had 6.3 pressor units per milligram; 4.6407 g. were run under the same conditions as CE 208-2,3. The weight recovery was 72%.

The MSH activities of these fractions were tested with the *in vivo* frog assay of Landgrebe and Waring (83). Because

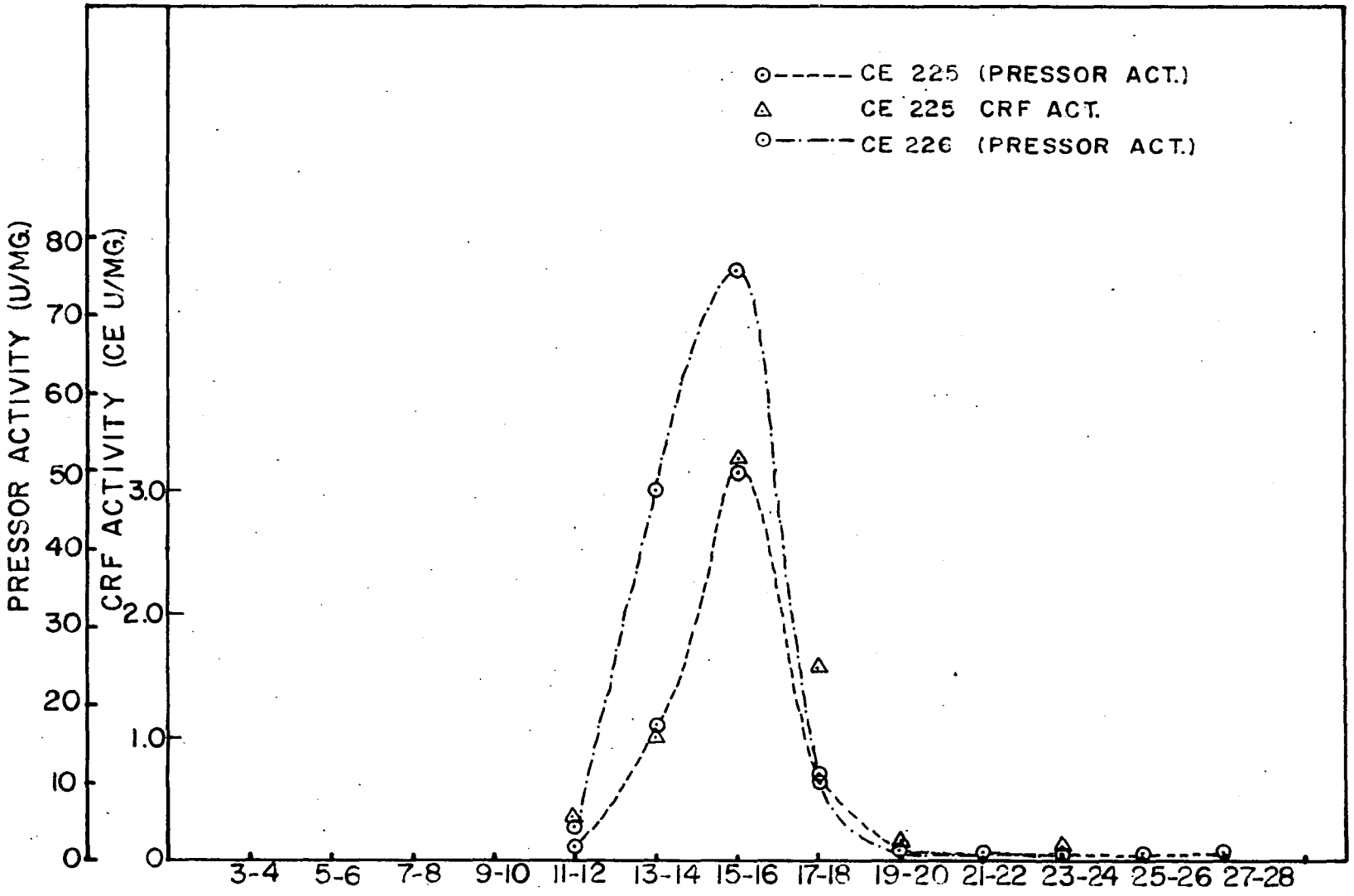
Table 14b. CRF assays of fractions from CE 225

| Fraction | Pressor activity U./mg. | µg./inj. | Pressor dosage mU./inj. | Plasma - cpd. B µg./100 ml. Control | Sample | Cpd. B incr. | CE mU. per inj. | CE U. per mg. | % incr. |
|---|---|---|---|---|---|---|---|---|---|
| 11-12 | 2.0 | 15 | 30 | (4)[a] 6.25±1.5[b] | (4) 26.6±2.8 | 20.35 | 5.5 | 0.37 | 330 |
| 13-14 | 17.4 | 1.8 | 31 | | (5) 15.3±1.8 | 9.05 | 1.85 | 1.0 | 145 |
| 15-16 | 50.0 | 0.6 | 30 | | (5) 15.45±2.3 | 9.20 | 1.87 | 3.12 | 150 |
| 17-18 | 12.4 | 2.4 | 30 | (5) 6.2±0.5 | (5) 22.2±2.3 | 16.0 | 3.6 | 1.5 | 260 |
| 19-20 | 1.6 | 18.0 | 29 | | (5) 17.6±0.7 | 11.4 | 2.3 | 0.13 | 180 |
| 21-22 | 1.4 | 21.0 | 29 | | (5) 16.2±2.7 | 10.0 | 2.03 | 0.10 | 160 |
| 23-24 | 1.1 | 27.0 | 31 | (4) 5.3±0.5 | (4) 19.6±2.2 | 14.3 | 3.08 | 0.11 | 270 |
| 25-26 | 0.8 | 36.0 | 29 | | (5) 18.2±1.4 | 12.9 | 2.67 | 0.08 | 240 |
| 27-28 | 1.2 | 24.0 | 29 | | (5) 13.3±1.7 | 8.0 | 1.7 | 0.07 | 150 |

[a]Number of rats.

[b]Standard error of the mean.

Figure 2. Pressor and CRF activities of continuous electrophoresis fractions (CE 225 and CE 226)

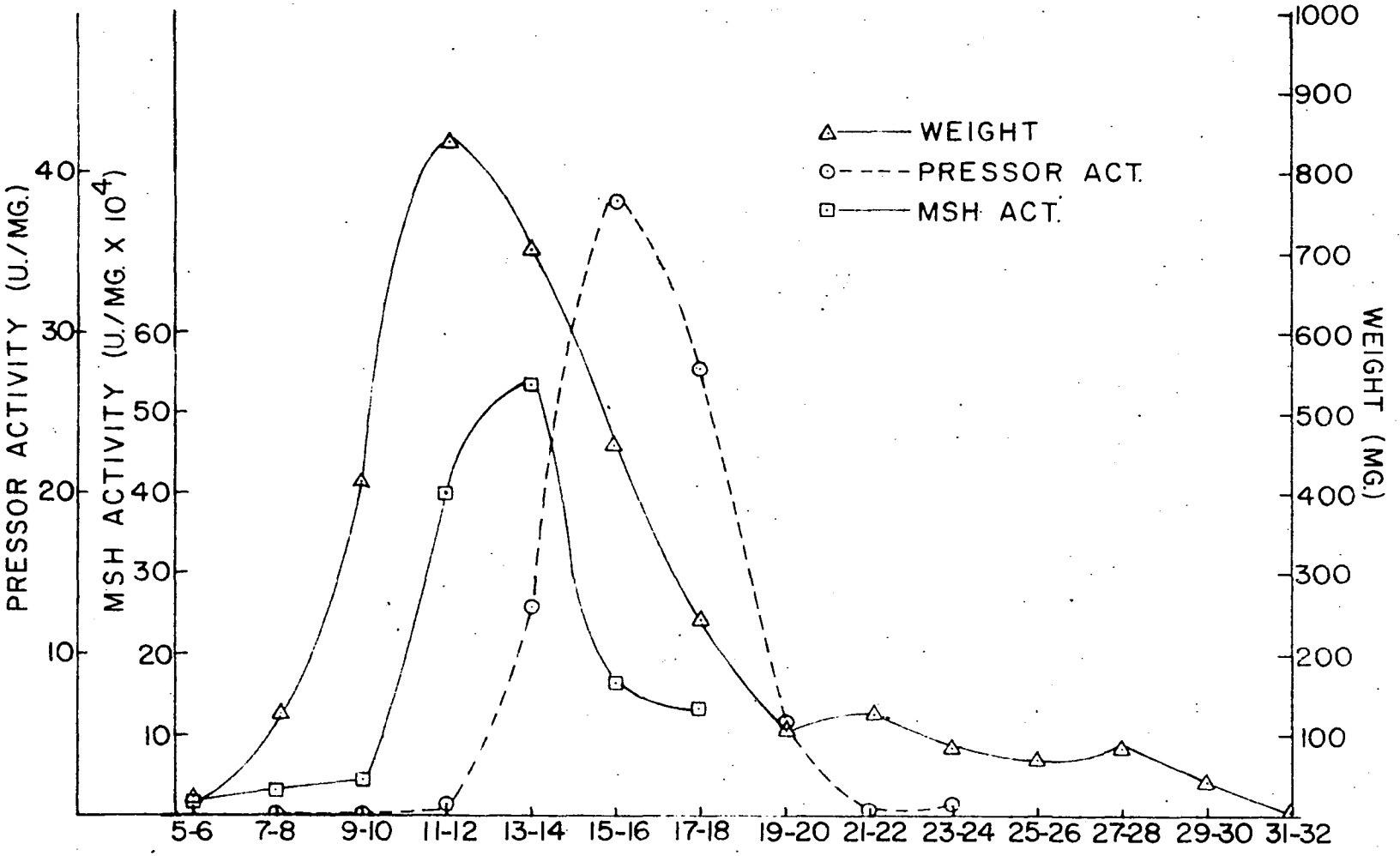

⊙----- CE 225 (PRESSOR ACT.)
△ CE 225 CRF ACT.
⊙-·-·- CE 226 (PRESSOR ACT.)
PRESSOR ACTIVITY (U/MG.)
CRF ACTIVITY (CE U/MG.)
0
10
20
30
40
50
60
70
80
1.0
2.0
3.0
3-4
5-6
7-8
9-10
11-12
13-14
15-16
17-18
19-20
21-22
23-24
25-26
27-28

this assay was only semiquantitative, the MSH activities were only approximations. The weight, pressor and MSH results for CE 236 are shown in Table 15 and graphed in Figure 3. The peak of pressor activity was at F15-16, and the peak of MSH

Table 15. Weight, pressor and MSH results of CE 236

| Fraction | Weight mg. | Pressor activity U./mg. | MSH activity U./mg. x $10^4$ |
|---|---|---|---|
| 1-2 | 11.1 | | |
| 3-4 | 11.0 | | |
| 5-6 | 16.0 | | 1.6 |
| 7-8 | 120.7 | 0.08 | 3.2 |
| 9-10 | 415.5 | 0.10 | 4.25 |
| 11-12 | 838.9 | 0.4 | 40 |
| 13-14 | 702.3 | 12.9 | 53.5 |
| 15-16 | 459.8 | 38.2 | 16.25 |
| 17-18 | 242.5 | 27.7 | 12.5 |
| 19-20 | 106.7 | 5.9 | |
| 21-22 | 127.6 | 0.16 | |
| 23-24 | 81.9 | 0.72 | |
| 25-26 | 68.1 | | |
| 27-28 | 86.5 | | |
| 29-30 | 44.7 | | |
| 31-32 | 1.9 | | |

Figure 3. Pressor and MSH activities of continuous electrophoresis fractions (CE 236)


PRESSOR ACTIVITY (U./MG.)
MSH ACTIVITY (U./MG. X $10^4$)
WEIGHT (MG.)
WEIGHT
PRESSOR ACT.
MSH ACT.
40
30
20
10
60
50
40
30
20
10
1000
900
800
700
600
500
400
300
200
100
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

activity was at F13-14. No CRF assays were performed on the fractions of this run.

Additional experiments on continuous electrophoresis fractions

Continuous electrophoresis samples were submitted to further fractionation by other methods to determine if the CRF activity could be separated from the vasopressin. These experiments were carried out by Eugene Lazzari and his assistant and are described in detail in his Ph. D. thesis (86).

Paper chromatography of CE 225, F17-18 (PC-120) An EDTA-washed Whatman No. 1 paper was streaked with a sample of CE 225, F17-18 and developed in butanol:acetic acid:water (4:1:5). The chromatogram was cut into segments which were eluted with 1% cold acetic acid. When pressor and CRF assays were performed on the eluates, the highest concentrations of pressor activity and CRF activity were found at the same Rf of 0.25.

Ion exchange chromatography of CE 225 and 226, F15-16 (IRC 50-19) The pressor peak (F15-16) was chromatographed on IRC 50 cation exchange resin. When the column was eluted with 0.5 M ammonium acetate at pH 6.4, the vasopressin peak came off at 3.2 holdup volumes. The maximum CRF activity also was found in this fraction.

The pressor and CRF peak from the IRC 50 column then was submitted to countercurrent distribution (CCD 52) in 0.5%

trichloroacetic acid equilibrated with an equal volume of sec-butanol. These CCD fractions were assayed twice for CRF on different days. Both assays gave the same result. The peak of CRF activity coincided with the pressor peak.

A sample from the pressor and CRF peak of the IRC 50 column also was streaked on paper. The chromatogram (PC 121) was developed in acetone:water (3:2 v/v), and segments of the paper were eluted with 0.1% acetic acid. Assays of the eluted samples again showed the CRF activity in the same location as the pressor activity at an Rf of 0.77.

For these experiments the CRF assays were carried out on samples with 30 mU. of pressor activity per injection. At this pressor dosage similar increases in cpd. B over controls were noticed for almost all the samples assayed. This observation and the failure to achieve separation of CRF activity from vasopressin by any of the fractionation procedures prompted an investigation of the inherent corticotropin-releasing activity of vasopressin.

## Corticotropin-releasing Activity of Synthetic Lysine Vasopressin

### du Vigneaud synthetic lysine vasopressin

Synthetic lysine vasopressin was chosen to test the corticotropin-releasing ability of vasopressin in the *in vivo* CRF assay. With synthetic material there was no possibility

of contaminants with CRF activity which might be present in vasopressin isolated from pituitary sources.

The synthetic vasopressin (JM V 68/16) had been prepared in the laboratories of Dr. V. du Vigneaud. The entire sample was weighed, dissolved in deionized water and assayed for pressor activity. Two pressor assays gave an average value of 268 U./mg. The solution was then divided into aliquots, lyophilized and stored in a deep freeze. Assays for CRF were carried out on solid samples which had been diluted in physiological saline just before each assay.

The results from the injection into nembutal-morphine clocked rats of four dose levels of synthetic lysine vasopressin are given in Table 16. The controls include both uninjected rats and saline injected rats, because statistical analyses of the two types of controls showed no significant difference between them. At the lowest dosage of vasopressin, 8.4 mU., the slight increase in plasma-cpd. B was insignificant. At the three higher doses, 16.8 mU., 33.5 mU. and 67 mU., a significant increase in cpd. B was found in each case. The data for these three dose levels were submitted to analysis of variance. A value of 11.4 was obtained for F significant at the 0.1% level.

Figure 4 shows the regression line calculated from these data. The regression equation was $y = -134 + 14.5(x)$. The per cent increase in plasma corticosterone is plotted versus

Table 16. Effect of du Vigneaud synthetic lysine vasopressin on the plasma corticosterone of rats

| Pressor dosage mU./inj. | Plasma - cpd. B µg./100 ml. Control | Sample | Cpd. B increase | p value[a] | % increase over control |
|---|---|---|---|---|---|
| 8.4 | (7)[b] 8.0 ± 0.8[c] | (10) 8.5 ± 1.1 | 0.5 | .8>p>.7 | -- |
| 16.8 | (18) 8.2 ± 0.7 | (19) 11.4 ± 0.7 | 3.2 | .01>p>.001 | 39 |
| 33.5 | (10) 8.1 ± 0.8 | (9) 14.7 ± 1.4 | 6.6 | <.001 | 81 |
| 67.0 | (7) 8.1 ± 1.4 | (10) 18.2 ± 1.4 | 10.1 | <.001 | 125 |

[a]p calculated from t on values for plasma cpd. B in controls and in sample injected rats.

[b]Number of rats.

[c]Standard error of the mean.

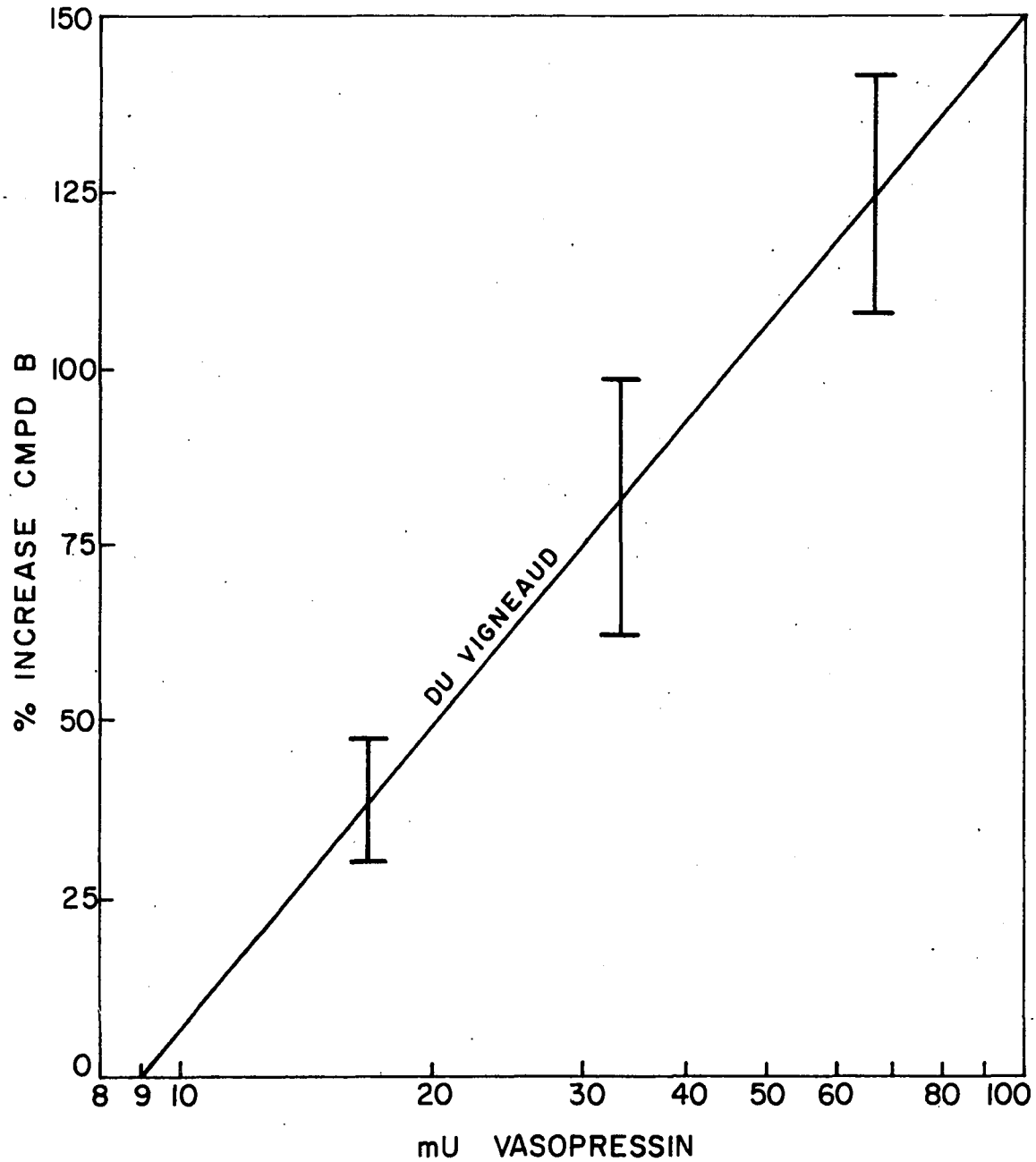



Figure 4. ACTH-releasing activity of du Vigneaud synthetic lysine vasopressin in nembutal-morphine blocked rats

the log of the vasopressin dose. The bars indicate the standard errors of the means at the three dose levels.

McCann and Fruit (95) investigated the ACTH-releasing activity of synthetic vasopressin, but they used rats with acute hypothalamic lesions and depletion of adrenal ascorbic acid as the index of ACTH release. With their assay system lysine vasopressin gave a linear log-dose versus response curve from 100 mU. to 1000 mU. When Guillemin et al. (51) tested highly purified lysine vasopressin (287 U./mg.) in the nembutal-morphine blocked rat, no significant increase in plasma-cpd. B was found at any dose level below 80 mU. Above 80 mU. a large discharge of ACTH was observed increasing linearly with the log-dose of vasopressin.

Rather than indicating a threshold at 80 mU., the present data extend the linear response curve of ACTH release versus log dose of vasopressin down to dose levels between 17.8 to 67 mU. of vasopressin. Synthetic lysine vasopressin is active in releasing ACTH at levels of .08 to .26 μg. The corticotropin-releasing activity of lysine vasopressin exceeds that reported by Schally et al. (140) for their purest preparations of α-CRF and β-CRF. The minimum effective dose in the in vivo assay of α-CRF was 2-3 μg. and of β-CRF was 0.1 μg.

After the high inherent corticotropin-releasing activity of lysine vasopressin was discovered, a re-evaluation was made of all the assays which had been carried out during the

unsuccessful CRF isolation work. According to the synthetic lysine vasopressin data when these samples were assayed at 30 mU. of pressor activity an increase of 6.1 μg. of cpd. B over the level of the controls was due to the CRF activity of the vasopressin present. If a β-CRF was also present in these samples, the additional CRF activity should be apparent in the assay results. Table 17 lists the assays performed on samples from the series of experiments which started with CE 226. For the 21 assays an average value of 8.5 was obtained for the controls and 15.2 for the samples. This gives an increase in cpd. B of 6.6 μg./100 ml. plasma. In view of the wide variation in controls and large standard errors of the means which occurred in many of these in vivo assays, this value shows remarkable agreement with the increase in cpd. B given by synthetic lysine vasopressin. It appears that essentially all the CRF activity of these samples was due to their pressor content. The only sample not included in these calculations was IRC 50-19, tube 1. This sample had MSH activity and therefore, its high CRF activity was probably due to both α-CRF and vasopressin. If β-CRF exists in porcine posterior pituitary preparations, it must have been inactivated early in the isolation procedure or it must be a weaker CRF than vasopressin.

Table 17. CRF assays of fractions obtained in purification procedure

| Fraction or tube | µg./inj. | Pressor U./ml. or mg.* | Pressor dosage mU./inj. | Plasma - cpd. B µg./100 ml. Control | Plasma - cpd. B µg./100 ml. Sample | Cpd. B incr. | % incr. |
|---|---|---|---|---|---|---|---|
| CE 226 | | | | | | | |
| 13-14 | 1.8 | 17.4* | 31 | (4)[a] 6.3±1.5[b] | (5) 15.3±1.8 | 9.05 | 145 |
| 15-16 | 0.6 | 50.0* | 30 | (4) 6.3±1.5 | (5) 15.5±2.3 | 9.2 | 150 |
| PC 120 | | | | | | | |
| 14 | | 10.5 | 25 | (5) 7.3±0.5 | (5) 10.8±0.4 | 3.5 | 50 |
| IRC 50-19 | | | | | | | |
| 1 | 60.0 | 0.48* | 30 | (5) 4.6±0.6 | (5) 24.6±1.3 | 20.0 | 435 |
| 2 | 3.0 | 8.0* | 24 | (3) 5.7±0.85 | (3) 11.75±0.3 | 6.05 | 108 |
| 3 | 0.17 | 160.0* | 27 | (5) 4.6±0.6 | (5) 13.1±2.5 | 8.05 | 185 |
| IRC 50-19 Peak 3 | | | | | | | |
| 86 | | 49 | 29 | (4) 6.0±0.9 | (4) 12.3±2.0 | 6.3 | 105 |
| 91 | | 66 | 31.5 | (4) 6.0±0.9 | (4) 11.1±1.2 | 5.1 | 85 |
| 96 | | 92 | 30 | (4) 6.0±0.9 | (4) 8.3±2.8 | 2.3 | 38 |
| 96 | | 91 | 30 | (5) 4.6±0.4 | (5) 9.0±1.3 | 4.75 | 95 |
| PC 121 | | | | | | | |
| 20 | | 0.7 | 30 | (2) 6.4±0.6 | (5) 8.4±0.8 | 2.0 | 31 |
| 22 | | 4.3 | 30 | (4) 7.6±0.4 | (4) 12.25±0.9 | 4.65 | 61 |
| 24 | | 0.2 | 30 | (2) 6.4±0.6 | (4) 18.0±2.5 | 11.6 | 181 |
| | | | | (4) 7.6±0.4 | (3) 11.8±1.6 | 4.2 | 55 |

[a]Number of rats.

[b]Standard error of the mean.

Table 17. (Continued)

| Fraction or tube | µg./inj. | Pressor U./ml. or mg.* | Pressor dosage mU./inj. | Plasma - cpd. B µg./100 ml. Control | Plasma - cpd. B µg./100 ml. Sample | Cpd. B incr. | % incr. |
|---|---|---|---|---|---|---|---|
| CCD 52 (7/29/60) | | | | | | | |
| 43 | | 10.6 | 30 | 6.6 (calc.) | (3) 12.0±1.65 | 5.4 | 82 |
| 47 | | 16.0 | 29 | " | (4) 12.7±1.3 | 6.1 | 92.5 |
| 51 | | 22.0 | 28 | " | (3) 12.3±1.4 | 5.7 | 86.5 |
| 55 | | 15.0 | 30 | " | (3) 11.0±2.8 | 4.4 | 66.8 |
| 59 | | 4.4 | 30 | " | (4) 11.6±0.6 | 5.0 | 76 |
| (8/19/60) | | | | | | | |
| 43 | | 10.6 | 30 | (4) 7.1±1.0 | (3) 14.1±2.4 | 7.0 | 98.5 |
| 47 | | 16.0 | 30 | " | (3) 19.5±2.3 | 12.4 | 175 |
| 51 | | 22.0 | 30 | " | (4) 17.9±2.4 | 10.8 | 152 |
| 55 | | 15.0 | 31 | " | (4) 17.6±4.7 | 10.5 | 148 |

Sandoz synthetic lysine vasopressin

Another preparation of synthetic lysine vasopressin was donated by Sandoz Pharmaceuticals Company. The material was received in solution in sealed vials. Each vial was stated to contain 10,000 mU. of pressor activity in 1 cc. of saline. Two assays in our laboratory gave 9,620 mU./cc. and 10,540 mU./cc. with a mean value of 10,080 mU./cc. The corticotropin-releasing activity of this preparation was tested in the in vivo assay. The results at nine different pressor levels are shown in Table 18.

An analysis of variance of these data gave a value of 3.6 for F, significant at the 0.5% level. The regression line calculated for Sandoz lysine vasopressin was $y = -280 + 214(x)$. The regression coefficient was significant at the 0.1% level. Figure 5 compares the regression line for Sandoz vasopressin with the line obtained for the du Vigneaud vasopressin. Both synthetic lysine vasopressins show CRF activity in the same range of pressor dosages. The greater variation of controls and sample values in the bioassays of the Sandoz vasopressin may account for the difference in the slopes of the two lines. The log-dose response curve obtained with the du Vigneaud vasopressin is probably the more reliable.

Table 18. Effect of Sandoz synthetic lysine vasopressin on plasma corticosterone of rats

| Pressor dosage mU./inj. | Plasma - cpd. B µg./100 ml. Control | Sample | Cpd. B increase | % increase over control |
|---|---|---|---|---|
| 22 | (8)[a] 9.5 ± 0.7[b] | (7) 10.5 ± 0.6 | 1.0 | 10.5 |
| 24 | (7) 9.3 ± 1.2 | (10) 12.3 ± 1.5 | 3.0 | 32 |
| 29 | (5) 8.5 ± 0.8 | (6) 17.4 ± 3.1 | 8.9 | 105 |
| 30 | (7) 9.6 ± 1.1 | (9) 13.5 ± 1.3 | 3.9 | 41 |
| 43.5 | (8) 10.3 ± 1.0 | (9) 17.6 ± 2.6 | 7.3 | 71 |
| 47 | (6) 10.85 ± 2.0 | (6) 20.0 ± 1.85 | 9.15 | 84 |
| 53 | (6) 9.7 ± 1.1 | (7) 21.0 ± 2.8 | 11.3 | 116 |
| 58 | (5) 8.5 ± 0.8 | (7) 19.2 ± 3.6 | 10.7 | 126 |
| 70 | (7) 11.6 ± 1.05 | (8) 27.6 ± 2.35 | 16.0 | 138 |

[a]Number of rats.

[b]Standard error of the mean.

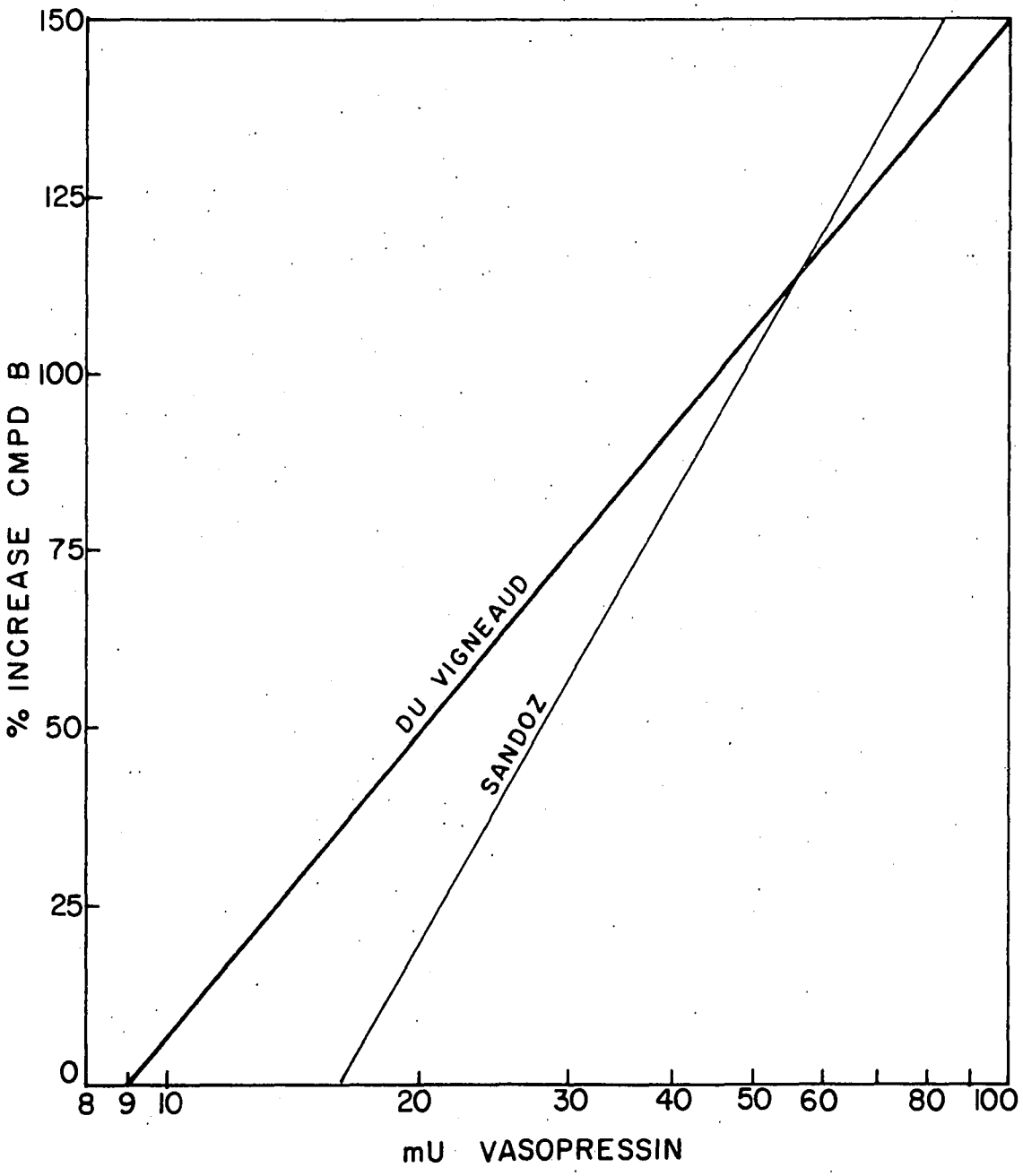



Figure 5. ACTH-releasing activities of du Vigneaud and Sandoz synthetic lysine vasopressins

ACTH assay of synthetic lysine vasopressin

The increase in plasma corticoids in the in vivo assay could have been the result of a direct ACTH-like effect on the adrenals by vasopressin. In order to test this possibility du Vigneaud lysine vasopressin was injected into hypophysectomized rats. A dose of 67 mU. was used. This was the highest dose that had been injected into the nembutal-morphine blocked rats. The results in the two types of assay animals are compared in Table 19. In the hypophysectomized rats the control

Table 19. Effect of synthetic lysine vasopressin in nembutal-morphine blocked and hypophysectomized rats

| Assay Animal | Pressor activity mU./dose | Plasma - cpd. B µg./100 ml. Control | Sample | Cpd. B increase |
|---|---|---|---|---|
| Nem.-mor. | 67 | (7)[a] 8.1±1.4[b] | (10) 18.2±1.4 | 10.1 |
| Hyp. | 67 | (11) 2.4±0.1 | (9) 2.5±0.1 | 0.1 |

[a]Number of rats.

[b]Standard error of the mean.

values were lower, but the injection of synthetic vasopressin gave no increase in cpd. B over the controls.

Previously Hilton et al. (63) obtained an increase in adrenal venous hydrocortisone during direct arterial infusion

of vasopressin into the adrenals of hypophysectomized dogs. Royce and Sayers (125) found that large intravenous doses (2.5-5.0 U.) of purified arginine vasopressin or of commercial Pitressin depleted adrenal ascorbic acid in hypophysectomized rats. However, in the hypophysectomized guinea pig 5 U. of Pitressin injected intraperitoneally had no effect (148). McCann and Haberland (96) were unable to demonstrate an extrapituitary effect of vasopressin in a decapitated rat with a dose of 500 mU. of Pitressin. Guillemin et al. (48) found no increase in plasma corticosteroid levels with intravenous injections of 300 mU. of arginine vasopressin into hypophysectomized rats. Lower doses of vasopressin injected intravenously appeared to have no direct effect on the adrenals.

The results with the du Vigneaud vasopressin support the latter findings and indicate that the observed increase in corticoids after injection of synthetic lysine vasopressin is a true CRF effect.

MSH assay of synthetic lysine vasopressin

The Sandoz synthetic lysine vasopressin was tested for MSH activity in the in vivo frog assay of Landgrebe and Waring (83). Four dose levels (25 mU., 100 mU., 400 mU. and 800 mU.) were injected. No MSH activity was observed.

## Pressor and Corticotropin-releasing Activities of Synthetic Lysine Vasopressin after Inactivation by Various Agents

### Temperature inactivation

One of the problems in this study was that it was almost impossible to do both the pressor and the CRF bioassays at the same time. The pressor determination on the CRF sample was made the afternoon following a CRF assay, if possible, but often 24 hours earlier or later. Sometimes, if the pressor rat or rats gave poor results, there was an even longer period of time between the two assays. Therefore, an investigation was made of the stabilities of the pressor and corticotropin-releasing activities under various temperatures and storage conditions.

In one experiment solutions of du Vigneaud vasopressin, which had been stored in the freezer for two to five weeks, were pooled. The pressor assay of the pooled solutions showed a loss of 28% of the original pressor activity. This solution then was left at room temperature for 48 hours. After this treatment the pressor activity remained the same. The solution was frozen and thawed three times in the next 48 hours, left at room temperature for 48 hours and then heated in an oven at 37° C. for 26 1/2 hours. At this time it had lost 23% of its pressor activity. Table 20 summarizes these results.

Table 20. Inactivation of synthetic vasopressin by storage and temperature treatments

| Treatment | Pressor activity mU./dose | Plasma - cpd. B µg./100 ml. Control | Sample | % incr. | % incr. from graph |
|---|---|---|---|---|---|
| None | 67 | (7)[a] 8.1±1.4[b] | (10) 18.2±1.4 | 125 | -- |
| Freezer inact. 2-5 wks. | 48 | (8) 8.5±0.9 | (6) 14.7±1.0 | 72 | 104 |
| Room temp. 48 hrs. | 48 | (8) 7.6±1.0 | (7) 14.4±0.8 | 89 | 104 |
| 37° C. 26 1/2 hrs. | 37 | (5) 5.3±0.6 | (7) 14.3±1.3 | 170 | 185[c] |

[a]Number of rats.

[b]Standard error of the mean.

[c]Taken from graph with 5.8 controls.

The loss of CRF activity appeared to parallel the loss of pressor activity under all these treatments. The last column in the table gives the value taken from the graph of per cent increase in cpd. B versus the log-dose of du Vigneaud vasopressin (Figure 3). This value represents the percentage increase that would be expected from the mU. of pressor activity injected. The experimental per cent increase was lower than the expected value in each case, but the small difference was probably a reflection of the variations encountered in the pressor and CRF assays rather than differential destruction of

the activities.

Another du Vigneaud vasopressin solution which had been frozen for seven weeks retained only 55% of its original pressor activity. There was no loss of activity after 46 hours of incubation at 37° C., but after an additional 50 hours only 60% of the activity was still present. Table 21 shows these results. Again the loss of CRF potency was consistent with the destruction of pressor activity.

Table 21. Inactivation of synthetic vasopressin at -15° C. and 37° C.

| Treatment | Pressor activity mU./dose | Plasma - cpd. B µg./100 ml. Control | Sample | % incr. | % incr. from graph |
|---|---|---|---|---|---|
| None | 67 | | | | |
| Freezer inact. 7 wks. | 37 | | | | |
| 37° C. 46 hrs. | 37 | (10)[a] 7.8±0.5[b] | (7) 13.3±1.5 | 71 | 88 |
| 37° C. 116 hrs. | 22 | (6) 6.9±0.8 | (4) 10.0±0.6 | 56 | 55.5 |

[a]Number of rats.

[b]Standard error of the mean.

These experiments indicated that both pressor and CRF activities were relatively stable for several days. Therefore, the pressor assay could be performed within a day or two of the CRF assay with accurate results. However, there

was a slow loss of both activities even when the samples were stored in the deep-freeze, and samples should be reassayed if frozen more than one to two weeks.

Acid and base inactivation

The inactivation of vasopressin in acidic and basic solutions was also studied. At room temperature the pressor activity appeared to be fairly stable for long periods of time in either a weakly acidic or basic solution. After 18 hours at pH 3 a solution of du Vigneaud vasopressin, which originally assayed 123 mU./cc., lost only 19% of its pressor activity. In alkaline solution the pressor activity was constant for 24 hours. Table 22 shows these results.

Table 22. Stability of pressor activity in alkaline solution at room temperature

| Solution no. | pH | Time hrs. | Pressor activity mU./cc. | Inactivation % |
|---|---|---|---|---|
| 70 | -- | 0 | 200 | -- |
| 73 | 11.5 | 1 | 196 | 2 |
| 74 | " | 5 3/4 | 172 | 14 |
| 75 | " | 18 | 193 | 35 |
| 76 | " | 24 | 194 | 3 |

The very slight variation in loss of activity may be due to the fact that a separate dilution of Sandoz vasopressin was prepared for each time period. Each solution was neutralized to a pH of 7 before the sample was injected into the pressor rat.

Sideman and Sobel (148) isolated a fraction from Pitressin that released ACTH in the guinea pig. They concluded that it was vasopressin, and part of their supporting evidence was that mild acid and alkaline hydrolysis altered the pressor activity and the CRF activity of the fraction to the same extent.

Sandoz vasopressin was hydrolyzed under conditions similar to those used by Sideman and Sobel. Physiological saline was acidified to a pH of 1.2 with hydrochloric acid. The acidic solution of 200 mU./cc. of Sandoz vasopressin was prepared and placed in a boiling water bath. After 30 minutes the solution was immediately cooled in ice water, and the pH was adjusted to 7.1 for injection. The alkaline solutions were handled in the same manner except that the pH was adjusted to 11.5 with sodium hydroxide, and one solution was left in the boiling water bath for 10 minutes and another for 20 minutes. The effects of basic and acidic hydrolysis on the corticotropin and pressor activities of synthetic lysine vasopressin are shown in Table 23. Sandoz vasopressin was much more resistant to acidic than basic hydrolysis. However,

Table 23. Effect of mild acid and alkaline hydrolysis on pressor and CRF activity of Sandoz vasopressin

| Treatment | Time min. | Pressor activity mU./dose | Plasma - cpd. B µg./100 ml. Control | Plasma - cpd. B µg./100 ml. Sample | % increase | % increase Sandoz graph |
|---|---|---|---|---|---|---|
| None | 0 | 60 | (6)[a] 8.5 ± 0.9[b] | (7) 24.9 ± 2.3 | 193 | 120 |
| pH 1.2 | 30 | 28.5 | (6) 8.5 ± 0.9 | (6) 13.4 ± 0.9 | 58 | 51 |
| pH 11.5 | 10 | 25 | (8) 10.2 ± 1.5 | (10) 16.3 ± 1.8 | 60 | 39 |
| " | 20 | 8.5 | -- | -- | | |

[a]Number of rats.

[b]Standard error of the mean.

under both conditions the destruction of CRF activity paralleled the decrease in pressor activity.

Thioglycolate reduction of vasopressin

Thioglycolate reduction of continuous electrophoresis pressor fraction (CE 208-2, F15-16) The effect of thioglycolate reduction on pressor activity was studied first on a crude electrophoresis sample. This fraction (F15-16) was the pressor peak of CE 208-2 and had a pressor activity of 50 U./mg.

A 0.05 M solution of thioglycolate was prepared, and the pH was adjusted to 7.9. Then 2.5 mg. of F15-16 were dissolved in 2.5 ml. of the thioglycolate solution. The calculated molar ratio was 275 moles of thioglycolate to one of lysine vasopressin. Every ten minutes a sample was taken from the thioglycolate solution and injected into the dibenamine-blocked rat. At the beginning of the experiment there was a rapid destruction of pressor activity, and 90% had been lost after two hours. At the end of four hours 97% had been destroyed. A plot of the inactivation versus time has been prepared in Figure 6.

Thioglycolate reduction of synthetic lysine vasopressin Thioglycolate reduction of synthetic lysine vasopressin was attempted under many different conditions of concentration and pH. A slow and reproducible method of pressor inactiva-

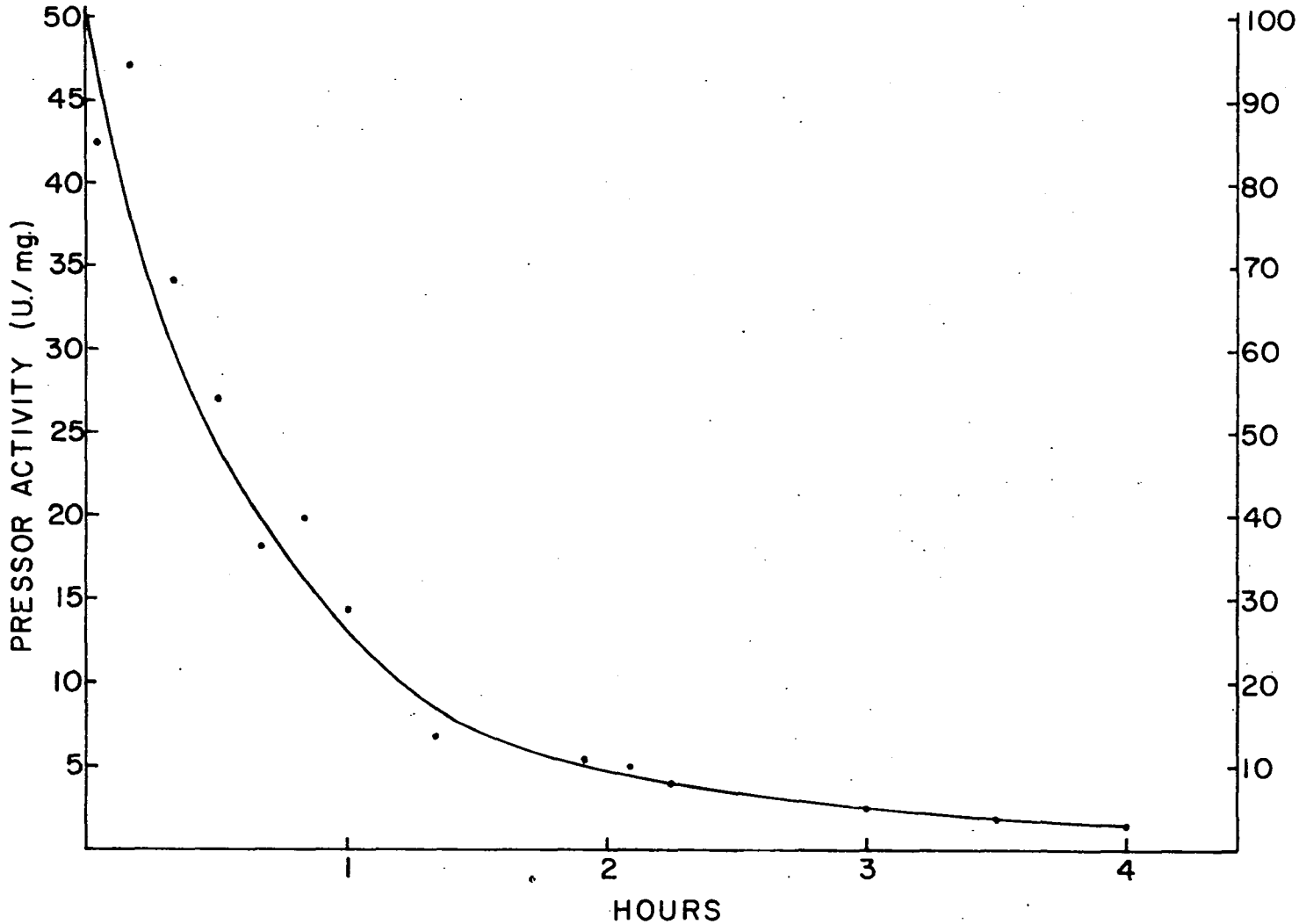



Figure 6. Thioglycolate inactivation of continuous electrophoresis pressor fraction (CE 208-2, F15-16)

tion was sought in order that the effect of disulfide bond rupture on the ACTH-releasing activity could be more carefully investigated.

Table 24 shows the results of experiments in which the number of moles of thioglycolate per mole of vasopressin was progressively increased. No adjustment was made in the pH of the solution.

Table 24. Effect of varying concentrations of thioglycolate on pressor and CRF activities

| Moles excess thio. | Pressor activity mU./dose | Plasma - cpd. B μg./100 ml. Control | Sample | % incr. | % incr. from graph |
|---|---|---|---|---|---|
| None | 51 | | | | |
| 1.25 | 46 | (6)[a] 11.0±1.1[b] | (7) 22.6±3.3 | 105 | 102 |
| 50 | ~46 | | | | |
| 150 | ~46 | (7) 9.5±1.4 | (9) 20.1±2.5 | 113 | 102 |

[a]Number of rats.

[b]Standard error of the mean.

The last two pressor assays were not reliable except as approximations, but it was clear that neither pressor nor CRF activity had been destroyed in these experiments.

The effects on the pressor activity of higher concentrations of thioglycolate and adjustment of the pH to 6.5 or 8

are shown in Table 25. The pressor activity was destroyed only after 96 hours with 500 moles excess thioglycolate at pH 8. During these experiments a solution of thioglycolate alone at the same concentration as in solution 13 was injected into the rat during the pressor assay. The thioglycolate had no effect on the blood pressure.

Table 25. Effect of concentration and pH on thioglycolate inactivation of vasopressin

| Sample | Moles thioglycolate per mole vasopressin | pH | Time hrs. | Pressor activity mU./μg. |
|---|---|---|---|---|
| Sol. B | None | | | 156 |
| Sol. 13 | 300 | 8.5 | 3 1/2 | ~185 |
| Sol. 14 | 500 | 8.5 | 8 | 138 |
| Sol. 15a | 500 | 8.0 | 24 | 156 |
| 15b | | | 96 | 9.5 |

Table 26 shows that there was also no decrease in the ACTH-releasing ability of these samples if the pressor activity was not destroyed by thioglycolate.

An alkaline solution and a high molar excess of thioglycolate apparently were necessary for inactivation of synthetic lysine vasopressin. With 600 moles of thioglycolate per mole of Sandoz vasopressin and a pH of 8 both pressor and CRF activities were destroyed. The data from these inactiva-

Table 26. Effect of pH and thioglycolate concentration on CRF activity

| Sample | Pressor activity mU./dose | Plasma - cpd. B µg./100 ml. Control | Sample | % incr. | % incr. from graph |
|---|---|---|---|---|---|
| Sol. 13 | 45 | (6)[a] 7.2±0.6[b] | (7) 15.6±1.4 | 91 | 100 |
| Sol. 14 | 54.5 | (6) 7.2±0.6 | (6) 11.0±1.1 | 54 | 83 |
| Sol. 15 | 39 | (7) 8.3±0.6 | (5) 16.2±2.0 | 95 | 91 |

[a]Number of rats.

[b]Standard error of the mean.

tions are shown in Table 27. The reduction by thioglycolate of the disulfide bond of vasopressin abolishes both pressor and CRF activities.

Pepsin inactivation studies

In 1958 Royce and Sayers (128) stated that the corticotropin-releasing activity of material they had isolated from the hypothalamus was destroyed by pepsin. The pressor activity, however, was retained. In order to determine if this interesting separation of activities could be shown with synthetic vasopressin, pepsin was incubated with synthetic lysine vasopressin in several experiments. The ratio of pepsin to milliunits of pressor activity (4 µg./cc. to 160 mU./cc.) was approximately that used by Royce and Sayers.

Table 27. Effect of 600 moles excess thioglycolate on pressor and CRF activity of synthetic vasopressin

| Sample | Pressor activity mU./dose | Time hrs. | Plasma - cpd. B µg./100 ml. Control | Sample | % increase | % increase Sandoz graph |
|---|---|---|---|---|---|---|
| Sol. 66-S | 75 | 0 | | | | |
| | 21 | 3 | (7)[a] 11.7 ± 0.4[b] | (8) 17.9 ± 2.0 | 53 | 23 |
| Sol. 34-V | 51 | 0 | | | | |
| | 3 | 24 | (6) 10.3 ± 1.7 | (10) 10.0 ± 0.9 | 0 | 0 |

[a]Number of rats.

[b]Standard error of the mean.

The vasopressin solutions were incubated with pepsin at 37° C. for 2 hours. Then the pH was changed to 7, and the solution was frozen until assayed. The CRF assays were performed first and then the pressor assays to determine if there had been any loss of pressor activity in storage or during the CRF bioassays. In Table 28 V represents du Vigneaud vasopressin and S Sandoz.

Table 28. Effect of pepsin on the CRF activity of synthetic lysine vasopressin

| Sample | Pressor dosage mU./dose | Plasma - cyd. B µg./100 ml. Control | Sample | % incr. | % incr. from graphs |
|---|---|---|---|---|---|
| Sol. 32V | 41 | (6)[a] 8.2±0.7[b] | (10) 17.3±1.2 | 110 | 95 |
| Sol. 49S | 47 | (8) 9.0±1.3 | (7) 20.3±1.4 | 126 | 98 |

[a]Number of rats.

[b]Standard error of the mean.

There was no loss of pressor activity after incubation with pepsin for two hours at 37° C. and also no destruction of CRF activity. Perhaps the pepsin-labile ACTH-releasing factor of Royce and Sayers was α-CRF. This factor appears to be structurally related to MSH and therefore would probably be degraded by pepsin.

## SUMMARY

1. Porcine posterior pituitary preparations were submitted to continuous electrophoresis, and the CE fractions were assayed for MSH, ACTH, pressor and CRF activities.
2. The CRF and pressor activities were not separated by continuous electrophoresis; the CE fraction with the highest pressor activity showed the greatest ACTH-releasing activity.
3. Corticotropin-releasing activity was measured in the Guillemin in vivo assay using nembutal-morphine blocked rats and fluorometric determination of plasma corticoids as the index of ACTH release.
4. All electrophoresis fractions, if assayed at a constant pressor dosage of 30 mU., gave increases in plasma level of corticosterone which were relatively constant within the range of the CRF assay.
5. du Vigneaud synthetic lysine vasopressin was assayed for ACTH-releasing activity; a linear response versus log-dose relationship was established between 16.8 and 67 mU. of vasopressin.
6. du Vigneaud vasopressin (262 U./mg.) was active in releasing ACTH at 0.06 $\mu$g, and is, therefore, more active than the purest preparations of $\alpha$-CRF and $\beta$-CRF reported in the literature.

7. Sandoz synthetic lysine vasopressin also gave a linear log-dose CRF response curve between pressor dosages of 22 mU. to 70 mU.
8. Injection of 67 mU. of du Vigneaud synthetic vasopressin into hypophysectomized rats gave no increase in plasma corticoids indicating that the effect of vasopressin in the in vivo CRF assay was a true corticotropin-releasing effect and not a direct action on the adrenals.
9. The CRF activity of the continuous electrophoresis fractions was accounted for by the inherent ACTH-releasing activity of the vasopressin present; no evidence for a $\beta$-CRF was found.
10. Solutions of synthetic lysine vasopressin showed no loss of CRF or pressor activity for 48 hours at -15° C., 25° C. or 37° C.; after 5-7 weeks of storage in a deep freeze 45-72% of both activities were recovered.
11. At 100° C., synthetic lysine vasopressin was more stable to hydrolysis at pH 1.2 than at pH 11.5; both pressor and corticotropin-releasing activities were destroyed to the same extent during hydrolysis.
12. Reduction of the disulfide ring of vasopressin at pH 8 by 600 molar excess thioglycolate abolished both CRF and pressor activities.
13. Pepsin did not destroy the pressor or the corticotropin-releasing activity of synthetic lysine vasopressin.

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# APPENDIX

## Abbreviations

| | |
|---|---|
| ACTH | Adrenocorticotropic hormone |
| ADH | Antidiuretic hormone (vasopressin) |
| CCD | Countercurrent distribution |
| CE | Continuous electrophoresis |
| Cpd. B | Corticosterone |
| CMC | Carboxymethyl cellulose |
| CRF | Corticotropin-releasing factor |
| MSH | Melanocyte-stimulating hormone |
| PC | Paper chromatogram |
| Rf | Ratio of the component mobility to the solvent front mobility |
| U | Units of biological activity |
| μg. | Micrograms |