PURIFICATION OF A PEPTIDE WITH UTERUS-STIMULATING ACTIVITY FROM BOVINE POSTERIOR PITUITARY

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HAROLD A. SURES

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ABSTRACT

Whole bovine posterior pituitary glands were homogenized and extracted with 0.1 N HCl. The protein component of the extract, neurophysin, was isolated by successive gel filtration on Sephadex G-25 and G-75. Only one component (estimated mol wt 13,000) was resolved, by chromatography of the crude neurophysin on Sephadex G-50 at pH 23. Chromatography of neurophysin on Sephadex G-50 at pH 9.6, resolved three subfractions of estimated mol wt $\geq$ 4,000, $<$ 4,000, and $<$ 2,000, respectively. The high mol wt components have been discussed as polymorphic forms of neurophysin which may aggregate by means of non-covalent bonding (LaBella, 1971). The lowest mol wt subfraction contained material which induced contraction of the isolated rat uterus. The active material from this subfraction was further purified by gel filtration on Sephadex G-10, paper chromatography, and ion-exchange chromatography on carboxy-methyl cellulose and QAE-Sephadex.

The active material was estimated, on the basis of ninhydrin reaction of the fraction, to be approximately one-third as potent as synthetic oxytocin on the rat uterus.

Amino acid analysis of an acid hydrolysate of the active fraction indicated the presence of aspartic acid, glutamic acid, glycine, alanine, valine, serine, threonine, as well as low concentrations of leucine, lysine, and histidine. The mol wt of the active material was estimated, by gel filtration on Sephadex G-10, to be 700-1,200. A value of 4,500 mol wt was calculated from the amino acid composition.

Homogeneity of the active material was indicated by thin layer chromatography, high voltage paper electrophoresis, and ion-exchange chromatography, and thus, did not favor consideration of the active material as being composed of a peptide mixture. The discrepancy in mol wt estimations was considered, therefore, in terms of a possible contamination (by amino acid residue(s)) of the active material, and/or the existence of heteroallelomorphic forms of the active peptide. If an amino acid residue is not a normal constituent of the peptide in question, then, the presence of that amino acid, in low concentration, could result in an erroneous high estimation of the mol wt of the peptide. The low concentrations of leucine, lysine, and histidine (detected in the initial amino acid analysis) were considered in the above context and a second calculation of the amino acid composition of the active substance was made.

The two possible amino acid compositions of the active material differ in mol wt, i.e. 4,500 and 1012. A calculated mol wt of 1012 for the active material was consistent with that of 700-1200, estimated by gel filtration. As evidence does not permit a clear choice as to which amino acid composition is representative of the true situation (4,500 or 1,000 mol wt), comparisons were made with respect to both possible compositions.

In terms of chromatographic characteristics and amino acid composition, the active substance is apparently distinct from oxytocin, vasopressin, angiotensin, bradykinin, substance P, and from oxytocic peptides isolated from the hypothalamus and posterior pituitary by other workers.

To my parents

Louis and Dena Sures

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LIST OF ABBREVIATIONS

Centigrade	C
Centimeter	cm
Gram	g
Hour	hr
Molar	M
Milligram	mg
Millimeter	ml
Minute	min
Molecular weight	Mol wt
Milliunit	mU
N	normal
No.	number

I. INTRODUCTION

INTRODUCTION

The pituitary gland, or hypophysis cerebri, lies at the base of the brain in the region of the diencephalon, and is connected to the brain by the hypophyseal or infundibular stalk. It is a compound organ situated in the sella turcica, a depression in the basal portion of the sphenoid bone (basisphenoid). The pituitary gland is composed of three main lobes, namely, the anterior, the intermediate and the posterior lobe. A fourth component, the pars tuberalis, is a modified portion of the anterior lobe.

The posterior lobe (neurohypophysis or pars nervosa) consists of three main parts, the infundibular process (neural lobe), the infundibular stalk, and the median eminence of the tuber cinereum. These take embryonic origin from the infundibulum, a ventral evagination of the embryonic diencephalon. The neurohypophysis remains attached to the brain via the infundibular stalk and median eminence.

A dorsal evagination called Rathke's pocket arises from the ectodermal epithelium of the stomodaeum (primitive mouth cavity). Rathke's pocket constricts off the stomodaeum becoming a closed vesicle in contact with the neurohypophysis. The anterior portion of Rathke's pocket is enlarged and modified to form the anterior lobe (adenohypophysis or pars distalis), from which the pars tuberalis is derived. The posterior portion of Rathke's pocket enlarges to a lesser extent to become the intermediate lobe (pars intermedia). The original cavity of Rathke's pocket remains as the hypophyseal cleft (Weichert, 1958; Harris, 1960).

While nerve supply to the anterior pituitary is virtually negligible (Green, 1951, 1966), and while the function of such innervation as exists does not seem to be concerned with direct regulation of

anterior pituitary hormonal output (Green, 1966), the nerve supply to the posterior pituitary is extensive and is intimately concerned with the hormonal output of the posterior lobe.

The innervation of the neurohypophysis is by way of the hypothalamohypophyseal nerve tract. The soma of this nerve tract lie in the supraoptic and paraventricular nuclei of the hypothalamus (Christ, 1966). Nerve fibers from these soma run to all three divisions of the neurohypophysis (Rasmussen, 1940). Section of the hypothalamohypophyseal tract in the region of the infundibular stalk (between the median eminence and neural lobe) results in atrophy and hypercellularity of the neural lobe and consequently, a loss of nerve fibers and of pressor, antidiuretic and oxytocic substances from the neural lobe (Fisher et al., 1938; Magoun et al., 1939). Above the level of the section however, there is an increase in Gomori stainable material in the infundibular stalk and median eminence (Stutinsky, 1951; Hild and Zettler, 1953a; Billenstein, 1955), as well as an increase in extractable antidiuretic and oxytocic substances in the hypothalamus (Lloyd et al., 1955; Sato, 1949; Moreno et al., 1955). The amount of material that was stainable with the chrome-alum haematoxylin stain of Gomori was shown to be correlated with antidiuretic content (Ortmann, 1951; Hild and Zettler, 1953 a,b). When the hypothalamohypophyseal tract is sectioned above the level of the median eminence (between the hypothalamus and the median eminence) neurohypophyseal activity ceases entirely (Harris, 1960).

It is generally accepted that the neurohypophyseal hormones are synthesized by the cells of the supraoptic and paraventricular nuclei of the hypothalamus. The hormones then travel along the hypo-

thalamohypophyseal nerve tract into the neural lobe where the hormones are stored in granules in association with a carrier protein "neurophysin" (Bargmann and Scharrer, 1951; Scharrer and Scharrer, 1954; Bargmann, 1960). Van Dyke and coworkers (1942) first isolated vasopressin and oxytocin from bovine posterior pituitaries in the form of a hormone-protein complex which came to be known as the "van Dyke protein". The protein portion of the van Dyke protein was termed "neurophysin" (Chauvet, Lenci and Acher, 1960). With regard to the synthesis of the neurohypophyseal hormones and neurophysin, Sachs (1960) infused ^{35}S-cysteine into the third ventricle of the dog and isolated labeled vasopressin from the hypothalamus and posterior pituitary. Sachs observed that the incorporation of ^{35}S-cysteine into vasopressin was approximately three times greater in the hypothalamus than in the posterior pituitary. Sachs also found (1963) that after 3-6 hours of continuous infusion of ^{35}S-cysteine into the third ventricle of the dog, the protein in the hypothalamic neurosecretory granule (NSG) fraction possessed the highest radioactive specific activity, while vasopressin associated with these hypothalamic NSGs had the lowest radioactive specific activity. Highly labeled vasopressin was not associated with the ribosomal fraction, but rather with the hypothalamic nuclear fraction, a large granule fraction and a microsomal membranous component. Sachs therefore hypothesized that vasopressin is synthesized on ribosomal RNA as a biologically inactive macromolecule, which is incorporated into the neurosecretory granule. It was proposed by Sachs that biologically active vasopressin is formed from the macromolecule during formation and maturation of the neurosecretory granule, and the active hormone is then associated with neurophysin within the granule. The

neurosecretory granules are transported along the nerve axons to the posterior pituitary. Further experiments conducted by Sachs (1968a) involving infusion of labeled amino acids into the third ventricle of the dog provided additional support for his hypothesis. Hypothalamic neurophysin was found to be labeled extensively immediately following infusion, while at the same time the posterior pituitary contained only minimal quantities of radioactivity. However, 10-21 days following the infusion of labeled amino acids the posterior pituitaries of experimental animals were found to contain significant quantities of labeled protein and hormones. The labeled protein, when examined, was found to possess solubility, gel filtration, electrophoretic, and hormone binding properties similar to bovine neurophysin. In addition, the labeled protein was immunologically identical to bovine neurophysin.

Studies demonstrating a similar distribution of neurophysin and the neurohypophyseal hormones in the posterior pituitary, favour the proposal that neurohypophyseal hormones are stored in the neurosecretory granule bound to, or associated with, neurophysin (LaBella, 1963, 1967, 1968; Ginsburg, 1965, 1966; Dean, 1968 a,b; Hollenberg, 1968). The investigations of Hope and his coworkers indicate that there are at least two neurohypophyseal hormone binding neurophysins, namely neurophysin-I and neurophysin-II (Dean, 1968 a,b; Hollenberg, 1968). Although both neurophysin-I and neurophysin-II are capable of binding both oxytocin and vasopressin (Hollenberg, 1968; Dean, 1968 b,c), evidence has been presented (Dean, 1968 b,c) suggesting a natural storage of oxytocin in association with neurophysin-I and of vasopressin in association with neurophysin-II. Evidence for the existence of a third neurophysin, neurophysin-III, has been presented by Hope and his

coworkers (Rauch, 1968, 1969; Uttenthal, 1970). These workers suggest that the presence of the third neurophysin may indicate that in addition to oxytocin and vasopressin, there exists a third neurohypophyseal peptide which is associated with neurophysin-III. The molecular weights of the neurophysins isolated by Hope and coworkers have been estimated to be; 19,000 mol wt for neurophysin-I; 21,000 mol wt for neurophysin-II; and 20,000 mol wt for neurophysin-III.

The findings of other workers in the field support the multiple neurophysin concept, but there is no agreement as to the number, the molecular weights, or the binding capacities of the neurophysins. Breslow and Abrash (1966) resolved bovine neurophysin into four components. One of these components of estimated mol wt 25,000 was found to bind 2 moles of oxytocin per mole neurophysin. Pickering (1968) subfractionated neurophysin isolated from cod posterior pituitary into four major components. One of these components (estimated mol wt 14,000) bound 2.2 moles of oxytocin per mole of neurophysin. A low mol wt neurophysin (9,170 mol wt) isolated from porcine posterior pituitary by Wuu and Saffran (1969) was found to have a binding capacity of 0.87 moles of oxytocin per mole neurophysin. Shin (1969) and LaBella et al. (1971) found that neurophysin (bovine posterior pituitary) could be reproducibly resolved by anion exchange chromatography into six major components. Rechromatography of each of the individual components gave rise to several of the other components. These results were interpreted to indicate the existence of an equilibrium state due to dissocation and association of the various species of protein. Burford et al. (1969) also reported on the behavior of porcine neurophysin as a "rapidly polymerizing equilibrium system". LaBella (1971) estimated,

by gel filtration, that the molecular weights of neurophysin 1 to 4 ranged from 10,000 to 12,000, and that neurophysins 5 and 6 were approximately 20,000 mol wt. The six polymorphs were found to have an identical maximum binding capacity of 100 Units oxytocin per mg protein (Shin, 1969). In addition, LaBella et al. (1971) and Shin (1969) observed that a large number of peptides were associated with bovine neurophysin. These peptides, partially fractionated by ion exchange chromatography, included two fractions distinct from vasopressin and oxytocin which exhibited uterus-stimulating activity, as well as several other fractions which selectively promoted the release of certain trophic hormones from the bovine anterior pituitary (in vitro). All of these peptides could be extracted from the neurohypophyseal granule fraction, and were shown to be complexed with the protein contained within the granules. On the basis of their experimental results, LaBella and coworkers concluded that the hypothesis of three neurohypophyseal neurophysins binding three hormones in specific granules was attractive but incomplete. LaBella considers the existence of multi-hormonal neurosecretory granule and its physiological significance to be a possibility worthy of further investigation.

NEUROHYPOPHYSEAL HORMONES AND PEPTIDES

i. History

The vasoconstrictive and hypertensive effects of mammalian pituitary extracts were first demonstrated by Oliver and Schäfer (1895). Howell (1898) localized this vasopressor activity in the neurohypophysis. The uterine contracting activity of neurohypophyseal extract was first observed by Dale (1906, 1909), who proposed that the action resulted from a direct stimulation of the uterine smooth muscle rather than by

way of nervous activity. Two years following the observations by Ott and Scott (1910) of the milk ejecting effect of neurohypophyseal extract on the lactating mammary gland, Frank (1912), Farmi (1913), and Van den Velden reported the antidiuretic effects of extracts of the neurohypophysis. The observations of oxytocic, vasopressor, and antidiuretic activities were not confined to mammalian pituitary extracts. Herring (1908, 1913) found vasopressor or milk ejecting activity present in the extracts of the pituitaries taken from cyclostomes, teleosts, reptiles and birds. Subsequently, oxytocic and antidiuretic activities were detected in pituitary extracts taken from representatives of these species.

With the discovery of three major physiological activities (antidiuretic, vasopressor and oxytocic) in extracts of the mammalian posterior pituitary, it became a matter of contention amongst workers in the field as to whether the neurohypophysis contained a single component responsible for all the observed activities, or whether the neurohypophysis contained several components each responsible for a single activity.

The idea of a unitary hormone with multiple activity is based upon the ability to isolate from the neurohypophysis, by means of mild conditions of extraction and purification, a pure product possessing oxytocic, antidiuretic, and vasopressor activities in the same proportion as exist in the gland itself. Abel and his coworkers (Abel et al., 1923; Abel, 1930) were proponents of this unitary hormone theory and persisted in their belief long after oxytocic and vasopressor activities had been separated. Abel claimed that excessively violent extraction methods could split the multiactive hormone into fragments, each possessing one

of the activities. Rosenfeld (1940), also a supporter of the unitary hormone theory, subjected the press juice of pituitaries to ultra-centrifugation and claimed that the active principle sedimented at rates suggestive of a mol wt of 20,000. If the press juice was then treated with acetic acid, boiled and centrifuged, biologically active substances of much lower mol wt were observed. Rosenfeld suggested that these were fragments cleaved from the natural hormone. Van Dyke and his colleagues (1952) added further support to the theory of a unitary hormone by isolating a homogeneous biologically active protein with an estimated mol wt of 30,000. Van Dyke's purified protein possessed the three major biological activities in the same ratio as occurred in the standard U.S.P. powder. The oxytocic, vasopressor and antidiuretic activities of the protein were equal (17 units per mg). On the basis of behaviour during extraction, electrophoretic and centrifugation characteristics and homogeneity of solubility, van Dyke proposed that these three biological activities were contained in a single protein.

In opposition to the unitary hormone theory was the theory which maintained that a number of hormones, with individual actions, were responsible for the multiplicity of actions shown by posterior pituitary extracts. The basis for this theory was the work of Dudley (1919). Dudley was able to separate the oxytocic and vasopressor substances and show that they differed in chemical properties. Kamm and coworkers (Kamm et al., 1928) were also able to obtain two preparations from the neurohypophysis, one possessing oxytocic activity, the other vasopressor activity. However, as Kamm's extraction procedure involved the use of 0.25 M acetic acid with heating, the possibility of cleavage of the 'true' hormone was not ruled out. Du Vigneaud (1954-55)

was able to isolate in pure form oxytocin having a mol wt of about 1,000. The main actions of this preparation were to promote uterine contraction and milk ejection. Vasopressin was also isolated, and showed similar mol wt. The main actions, however, were vasopressor and antidiuretic. The point at issue was ultimately decided by the work of Acher et al. (1953, 1956, 1958) which negated the existence of a pure protein possessing several activities and demonstrated the existence of a complex formed in part by an inactive protein neurophysin, to which the neurohypophyseal hormones oxytocin and vasopressin were non-covalently bound.

ii. Distribution and species differences

The neurophysin-hormone complex has been found in all species of mammals that have been studied (Acher, 1963). The neurohypophyseal hormones have been isolated not only from the posterior pituitaries of mammals but also from the glands of lower vertebrates. All of these hormones contain eight amino acids, and a ring is formed in each through a disulfide bridge between the cysteine molecule in position 1 and the cysteine in position 6 (numbering system of Konzett and Berde, 1959; Bodanszky and du Vigneaud, 1959). The hormones of the various species are structurally dissimilar by virtue of a difference in amino acids in positions 3, 4 and 8 (TABLE I).

Although hormones possessing oxytocic activity but differing in structure from mammalian oxytocin have been isolated from non-mammalian species, the term 'oxytocin' is reserved for a specific chemical structure. The use of the term 'oxytocin' is confined to the hormone of mammals that promotes contraction of the uterus.

The term 'vasopressin' arises from a more pharmacological

TABLE I

NEUROHYPOPHYSEAL HORMONES OF VARIOUS ANIMAL SPECIES

Hormone	Species	Structure	References
		S ——————————— S |1 2 3 4 5 |6 7 8 9	
Oxytocin	bovine porcine	Cys-Tyr-Ile-Glu(NH_2)-Asp(NH_2)-Cys-Pro-Leu-Gly(NH_2)	du Vigneaud, 1954-1955; Acher et al., 1958.
Arg-Vasopressin	bovine	-Phe-Glu(NH_2)- -Arg-	du Vigneaud, 1954-1955; Acher et al., 1958.
Lys-Vasopressin	porcine	-Phe-Glu(NH_2)- -Lys-	du Vigneaud, 1954-1955; Acher et al., 1958.
Glumitocin	rayfish, dogfish	-Ile-Ser- -Glu(NH_2)-	Acher et al., 1965.
Isotocin	teleost	-Ile-Ser- -Ile-	Acher et al., 1962.
Mesotocin	frog	-Ile-Glu(NH_2)- -Ile-	Acher et al., 1964.
Vasotocin	birds, fish, amphibian	-Ile-Glu(NH_2)- -Arg-	Acher et al., 1960; Munsick et al.,1960.

activity of the hormone as opposed to its truer hormonal antidiuretic activity. It is usual to differentiate between lysine-vasopressin (the vasopressor hormone isolated from pig), and arginine-vasopressin (the vasopressor hormone isolated from most animal species).

iii. Chemical properties

(1) Oxytocin:

The amino-acid composition and structure of oxytocin was determined independently by du Vigneaud (1954-1955) and Tuppy and Michl (1953). The mol wt of oxytocin is 1,007 and the isoelectric point is 7.7. The ampholytic behaviour of oxytocin, detected by electrophoresis, indicates the presence of both acidic and basic groups in the hormone. Oxytocin contains no basic amino acids, so that the basic group assumed to be a free amino group. Analysis after acid hydrolysis indicates the presence of three molecules of ammonia which originate from amide groups of glutamic acid, aspartic acid, and carboxy-terminal glycine amide (TABLE I). The acidic groups of the component amino acids of oxytocin are carboxylic and phenolic groups. However, the isoelectric point of 7.7 indicates an absence of free carboxylic groups in the hormone and implies an involvement of the phenolic group in creating the ampholytic properties of the hormone.

(2) Vasopressin:

The structure of arginine-vasopressin was determined by du Vigneaud (1954-1955), Turner et al. (1951), Acher and Chauvet (1953, 1954), du Vigneaud et al. (1953) and Popenoe and due Vigneaud (1953, 1954). Vasopressin has a mol wt of 1,084 and an isoelectric point of 10.9. It is an octapeptide (TABLE I).

OTHER NEUROHYPOPHYSEAL PEPTIDES

Abel and Pincoff (1917) demonstrated that the neurohypophysis contains high concentrations of "proteoses" and "peptones". While the peptide hormones oxytocin and vasopressin are thought to be the major physiological products of the posterior pituitary, examination of other components contained in the neurohypophyseal peptide pool has provided, in more recent years, information suggesting further possibilities as to the function of the neurohypophysis.

Winnick et al (1953) examined the amino acids and peptides of bovine and porcine posterior pituitary. The extracts of fresh glands were fractionated by charcoal adsorption and ionophoresis. Peptides were quantitated by both the ninhydrin and biuret reactions. The peptide concentration contained in the bovine and porcine posterior pituitaries was found to be four to eight times greater than the peptide concentration attributable to the known peptide hormones. Winnick estimated that peptides comprise three to four percent of the dry weight of the neurohypophysis.

Ramachandran and Winnick (1957) continued the systematic examination of the large peptide pool of the neurohypophysis. These workers extracted acetone dried porcine posterior pituitaries with dilute acetic acid and precipitated the protein from the extract with trichloracetic acid. The peptides of the deproteinized solution were fractionated and characterized as to mobility on paper electrophoresis, amino acid composition, molecular size, carboxyl and amino terminals and abundance in the original tissue. The most abundant amino acids found were glutamic acid, glycine, alanine, aspartic acid, serine, threonine, valine and leucine.

Isolation from the neurohypophyseal peptide pool of both biologically active peptides and peptides of unknown biological significance has been achieved. However, the physiological significance of the extensive peptide pool and the function of the majority of peptides contained therein remains unknown.

i. Releasing factors

The connection between hypothalamus and posterior pituitary has been described earlier in this report as consisting mainly of nerve tracts. In the case of the anterior pituitary, physiological communication with the hypothalamus is by way of a vascular network. Harris (1948) reviewed the hypothalamic control of anterior pituitary hormone secretion. In the hypothalamus, neurohumoral transmitters (releasing factors) are synthesized and released into portal vessels which run through the pituitary stalk and supply blood to the anterior pituitary. The releasing factors control the rate of release of anterior pituitary hormones (Meites and Nicoll, 1966; Martini, 1966; McCann and Dhariwal, 1966; Guillemin, 1967; McCann et al., 1968). To date, study of the releasing factors has concentrated in the area of the hypothalamus, however, there exists the possibility of an involvement of the peptide pool of the neurohypophysis in control of anterior pituitary hormone secretion (Martini, 1966).

Saffran et al. (1955) isolated from posterior pituitary extract a component capable of stimulating the release of adrenocorticotrophin (ACTH) at doses as low as 1 nanogram. This component, named "Corticotrophin Releasing Factor (CRF)", was shown to be a peptide distinct from vasopressin and oxytocin. The discovery of this releasing factor in the neurohypophysis prompted the suggestion that the posterior

pituitary could form an important link between the hypothalamic nuclei and the anterior pituitary for the storage and secretion of CRF as well as the hormones oxytocin and vasopressin (Saffran and Schally, 1955 a,b). The purified CRF contained the amino acids cystine, aspartic acid, glutamic acid, glycine, proline, lysine, phenylalanine, serine, and histidine (Schally et al., 1958).

Guillemin et al. (1957) isolated another CRF from a 90% methanol extract of commercial posterior pituitary preparation (Proto-pituitrin^{r}, Parke Davis). This active peptide was shown to differ from oxytocin, vasopressin and ACTH by paper chromatography.

Schally et al. (1962) isolated from porcine neurohypophysis yet another CRF which differed from the compounds isolated by Guillemin et al. (1957) and Schally et al. (1958).

Subsequent investigation (Guillemin et al., 1960 a,b; Schally et al., 1960; Schally and Guillemin, 1963; Guillemin, 1964; Schally and Bowers, 1964) demonstrated the existence of two distinct peptides or families of peptides contained in the neurohypophysis possessing ACTH-releasing activity.

The first is a potent ACTH-releasing peptide related structurally to lysine vasopressin and containing cystine, serine, glycine, lysine, histidine, tyrosine, valine, phenylalanine, proline, aspartic and glutamic acid but no methionine. This peptide has been designated "beta-CRF". The N-terminal and C-terminal amino acid sequences of this peptide show sequential similarities to those of alpha-melanocyte stimulating hormone (alpha-MSH).

The second family of ACTH-releasing peptides is also related structurally to alpha-MSH but contain methionine (beta-CRF contains no

methionine). These peptides have been classified as "alpha-CRFs".

Alpha$_1$-CRF (Guillemin et al., 1960; Gros and Privat de Garilhe, 1959; Privat de Garilhe, 1960) contains all the amino acids of alpha-MSH plus threonine, alanine, and leucine. Alpha$_1$-CRF contains a hepta-peptide sequence which also appears in ACTH. This sequence probably accounts for the MSH-like and ACTH-like activities shown by alpha$_1$-CRF.

Alpha$_2$-CRF (Schally et al., 1962) has identical melanophoretic activity and reduced ACTH-like activity as compared with alpha-MSH. The amino acid sequence of alpha$_2$-CRF is identical to alpha-MSH with the exception of a difference at the terminal amino group. It is postulated that the structural difference at the terminal nitrogen of alpha$_2$-CRF may be responsible for the CRF activity (Schally et al., 1962).

The release of melanocyte-stimulating hormone (MSH) from the intermediate lobe of the pituitary gland is mediated by a hypothalamic neurohumoral substance, MSH-release inhibiting hormone (MRIH)(Schally et al., 1968). Most recently, Nair, Kastin and Schally (1971) successfully isolated and determined the structure of a bovine hypothalamic MRIH-active peptide. The amino acid sequence of this MRIH-active peptide was shown to be prolyl-leucyl-glycine amide. This sequence determined by the combined Edman degradataion and dansyl method was confirmed by mass spectrometry. The MRIH-active peptide was shown to be similar to synthetic L-Pro-L-Leu-glycine amide in terms of biological activity, chromatographic and electrophoretic mobility and mass spectral fragmentation pattern. On the basis of these observations, Nair, Kastin and Schally conclude that the structure of bovine MRIH is: Pro-Leu-Gly-NH$_2$.

It is of particular interest that the tripeptide Pro-Leu-Glycine amide also forms the side chain of the neurohypophyseal hormone oxytocin. Celis et al. (1971) report that microsomal preparations of rat hypothalmi

contain an enzyme which may be concerned with the release of MRIH. It is suggested that the action of this enzyme is to cleave the side chain from oxytocin, with the result that the tripeptide acts to inhibit the release of pituitary MSH.

Additional evidence, indicating a possible role of the posterior pituitary in the control of secretion of anterior pituitary hormones, has been provided by Franchimont and Legros (1969). The administration of total neurohypophyseal extracts to man was followed by an increase of serum levels of the trophic hormones, prolactin (LH), growth hormone (GH), and folicle stimulating hormone (FSH) (Franchimont and Legros, 1969). The administration of lysine-vasopressin had no effect on the serum levels of the trophic hormones, while oxytocin raised only the FSH levels.

Continued examination of the neurohypophyseal peptide pool as a source of releasing factors may serve to clarify the as yet unestablished physiological relationship between the anterior and posterior pituitary.

ii. Other peptides

a) inactive

Witter et al. (1964) demonstrated that in addition to the peptide hormones oxytocin and vasopressin, many amino acids and peptides can be dissociated from "van Dyke protein" prepared from dessicated porcine neurohyophysis powder. The distribution of amino acids dissociated from the "van Dyke protein" was in agreement with the findings of Winnick et al. (1955). However, Witter did not attempt to assign any biological significance to the large number of peptides which were obtained by his dissociation procedure.

More recently several peptides, also of unknown biological significance, have been isolated from the posterior pituitary (Penders and Arens, 1966; Schally and Barrett, 1968). These investigators suggest that although the isolated structures lack demonstrable biological activity, they may prove significant as the precursors or metabolites of important molecules and provide useful information as to the function of the hypophyseal complex.

b) active

The investigations of several workers have resulted in the discovery of lipolytic substances contained in the hypothalamus and pituitary, thyroid, pineal and parotid glands (Li et al., 1965, 1966; Chretien and Li, 1967 a,b; Lohmar and Li, 1967; Redding and Schally, 1970; Rudman et al., 1970; Gilardeau and Chretien, 1970). Although the activity of a hypothalamic lipid mobilizing factor, isolated by Redding and Schally (1970), has been attributed to ACTH or an ACTH analogue, Li and coworkers have determined the amino acid sequences of two sheep hypophyseal lipolytic peptides (Li et al., 1965, 1966; Chretien and Li, 1967 a,b). These peptides have been called sheep beta- and gamma-lipolytic hormones (LPH). Lohmar and Li (1967) also report the isolation of a bovine hypophyseal lipolytic peptide identical to sheep beta-LPH. More recently, Graf and Cseh (1968) and Gilardeau and Chretien (1970) have isolated and determined the amino acid composition of a porcine hypophyseal LPH. The mol wt, as determined from the amino acid composition of porcine LPH (9971) is similar to that determined for sheep beta-LPH (9958). The sheep and porcine beta-LPH's differ somewhat in amino acid composition as well as in electrophoretic mobility in polyacrylamide gel at pH 8.3. The

isoelectric points of porcine and ovine beta-LPH were determined by electrofocusing studies to be 6.43 and 8.30 respectively. On the basis of the differences in amino acid compositions and isoelectric points, Gilardeau and Chretien suggest that the primary structure of porcine beta-LPH should be at least partially different from that of sheep beta-LPH.

Smith and Rosenfeld (1962) isolated a peptide possessing oxytocin-like activity from an extract of bovine posterior pituitary. The pituitary extract, chromatographed on carboxymethyl cellulose, was resolved into three distinct peaks of oxytocic activity. Two of these peaks were found to correspond to protein bound and free oxytocin, while the third peak differed from the hormone oxytocin in terms of elution pattern from CM-cellulose and ultrafiltration characteristics. The molecular size of the unknown peptide was estimated (by comparison of ultrafiltration rates) to be intermediate between oxytocin (mol wt 1,000) and ribonuclease (mol wt 13,700). Smith asserted that the complete removal of the peptide from the gland extract in the first passage through the CM-cellulose column, and the subsequent failure to generate additional peptide with rechromatography of the large protein component of the extract was evidence that the oxytocin-like peptide was a natural constituent in the gland. Smith and Rosenfeld suggested that such a constituent might serve as an intermediate in the pathways concerned with synthesis, transport from hypothalamic centers, or in release of hormonal activity into the blood.

A large biologically active polypeptide was isolated from bovine posterior pituitary by Preddie and Saffran (1965). The oxytocic activity was described as minimal and the peptide was devoid of

detectable pressor or adrenocorticotrophic hormone releasing activities. Structurally (Preddie, 1965) the peptide consists of 48 amino acids including 5 cystein residues, 5 lysine and arginine and 9 aspartic plus glutamic acids. Histidine, tyrosine and methionine are not present in the molecule. The polypeptide with an estimated mol wt of 5,500 is approximately six times as large as the known neurohypophyseal hormones, and the same order of size as the polypeptides ACTH, insulin and parathyroid hormone. The carboxyl-terminal portion of the polypeptide includes a cyclic octapeptide which resembles in form the structure of oxytocin. It is suggested that this cyclic structure might account for the observed oxytocin-like activity of the peptide. Preddie, on the basis of the carboxyl-terminal structure, also suggests the possibility that the polypeptide may be a naturally occurring analogue of the hypothetical precursor of the neurohypophyseal hormones.

A small peptide, possessing oxytocin-like activity, has recently been isolated from ox hypothalamus (North et al., 1968). The isolated substance, tested on three smooth muscle preparations, rat uterus, guinea pig ileum and hen rectal caecum, was found to be equipotent in stimulating contraction in each of the preparations (Hawker et al., 1969). Treatment of the peptide with thioglycolate, a procedure which abolishes the effect of oxytocin on the rat uterus, did not affect the activity of the unknown peptide in that smooth muscle preparation. Atropine, phenoxybenzamine and mepyramine also failed to block the smooth muscle actions of the peptide. Examination of gel-filtration characteristics, drug action, relative potency and log dose-response relationships has differentiated the peptide from 5-hydroxytryptamine, acetylcholine, oxytocin, vasopressin, angiotensin amide, bradykinin and

purified preparations of substance P. Hawker and coworkers believe that a single low mol wt peptide is responsible for the observed activity of their isolated substance. To date, isolation of the peptide in quantities sufficient for chemical characterization and physiological evaluation has not been achieved.

The polypeptide hormones of the neurohypophysis can readily be dissociated from the neurophysins and separated from each other by Sephadex G-25 chromatography (Frankland, Hollenberg, Hope and Schacter, 1966). While the vasopressin that is obtained by this procedure is relatively pure, the oxytocin is contaminated with other materials. Hope and Watkins (1969) found that rechromatography of this impure oxytocin preparation on Sephadex G-25 resulted in the separation of an oxytocic peptide, the mol wt of which was estimated by amino acid analysis to be 3,000. Uterine stimulating activity was not abolished by incubation of the peptide in thioglycollate, nor was the action of the peptide antagonized by phenoxybenzamine or atropine. The peptide was found to possess both pressor and antidiuretic activities. Comparison of elution volume on Sephadex G-25 distinguished the peptide from angiotensin, acetylcholine, oxytocin, vasopressin and bradykinin. ACTH and CRF activities were considered to be minimal, as intravenous administration of the peptide to the rat did not result in significant depletion of adrenal ascorbic acid, nor did it cause a rise in plasma corticoid levels.

In comparison to the hypothalamic oxytocic material isolated by Hawker et al. (1969), Hope (1969) has drawn attention to similar characteristics demonstrated by the oxytocic peptide isolated from the neurohypophysis (Hope and Watkins, 1969). Both oxytocic substances were separable from oxytocin by gel filtration on Sephadex G-25 and the

elution volumes of the oxytocic materials on Sephadex G-25 were less than that of oxytocin. The ratio of the elution volume (on Sephadex G-25) of oxytocin to the elution volume of the hypothalamic oxytocic material (Hawker et al., 1969) was 1:12. The ratio of the elution volume of oxytocin to the elution volume of the neurohypophyseal peptide isolated by Hope and Watkins (1969), has a similar value of 1:17. Another property common to both oxytocic materials is their resistance to inactivation by thioglycollate.

The biological complexities of the neurohypophysis remain to date largely unexplained and the seemingly limitless wealth of material, protein and peptide contained therein, has rested relatively untouched as a source of scientific information. There is no doubt as to the coincident importance of biologically active and inactive substances in delineating a total concept of pituitary function. Both are areas of study and research that only in more recent times have received the attention that will be necessary to elucidate the full physiological significance of the neurohypophysis. The present report is concerned with oxytocic activity of the neurohypophysis, in particular, that oxytocic activity which is attributable to a peptide distinct from the hormone oxytocin.

II. MATERIALS AND METHODS

MATERIALS AND METHODS

Bovine posterior pituitary glands

Whole bovine pituitary glands were obtained from the slaughterhouse and transported to the laboratory in ice bathed vessels. The posterior pituitary glands were separated from the anterior pituitary lobes, and stored in the deep freeze at-20°C. The time elapsing from the death of the animals to tissue freezing was approximately 2 hours. Fifty posterior pituitary lobes (12.5 g) were collected in this fashion daily for one week, so that 250 glands (62.5 g) served as starting material for a given extraction.

Extraction procedure

The posterior lobes were minced with scissors. The tissue mince was suspended in 0.1 N NCl (pH 1.5) that had been precooled to 4°C, and homogenized in a glass tube with a motor driven teflon pestle (LaBella et al., 1963). The homogenate suspended in 0.1 N HCl (100 ml/4g whole tissue) was stirred for 36 hours at 4°C. The suspension was allowed to settle and the supernatant was decanted and centrifuged at 15,000 "G" for 30 min in an International B-20 Refrigerated Centrifuge. The resultant pellet, consisting of nuclei and cell debris, was discarded. The pH of the supernatant was adjusted to pH 2.0 with sodium hydroxide and concentrated under reduced pressure at 35°C to a volume of approximately 40 ml in a rotary evaporator (Buchler Instruments, N.J.). The concentrate was centrifuged at 100,000 G for 30 min in an International Model B-60 Preparative Ultracentrifuge to remove the particulate fraction. The pellet from this centrifugation was discarded and the supernatant retained.

Gel filtration

Columns of Sephadex G-10, G-25 superfine, G-50 fine and G-75 fine (Pharmacia, Uppsala, Sweden) were prepared in accordance with the manufacturer's specifications. "Fines" were removed from the gels by decantation and the sediments were resuspended in distilled water. 12 hours later this water was replaced with; 1.0 M acetic acid pH 2.3 (G-25, G-50 and G-75), 0.2 M acetic acid pH 2.5 (G-10), or 0.025 M sodium carbonate-bicarbonate buffer pH 9.6 (G-50). The respective gels subsequently were eluted with these same eluants. In preparing the column for use, a small amount of eluant was poured into the empty glass column. The glass column was then filled with the gel slurry. Column dimensions were; 2.25 x 51 cm for G-10, 3.5 x 90 cm for G-25, 2.5 x 100 cm for G-50 (pH 2.3), 4.0 x 80 cm for G-75, and 2.5 x 100 cm for G-50 (pH 9.6). Samples were dissolved in a minimum amount (within limits specified by manufacturer) of column eluant and applied to the Sephadex columns without disturbing the gel. The respective eluant for the gel column was then applied and fractions were collected sequentially. The volume per fraction was; 6.0 ml for G-10, 12 ml for G-25, 12 ml for G-75, and 6.0 ml for G-50. The flow rate was; 50 ml/hr for G-10, 50 ml/hr for G-25, 40 ml/hr for G-50 (pH 2.3), 50 ml/hr for G-75, and 40 ml/hr for G-50 (pH).6).

Ion exchange chromatography

CM-cellulose (Bio-Rad, Calif). and QAE-Sephadex A-25 (Pharmacia, Uppsala, Sweden) columns were prepared, packed and regenerated according to the specifications of the manufacturers, or in accordance with the method of Schroeder et al. (1962). Column dimensions for CM-cellulose were 1.5 x 36 cm, and for QAE-Sephadex 1.5 x 16 cm. Samples to be chromatographed were evaporated at 35°C to dryness on a rotary evaporator

under reduced pressure. Samples to be chromatographed on QAE-Sephadex were redissolved in 2 ml of pyridine:collidine:acetic acid (glacial): water buffer (40:40:1.5:3918.5 by volume) pH 8.3 (Schroeder et al., 1962). Samples to be chromatographed on CM-cellulose were redissolved in 2 ml of 0.01 M sodium acetate pH 4.5. Dissolved samples were applied to the respective column and washed on to the column with a small portion of the same buffer in which they had been dissolved.

Elution from QAE-Sephadex was carried out using a nine chamber Technicon Autograd (Technicon Instrument Corp., New York) as a gradient device. Elution was continuous, starting with pyridine:collidine:acetic acid:water buffer, followed by 0.1 M, 0.5 M, 1.0 M, and 2.0 M acetic acid (TABLE II).

Elution from CM-cellulose was carried out using a two chamber linear gradient device (Kontes Glass Co., N.J.). Elution was continuous, starting with 0.01 M sodium acetate (pH 4.5) to 0.2 M sodium acetate (pH 7.0) (TABLE II). Following completion of the gradient, a further quantity of 0.2 M sodium acetate (pH 7.0) was applied to the CM-cellulose column.

The flow rate for the QAE-Sephadex column was 24 ml/hr and 4 ml fractions were collected. The flow rate for the CM-cellulose column was 30 ml/hr and 3 ml fractions were collected. Fractions were reacted with ninhydrin (Cadavid and Paladini, 1964), or with standard protein reagent (Lowry et al., 1951) in a Technicon Autoanalyser (Technicon Instrument Corp., N.Y.).

Bioassay

Uterus-contracting activity of chromatographic fractions was determined with the isolated rat uterus (Holten, 1948). Syntocinon-r

TABLE II

GRADIENT SYSTEMS FOR ION-EXCHANGE CHROMATOGRAPHY

QAE SEPHADEX

Chamber No.	pyridine:collidine: acetic acid:water (40:40:1.5:3918) pH 8.3	acetic acid (M) 0.1	0.5	1.0	2.0
1	50	0	0	0	0
2	0	50	0	0	0
3	0	50	0	0	0
4	0	0	50	0	0
5	0	0	50	0	0
6	0	0	0	50	0
7	0	0	0	50	0
8	0	0	0	0	50
9	0	0	0	0	50

Figures above represent the number of ml in each chamber of the nine chamber Technicon Autograd.

CM-Cellulose

Chamber No.	0.01M sodium acetate pH 4.5	0.1M sodium acetate pH 7.0
1	150	0
2	0	150

A two chamber linear gradient device was used with CM-Cellulose.

(Sandoz, Quebec) was used as a standard for the assay of this ocytocin-like activity. The usual dose of Syntocinon given to produce the standard contraction was 10 mU. The uterus-contracting activity of chromatographic fractions will be referred to in this text as such, or as "oxytocin-like activity".

Amino acid analysis

Purified peptides were hydrolyzed for 24 hours at 110°C in 6 N HCl under nitrogen in sealed glass vials.

Analysis of amino acids was performed with a Technicon amino acid analyser (Technicon Instrument Corp., N.Y.) as described by LaBella et al. (1967). The values determined for the amino acids were not corrected for the destruction that occurred during hydrolysis.

Thin layer chromatography and electrophoresis

As an estimation of homogeneity and purity, peptides were examined by ascending thin layer chromatography (TLC) on MN-polygram cellulose 300/UV_{254} (Macherey-Nagel and Co., Dueren, Germany). The solvent systems used were; t-butanol:acetic acid (glacial):water (4:1:1, by volume), n-propanol:ammonium hydroxide (conc.):water (100:1:50, by volume), and ethyl acetate:t-butanol:acetic acid (glacial):water (1:1:1:1, by volume).

Peptides were examined as well by paper (Whatman Chromatography Paper #1, W.R. Balston Ld., England) electrophoresis (Camac, Mutenz/Schweiz). Electrophoresis was carried out on paper strips (6 x 45 cm) in; 0.1 M di-sodium hydrogen phosphate buffer (pH 7.00) at 3,000 V/15 min, 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6) at 4,000 V/10 min, and formic acid:acetic acid:water (15.6:29.6:455, by volume) (pH 1.99) at

4,000 V/15 min.

Ninhydrin Aerosol spray NIN-3 (Sigma Chemical Co., St. Louis, Mo.) was used to visualize the peptides.

Paper chromatography

The purification procedure included the use of descending paper chromatography to achieve a partial separation of peptide mixtures. Samples were applied to the paper as strips perpendicular to the direction of solvent flow and eluted with t-butanol:acetic acid:water (4:1:1, by volume). 18 x 45 cm sheets of Whatman Chromatography paper # 3 (W.R. Balston Ltd., England) were used as the stationary phase.

The separation peptide bands were visualized by development with ninhydrin of 1 x 45 cm strips of chromatography paper cut from both lateral edges of the main sheet. Paper strips containing the corresponding separated peptide bands were cut from the main paper, and homogenized in a Virtis Homogenizer (The Virtis Co. Inc., N.Y.) in the presence of 0.1 M acetic acid to extract the peptides. Extracts were concentrated under reduced pressure in a rotary evaporator.

III. RESULTS

RESULTS

A flow chart outlining the purification procedure is shown in Figure 1. The concentrated acid extract of posterior pituitary was separated by chromatography on Sephadex G-25 into three ninhydrin-positive peaks (Figure 2). Fraction I was eluted in the exclusion volume, whereas Fractions II and III were retarded. Molecular weights of the components contained in Fractions I, II and III have been estimated (Shin and LaBella, 1970). Fraction I was shown to contain components of estimated mol wt greater than 4,000. Fraction II included components of estimated mol wt less than 4,000 and Fraction III contained components of mol wt less than 2,000. Shin and LaBella (1970) subfractionated and characterized extensively the low mol wt components contained in Fraction III. The work described in this report is concerned with components derived from the higher mol wt constituents of Fraction I, specifically a component possessing oxytocin-like activity.

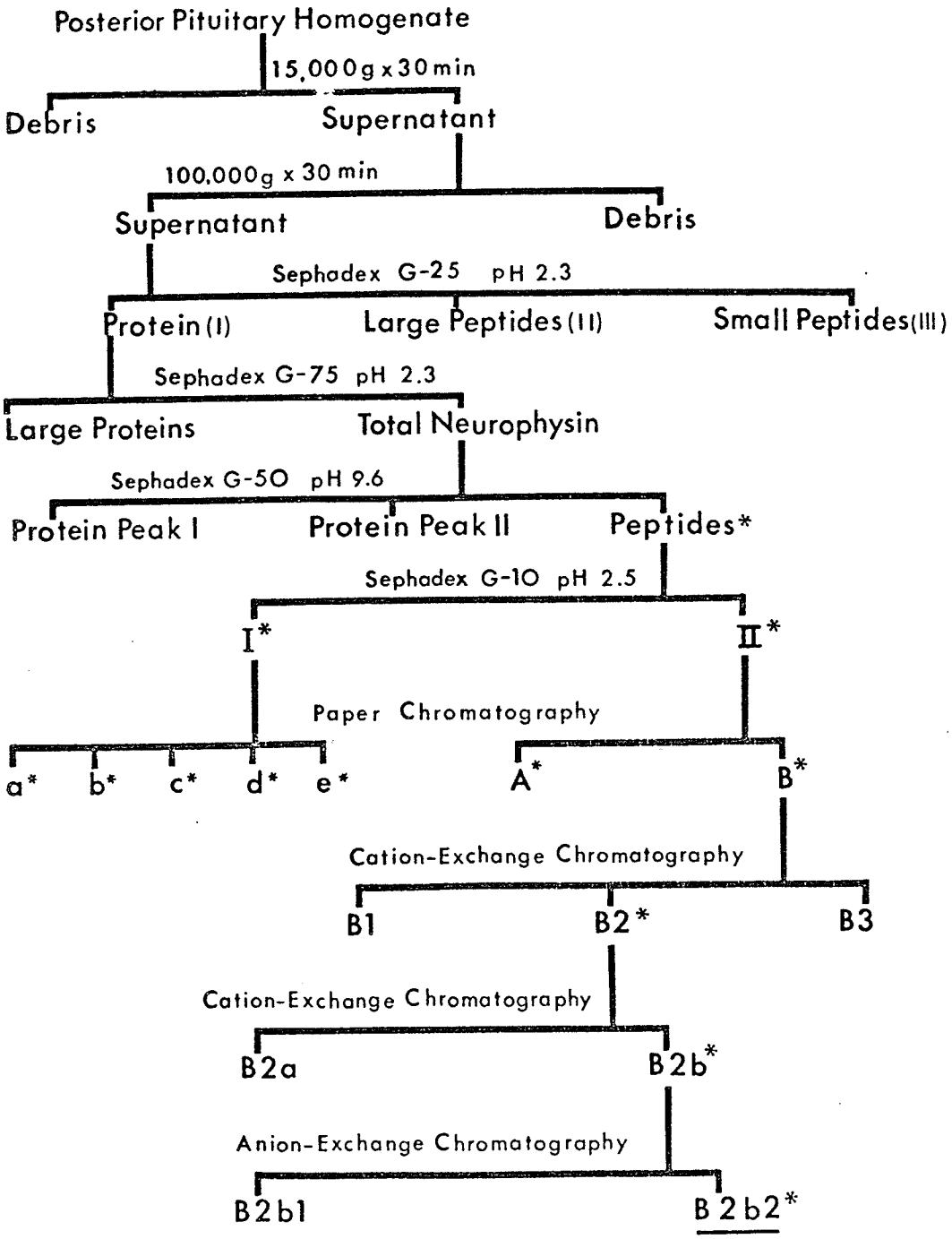
Chromatography on Sephadex G-75

Fraction I from Sephadex G-25 was concentrated and chromatographed on Sephadex G-75. Ninhydrin analysis of the fraction collected from Sephadex G-75 showed resolution of Fraction I from Sephadex G-25 into two major peaks (Figure 3). The strongly retarded second peak eluted from Sephadex G-75 composed the "total neurophysin" fraction described by Hollenberg and Hope (1967). The fractions included in this peak were pooled, concentrated and chromatographed on Sephadex G-50.

Chromatography on Sephadex G-50 at pH 2.3 and pH 9.6

Chromatography of the second peak obtained from Sephadex G-75 on Sephadex G-50 with 1.0 M acetic acid as eluant disclosed one

Figure 1. Flow chart of purification of uterus-stimulating peptide B2b2 from bovine posterior pituitary



* Indicates Oxytocic-Like Activity

Figure 1.

Figure 2. Elution profile from Sephadex G-25 (3.5 x 90 cm), 25°C, of an acid extract of bovine posterior pituitary. Flow rate was 50 ml/hr and 12 ml fractions were collected. Each fraction was measured after reaction with ninhydrin. The eluant was 1.0 M acetic acid.

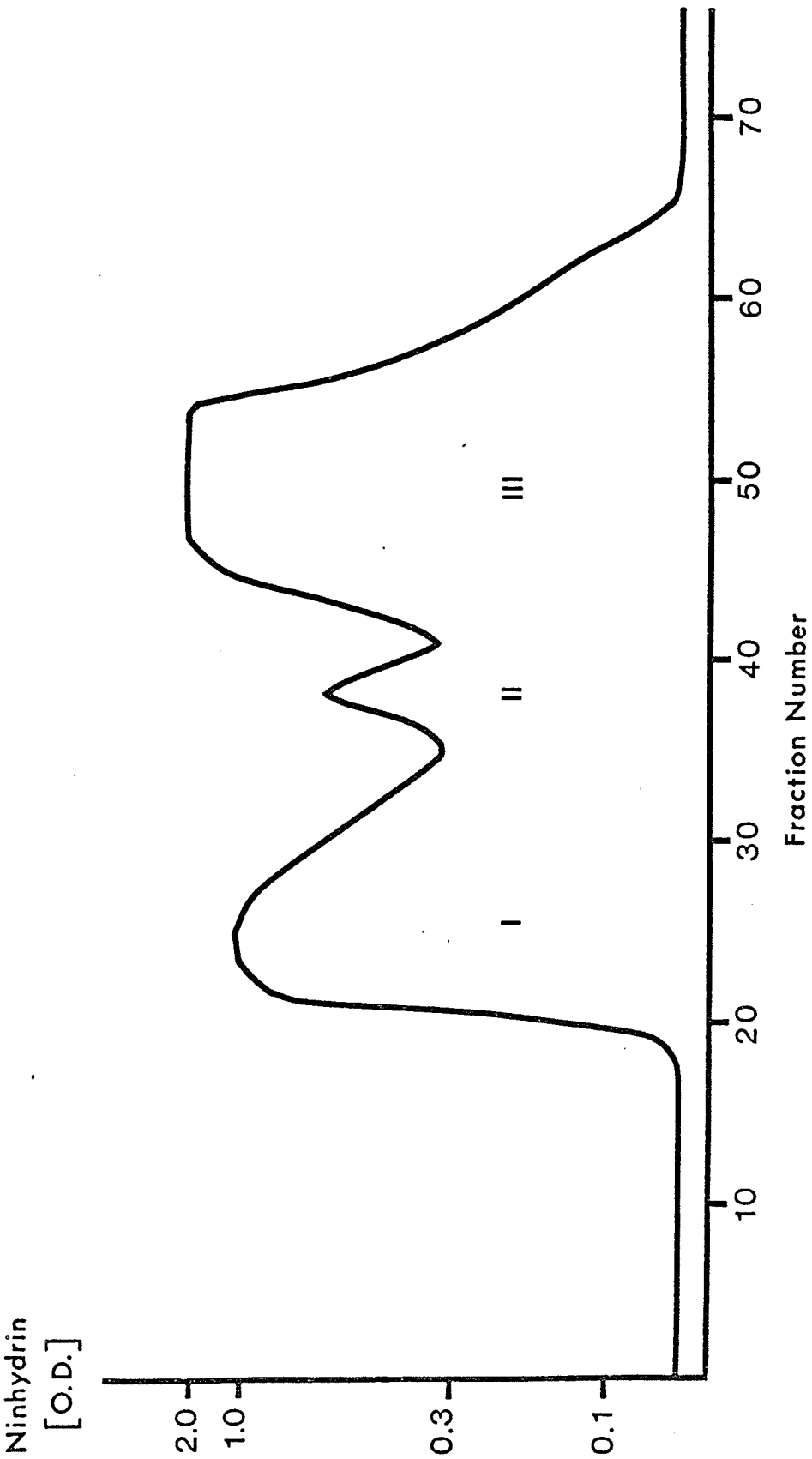

Ninhydrin
[O.D.]
2.0
1.0
0.3
0.1
10
20
30
40
50
60
70
Fraction Number
I
II
III

Figure 2.

Figure 3. Elution profile of Fraction I from Sephadex G-25 (Figure 2) on Sephadex G-75 (4.0 x 80 cm). Flow rate was 50 ml/hr and 12 ml fractions were collected. Each fraction was measured after reaction with ninhydrin. The eluant was 1.0 M acetic acid.

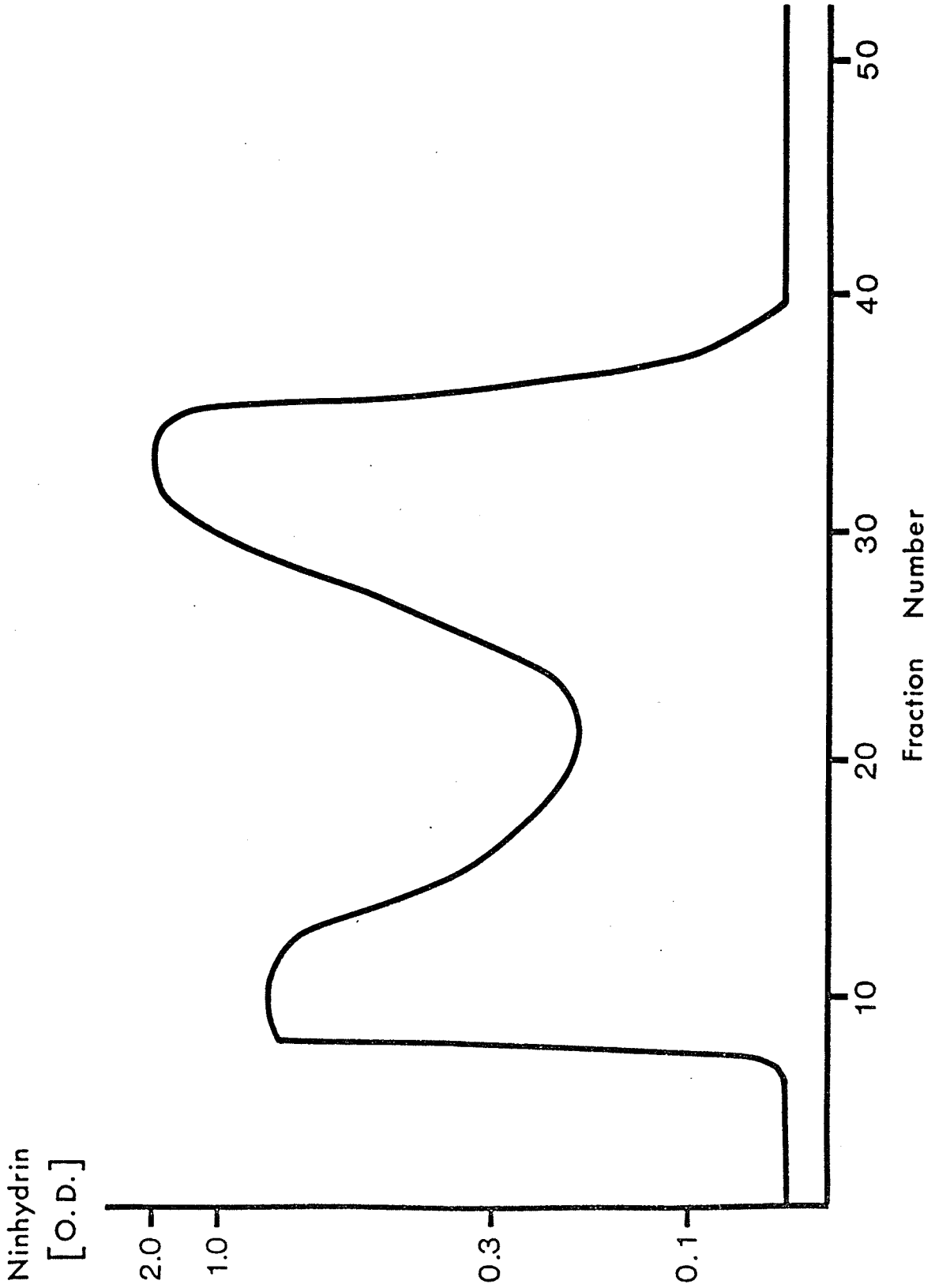


Figure 3.

ninhydrin positive peak (Figure 4). However, chromatography of the second peak obtained from Sephadex G-75 on Sephadex G-50 with 0.025 M sodium carbonate-bicarbonate buffer (pH 9.6) as eluant, resulted in separation of the peak into three incompletely resolved fractions (Figure 5). Shin and LaBella (1970) observed a similar phenomenon using 0.1 M choline in acetic acid to dissociate the "total neurophysin" peak obtained from Sephadex G-75. The range of the molecular weights of the components of the separated peaks was estimated to be from 15,000 for the components of the least retarded peak, to less than 4,000 for the components of the most retarded peak eluted from Sephadex G-50. Peaks I and II (Figure 5) were thought to be polymeric forms of neurophysin which are normally non-covalently bound to each other. Peak III (Figure 5) was found to be composed of a mixture of peptides, of which at least one possessed the ability to stimulate contraction of the rat uterus. The isolation of the uterus-stimulating substance(s) contained in peak III is the concern of this investigation.

In order to isolate the peptide(s) with uterus-contracting activity, it was necessary at each purification step to examine each peptide subfraction with respect to this activity. Uterus-stimulating activity was determined as described in the Methods section of this report.

Chromatography on Sephadex G-10

Gel filtration on Sephadex G-10 resulted in a subfractionation of peak III obtained from Sephadex G-50 into two ninhydrin positive peaks (Figure 6). The first of the two peaks obtained from Sephadex G-10 was contained in a volume corresponding approximately to the elution volume of a substance of mol wt 1,300. The estimation of mol wt for the second

Figure 4. Elution profile of second peak of Figure 3 on Sephadex G-50 (2.5 x 100 cm) with 1.0 M acetic acid as eluant. Flow rate was 40 ml/hr and 6 ml fractions were collected. Each fraction was measured after reaction with ninhydrin.

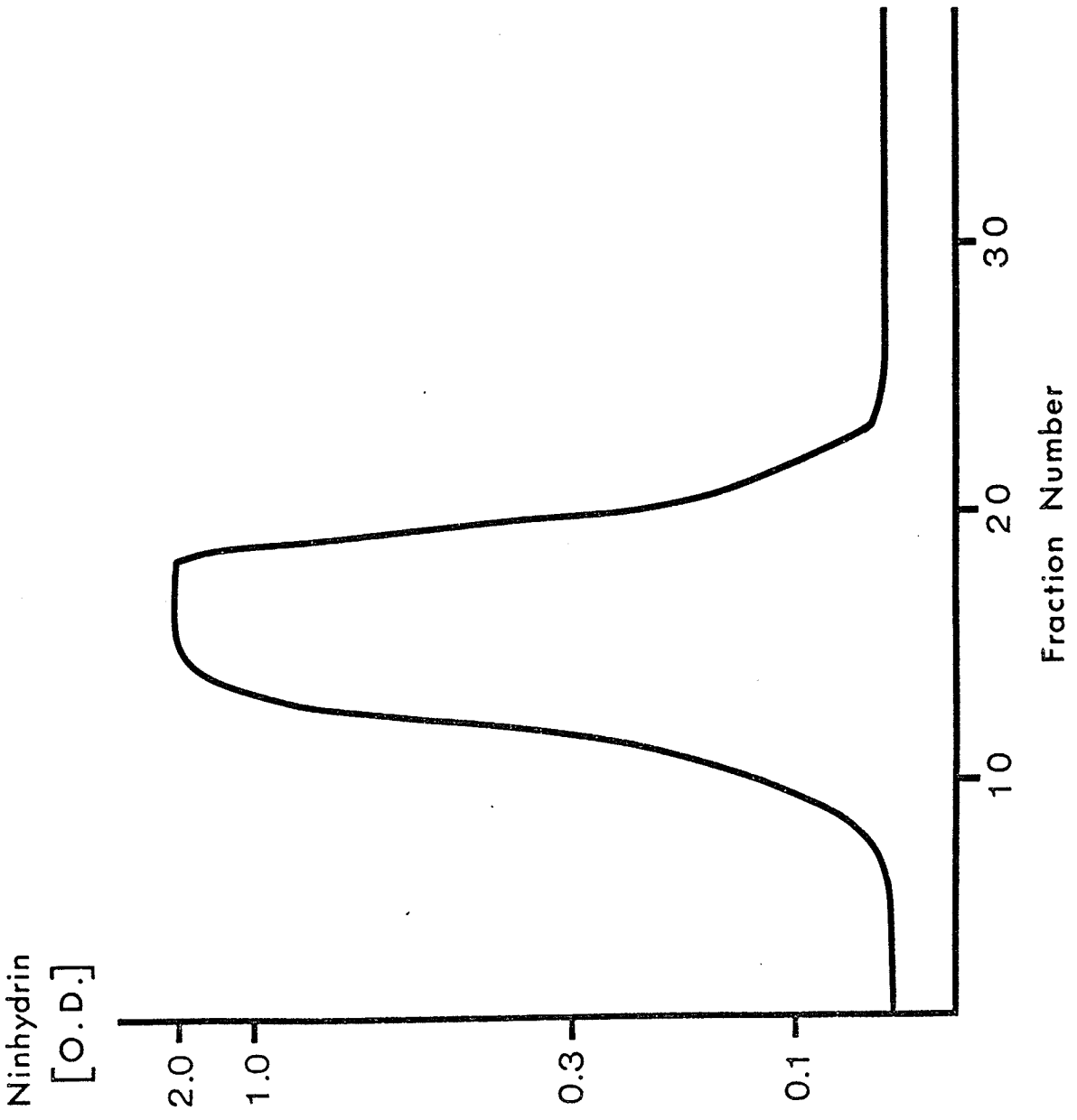


Figure 4.

Figure 5. Elution profile of second peak of Figure 3 on Sephadex G-50 (2.5 x 100 cm) with sodium carbonate-bicarbonate buffer pH 9.6 as eluant. Flow rate was 40 ml/hr and 6 ml fractions were collected. Each fraction was measured after reaction with ninhydrin.

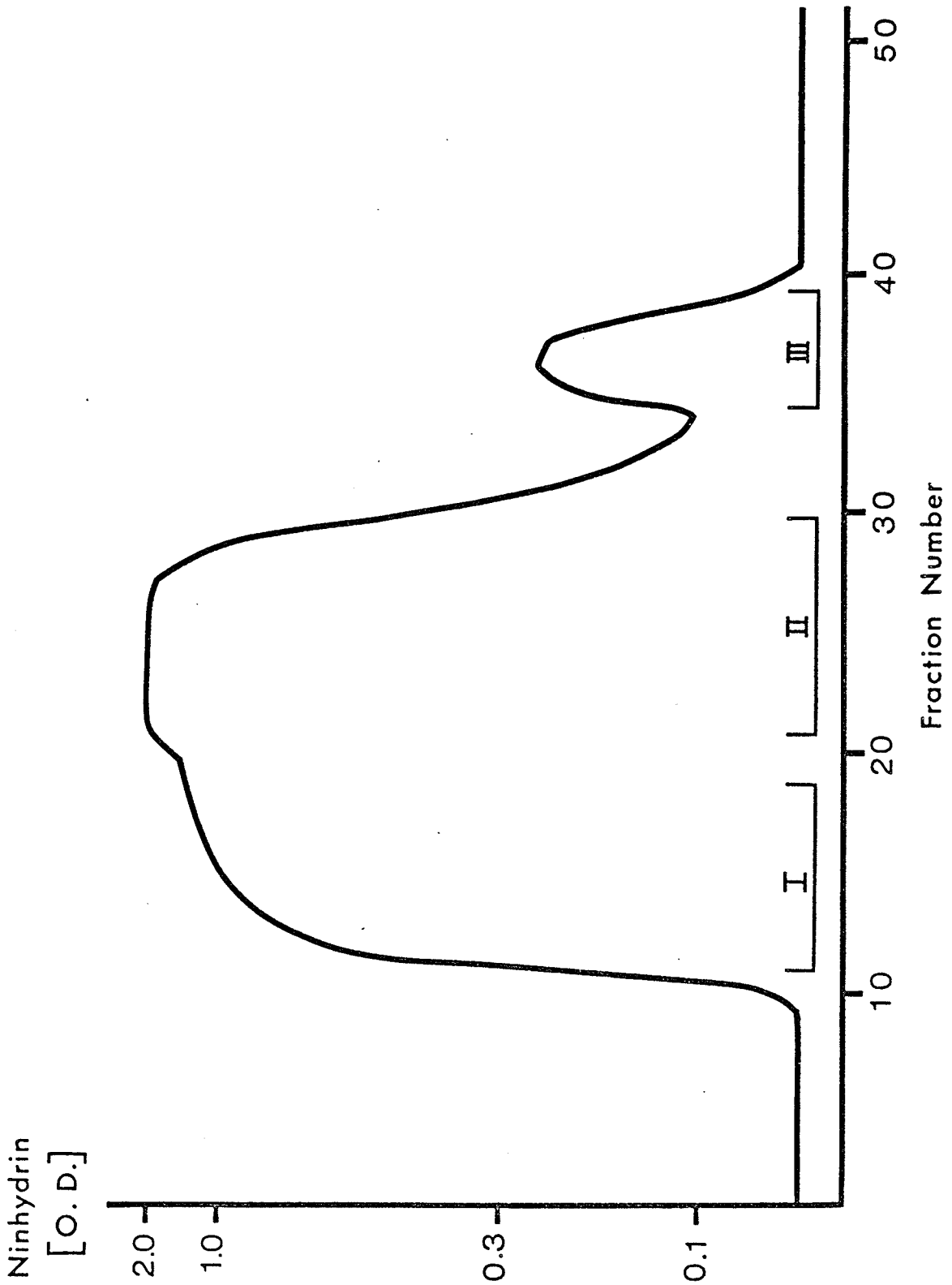


Figure 5.

Figure 6. Elution profile of peak III of Figure 5 on Sephadex G-10 (2.25 x 51 cm) with 0.2 M acetic acid as eluant. Flow rate was 50 ml/hr and 6 ml fractions were collected. Each fraction was measured after reaction with ninhydrin.

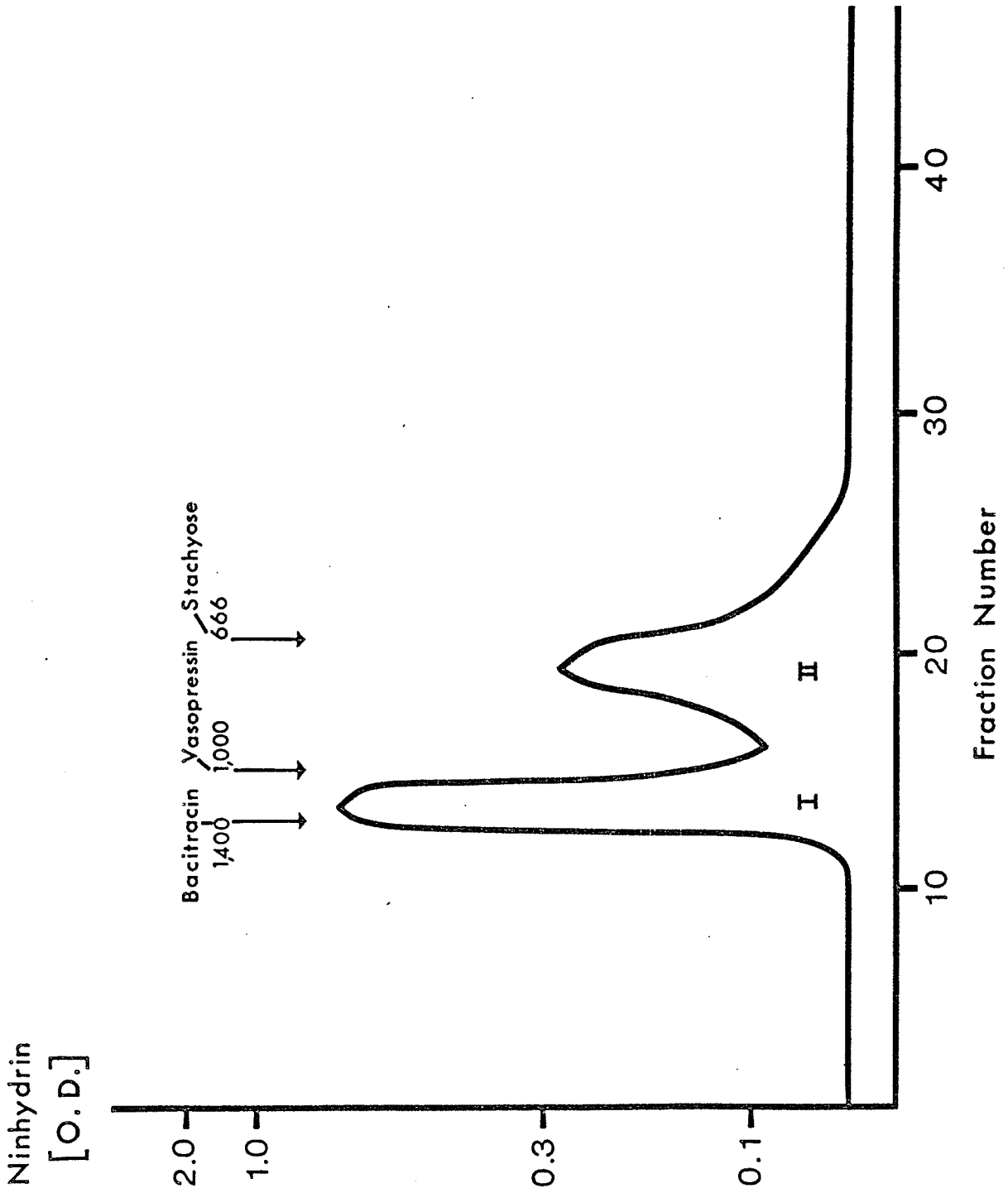

Ninhydrin
[O. D.]
2.0
1.0
0.3
0.1
Bacitracin
1,400
Vasopressin
1,000
Stachyose
666
I
II
10
20
30
40
Fraction Number

Figure 6.

peak was 700. The mol wt estimations were based upon the elution volumes of substances of known mol wt on the same column (Figure 6).

Paper chromatography

Peaks I and II obtained from Sephadex G-10 both demonstrated uterus-stimulating activity. Both peaks were rechromatographed separately on Sephadex G-10 to reduce cross contamination and applied to Whatman Chromatography paper # 3. Descending paper chromatography was carried out, developing both peak I and peak II with t-butanol:acetic acid:water (4:1:1, by volume) for 18 to 24 hours. Peak I from Sephadex G-10 was resolved into 5 bands by paper chromatography (see flow chart Figure 1). Each of these bands, when extracted from the paper and concentrated, showed uterus stimulating activity as indicated by the in vitro bioassay. Although further purification of the peptides obtained from peak I from Sephadex has been done, that work will not be described further in this report.

Peak II obtained from Sephadex G-10 was resolved into 2 ninhydrin positive bands by paper chromatography on Whatman Chromatography paper # 3. Whereas both the slower migrating band (A), and the faster migrating band (B) showed uterus-stimulating activity, the faster migrating band (B) was judged to be more potent in producing a uterine contraction in the isolated rat uterus when A and B were tested in equimolar quantities (as estimated by ninhydrin).

Ion-exchange chromatography

Band B obtained from paper chromatography was chromatographed by cation-exchange on CM-cellulose to yield three ninhydrin positive peaks B1, B2 and B3 (Figure 7). Peak B2 was shown to possess uterus-

Figure 7. Elution profile of band B (from paper chromatography of peak II from Sephadex G-10, Figure 1) on CM-cellulose (1.5 x 36 cm). Flow rate was 30 ml/hr and 3 ml fractions were collected. Each fraction was measured after reaction with ninhydrin. The cross-hatched bar indicates those fractions showing oxytocic-like activity.

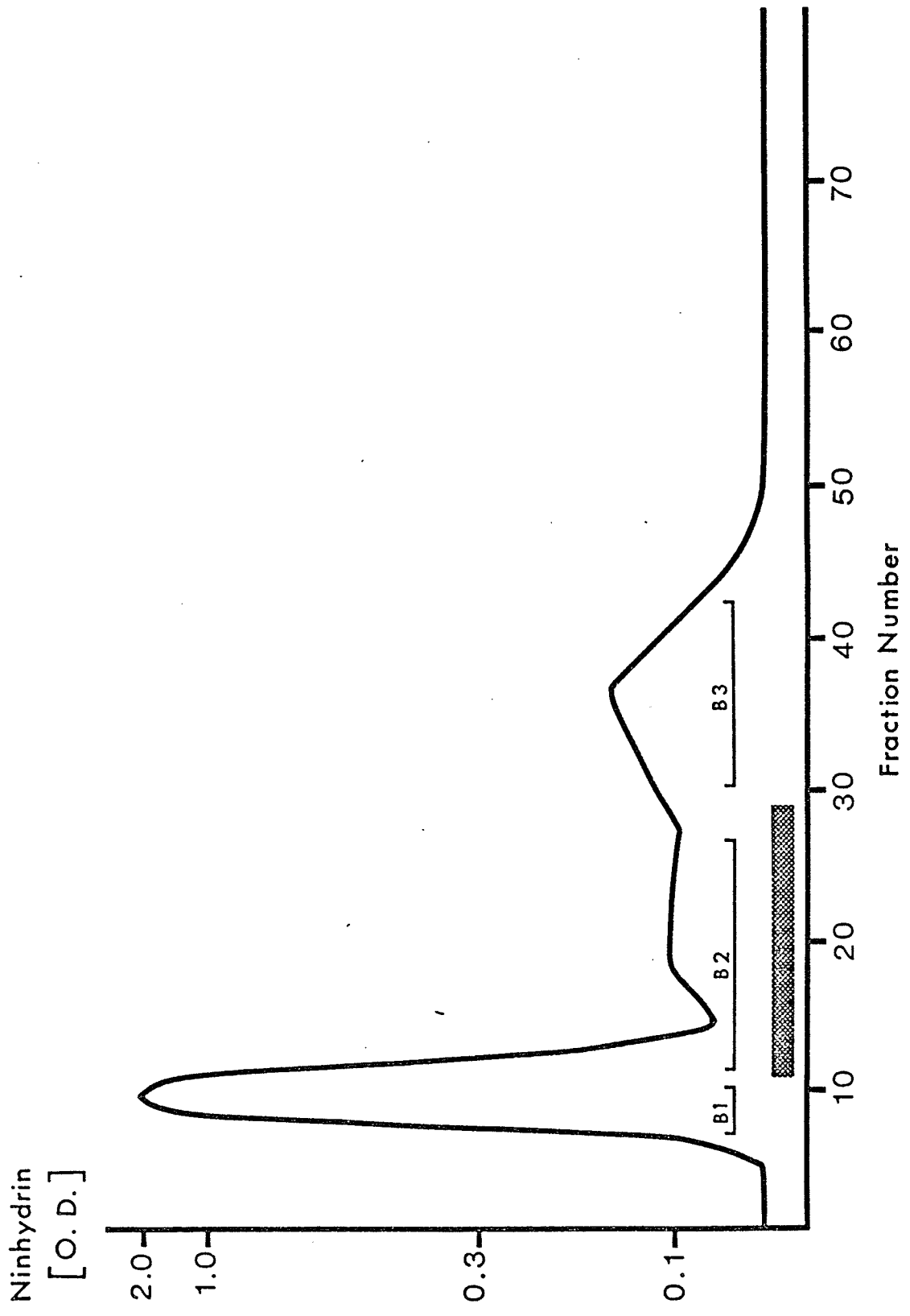

Ninhydrin
[O. D.]
2.0
1.0
0.3
0.1
B1
B2
B3
10
20
30
40
50
60
70
Fraction Number

Figure 7.

stimulating activity, while peaks B1 and B3 were inactive. B2 was rechromatographed by cation-exchange chromatography and resolved into two ninhydrin positive peaks, B2a and B2b (Figure 8). Uterus-stimulating activity was confined to peak B2b. High voltage paper electrophoresis of a sample of concentrated peak B2b (0.1 M di-sodium hydrogen phosphate-sodium dihydrogen phosphate buffer pH 7.0, at 3,000 V/15 min) resolved B2b into two ninhydrin positive sports which migrated in opposite directions from the central point of sample application.

Peak B2b was resolved into two ninhydrin positive fractions B2b1 and B2b2 by anion-exchange chromatography on QAE Sephadex (Figure 9). Uterus-stimulating activity was concentrated in the retarded peak B2b2 obtained from QAE Sephadex. Peak B2b1 showed only slight uterus-stimulating activity.

Uterus-stimulating activity of peptide B2b2

In order to gauge the potency of B2b2 an "activity coefficient" was defined as: the nanomoles (expressed as norleucine equivalents of intact peptide) of B2b2 required to produce a uterine contraction equivalent to the uterine contraction produced by a known dose of synthetic oxytocin, divided by the nanomoles of synthetic oxytocin in the standard dose. B2b2 and the synthetic oxytocin were tested in the same rat uterus. The standard dose of synthetic oxytocin used was 0.2 nanomoles (10 mU). It was found that 0.625 nanomoles of B2b2 were equivalent to 10 mU oxytocin. The "activity coefficient" of B2b2 was therefore calculated to be 3.5, indicating that B2b2 was approximately one third as potent as oxytocin in ability to stimulate contraction of the rat uterus.

Figure 8. Elution profile of peak B2 of Figure 7 on CM-cellulose (1.5 x 36 cm). Flow rate was 30 ml/hr and 3 ml fractions were collected. Each fraction was measured after reaction with ninhydrin. The cross-hatched bar indicates those fractions showing oxytocic-like activity.

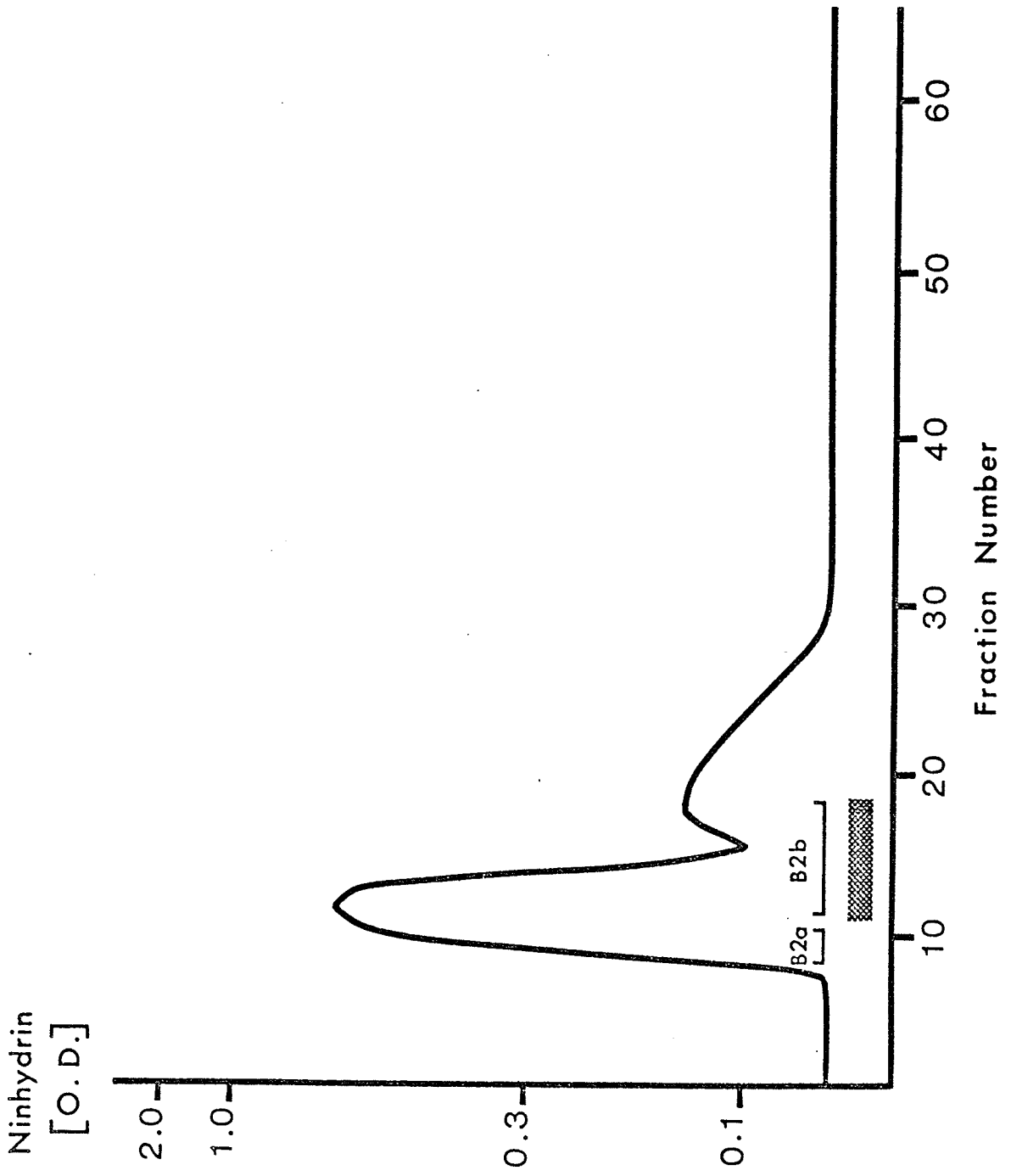

Ninhydrin
[O. D.]
2.0
1.0
0.3
0.1
B2a
B2b
10
20
30
40
50
60
Fraction Number

Figure 8.

Figure 9. Elution profile of peak B2b of Figure 8 on QAE-Sephadex (1.5 x 16 cm). Flow rate was 24 ml/hr and 4 ml fractions were collected. Each fraction was measured after reaction with ninhydrin. The cross-hatched bar indicates those fractions showing oxytocic-like activity.

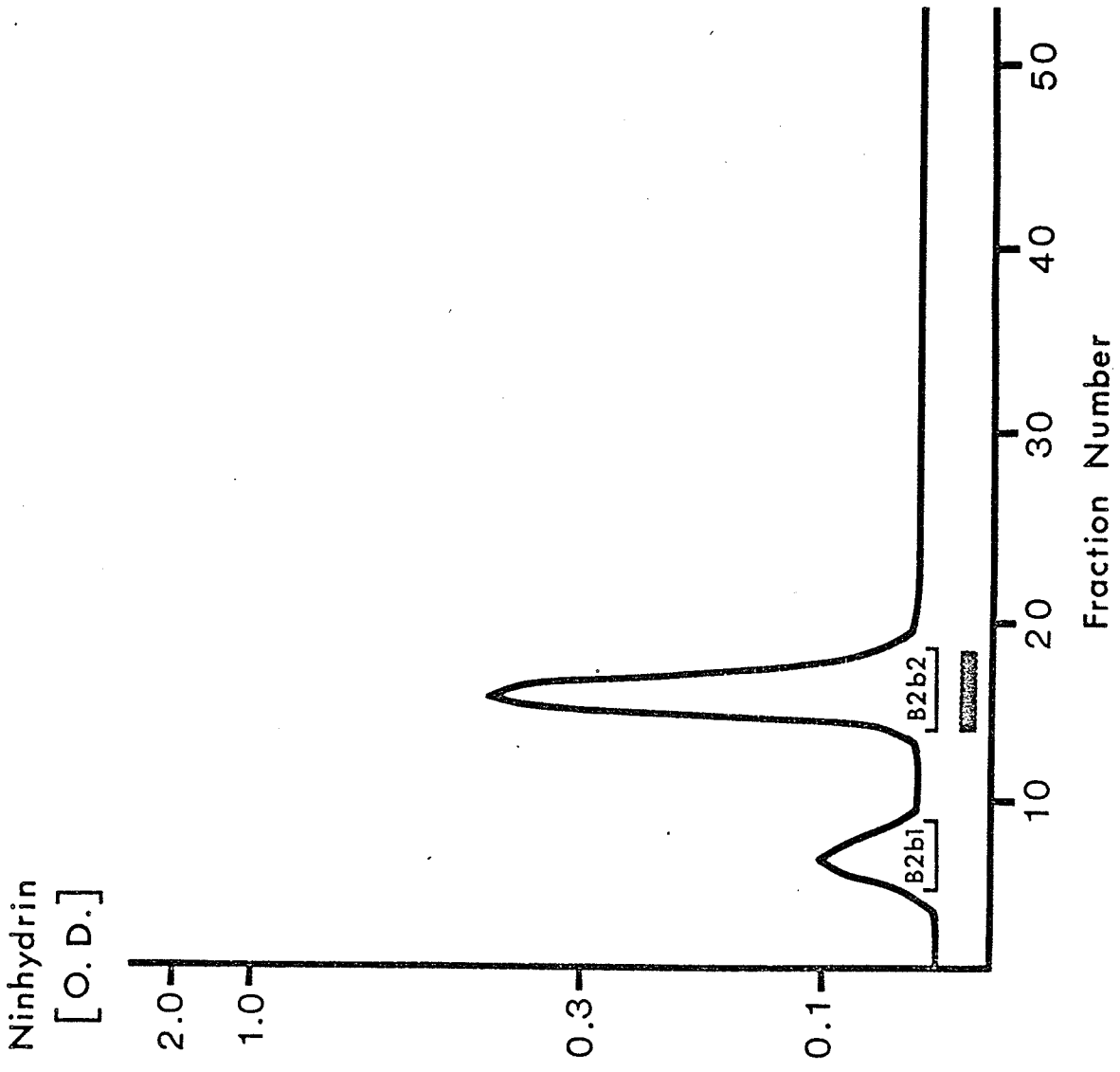

Ninhydrin
[O. D.]
2.0
1.0
0.3
0.1
B2b1
B2b2
10
20
30
40
50
Fraction Number

Figure 9.

Thin layer chromatography, paper electrophoresis and amino acid composition of peptide B2b2

Thin layer chromatography in three buffer systems was carried out on Peak B2b2, and R_f values of B2b2 in each of the systems were calculated (Figure 10). B2b2 migrated as a single ninhydrin positive spot in each of three buffer systems used.

Peak B2b2 was examined by high voltage paper electrophoresis. In all buffer systems (Figure 11) B2b2 migrated as a single ninhydrin positive spot.

In all systems B2b2 was spotted at a concentration fifty times the minimum concentration of B2b2 detected by ninhydrin reaction.

On the basis of the results of ion-exchange chromatography, thin layer chromatography, and high voltage paper electrophoresis, peak B2b2 was judged to be homogeneous. B2b2 was hydrolysed and amino acid composition was determined (TABLE III). TABLE III also compares the amino acid composition of B2b2 with that of oxytocin and vasopressin.

Figure 10. Thin layer chromatography (ascending) of B2b2 in three buffer systems: Plates A, B and C are tracings of the original developed thin layer chromatogrphy plates. The three buffer systems used were:

plate A: ethyl acetate:water:t-butanol:acetic acid (1:1:1:1)

plate B: t-butanol:water:acetic acid (4:1:1)

plate C: ammonium hydroxide:n-propanol:water (1:100:50)

Samples of B2b2 were applied to each TLC plate as single spots (P). The direction of buffer flow is indicated by the arrows adjacent to the line denoting the final position of the solvent front (S F).

R_f values were calculated using the formula:

$$R_f = \frac{\text{distance migrated by B2b2 (cm)}^*}{\text{distance migrated by solvent front (cm)}^{**}}$$

* measured from the origin (P) to the leading edge of the migrating sample (M S).

** measured from the origin (P) to the final position of the solvent front.

Figure 10.

THIN LAYER CHROMATOGRAPHY OF B2b2 IN THREE BUFFER SYSTEMS

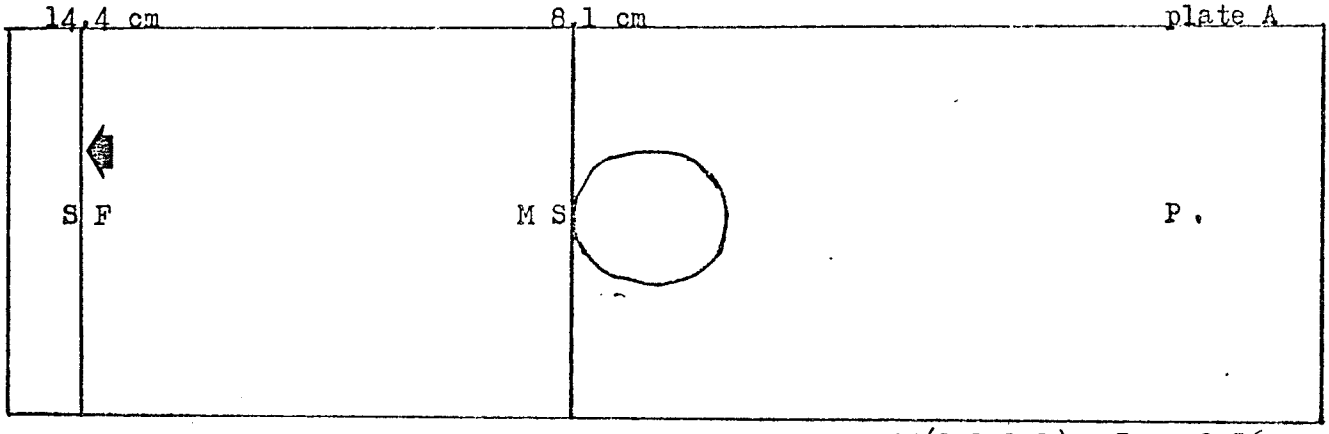


plate A: ethyl acetate:water:t-butanol:acetic acid(1:1:1:1) $R_f = 0.56$

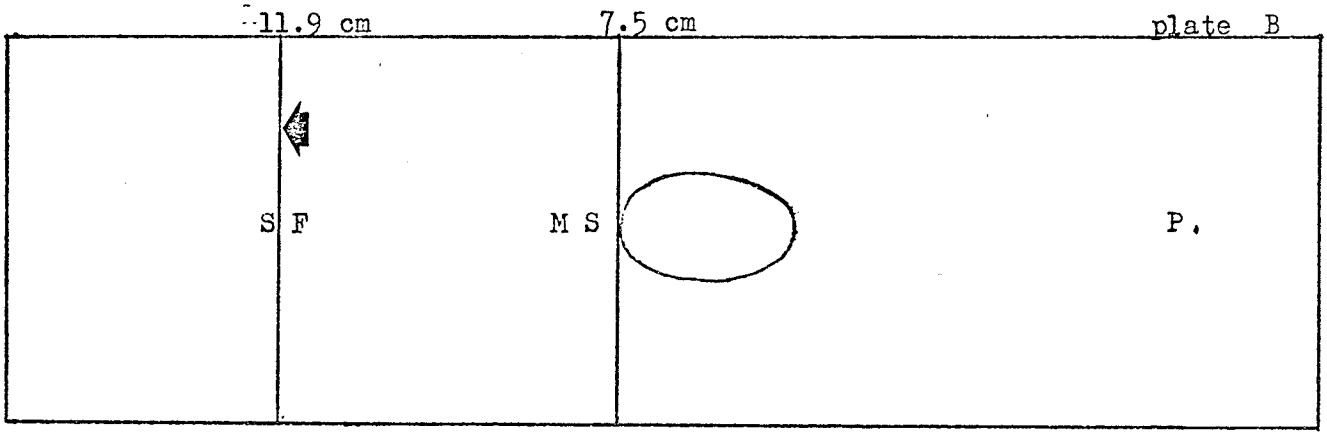


plate B: t-butanol:water:acetic acid(4:1:1) $R_f = 0.63$

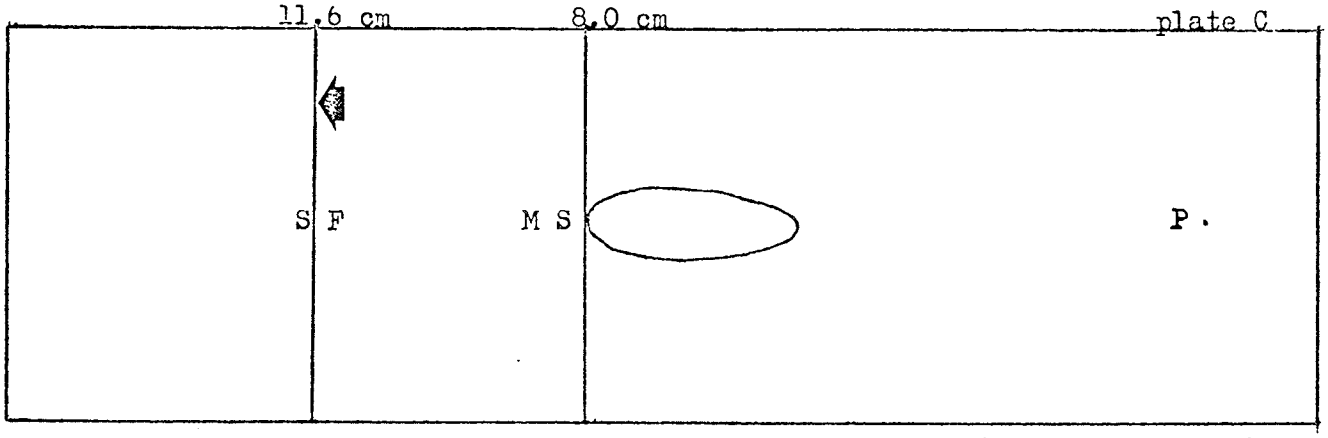


plate C: ammonium hydroxide:n-propanol:water(1:100:50) $R_f = 0.69$

Figure 11. High voltage paper electrophoresis of B2b2 at three different pHs. Papers A, B and C are tracings of the original developed electrophoresis papers. The three buffer systems used were:

paper A: formic acid:water:acetic acid (15.6:455,0:29.6)

paper B: di-sodium hydrogen phosphate-sodium dihydrogen phosphate 0.1 M.

paper C: sodium carbonate-sodium bicarbonate 0.1 M.

P indicates the point of application of samples of B2b2.

- and + denote the positions of the anode and cathode respectively.

kV = applied voltage in kilovolts.

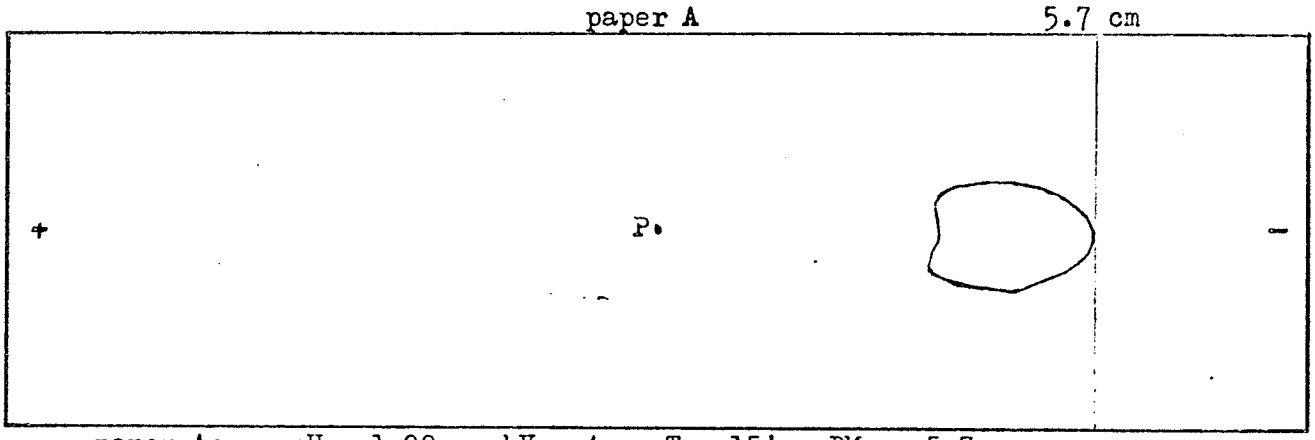
T = duration of applied voltage in minutes.

DM* = distance (cm) migrated by the sample from the origin (P).

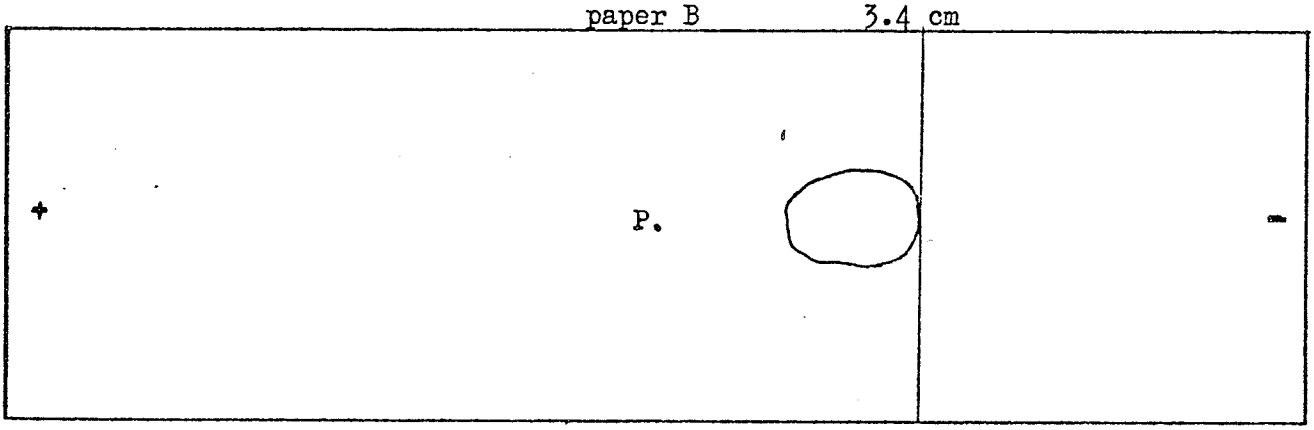
* The sign (- or +) prefixed to this figure denotes directional migration of the sample towards the anode (-) or cathode (+).

Figure 11.

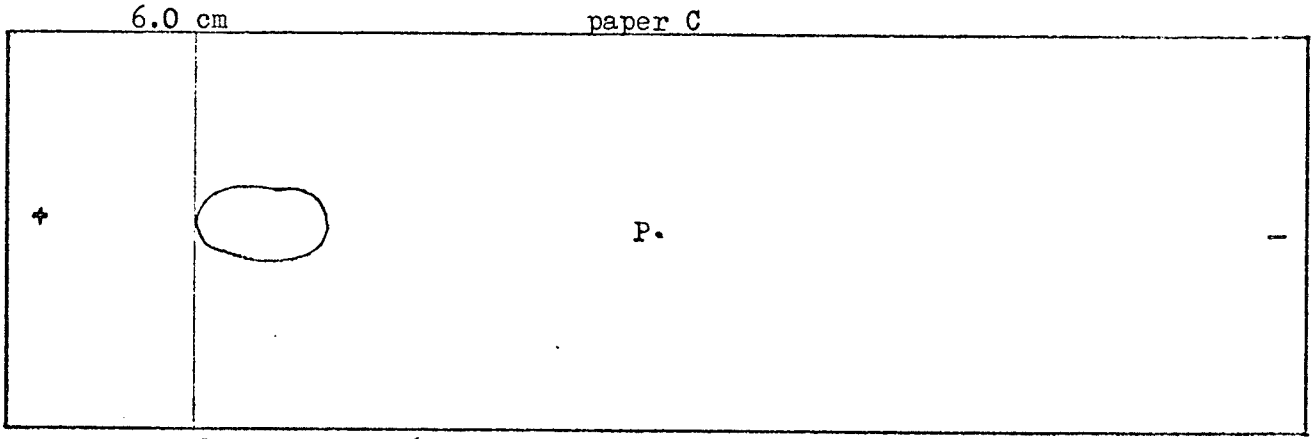
HIGH VOLTAGE PAPER ELECTROPHORESIS OF B2b2



paper A: pH = 1.99 kV = 4 T = 15' DM = -5.7 cm



paper B: pH = 7.01 kV = 3 T = 15' DM = -3.4 cm



paper C: pH = 9.60 kV = 4 T = 10' DM = +6.0 cm

TABLE III.

AMINO ACID COMPOSITION OF B2b2 COMPARED WITH OXYTOCIN AND VASOPRESSIN**

Amino acid	Residues/1000	No. of residues per minimum molecular weight		
		B2b2	Oxytocin	Vasopressin
ASP	78	4	1 (ASN)	1 (ASN)
GLU	178	8	1 (GLN)	1 (GLN)
GLY	172	8	1	1
ALA	87	4	---	---
VAL	70	3	---	---
LEU	22	1	1	---
ILE	---	---	1	---
PRO	---	---	1	1
LYS	33	1	---	1(porcine)
HIS	27	1	---	---
ARG	---	---	---	1(bovine)
CYS	---*	---*	2	2
SER	191	9	---	---
THR	69	3	---	---
TYR	---	---	1	1
PHE	---	---	---	1
	TOTAL	42	9	9

*determined from absence of cysteic acid

**not corrected for destruction that occurred during hydrolysis.

IV. DISCUSSION

DISCUSSION

The posterior pituitary gland contains an extensive concentration and variety of peptides (Winnick et al., 1955). In addition to the peptide hormones oxytocin and vasopressin, other peptides have been found to be associated with the neurosecretory granules and the protein neurophysin contained therein. Evidence has been presented suggesting storage of oxytocin and vasopressin in separate granules and neurons (LaBella et al., 1963; Bindler et al., 1967), possibly associated with different neurophysin components (Dean et al., 1968).

The concern of the present investigation is with the isolation and identification of a neurohypophyseal peptide distinct from oxytocin and vasopressin, yet possessing uterus-stimulating activity, and which is associated with the neurophysin component of the neurosecretory granule.

Molecular weight and amino acid composition of peptide B2b2

The amino acid composition of peptide B2b2 is indicated in TABLE III of the results section. The approximate mol wt of peptide B2b2 as estimated from this amino acid composition is 4500. Although ion-exchange chromatography, thin layer chromatography and high voltage electrophoresis are techniques often used to demonstrate the fulfillment of the criteria of homogeneity, and in the case of peptide B2b2, did indicate a homogeneous peptide fraction, positive results obtained with these techniques do not necessarily constitute absolute proof that the substance in question is indeed homogeneous. Other results obtained in this study are not consistent with the mol wt of peptide B2b2 as estimated from its amino acid composition. The amino acid composition

of peptide B2b2 (TABLE III) indicates that peptide B2b2 is composed of forty-one amino acid residues and has an approximate mol wt of 4500. In contrast, the mol wt of B2b2 was estimated by gel filtration chromatography on Sephadex G-10 to be between 700 and 1200 mol wt. The apparent inconsistency between the mol wt estimations may be the result of several considerations.

Estimation of molecular weight by gel filtration

The reliability of gel filtration techniques as a means of estimation of mol wt is in many instances questionable. The presence of aromatic amino acid residues in proteins and peptides can result in the retardation of movement of these substances through the gel filtration column. The result is an erroneously undervalued estimate of the substance's true mol wt. The importance of the above consideration in explaining the discrepancy in mol wt estimation of peptide B2b2 would appear to be negligible in view of the absence of the aromatic amino acid residues in peptide B2b2.

Peptide B2b2 as a peptide mixture

The possibility exists that peptide fraction B2b2 is composed of a mixture of peptides of similar mol wt. These peptides could conceivably move as a single band during gel filtration, with the differences in the molecular compositions becoming apparent with the determination of the amino acid composition of individual bands. With respect to peptide B2b2, the occurrence of a mixture could reconcile the low molecular weight estimation by gel filtration with the large molecular size indicated by the amino acid composition. It is, however, unlikely that such a situation could occur and remain undetected

throughout the procedures and varied conditions of thin layer chromatography, electrophoresis and ion-exchange chromatography. As well, under the above conditions the complete formation of a single peptide aggregate from a mixture of peptides is improbable.

Interpretation of amino acid composition

The amino acid composition of peptide B2b2 shown in TABLE III is expressed in terms of amino acid residues per minimum mol wt. When initial analysis of the amino acid composition of a peptide is performed, the concentrations of the individual amino acids are expressed in terms of amino acid residues present per thousand total amino acid residues examined. The amino acid, which is detected as having the least number of residues present, forms the base for the calculation of the number of residues of any given amino acid present in one molecule of the unknown peptide. One molecule of the unknown peptide is assumed to contain only one residue of this chosen amino acid. The concentration (# residues/ 1,000 total amino acids) of this amino acid is compared to the concentrations of the other amino acids and the number of residues of each amino acid present in one peptide molecule is determined by proportion. It is evident, then, that the amino acid present in the peptide hydrolysate in minimum concentration is critical in the determination of total amino acid composition. The presence of trace contaminations of amino acids in the peptide hydrolysate, or in the analysis system can result in misinterpretation of amino acid analysis and lead to a distorted concept of the peptide's amino acid composition. The sources of contamination in preparative procedures and amino acid analysis systems can be minimized, but are seldom entirely eliminated. It is, therefore, necessary that critical decisions be made as to whether an amino acid,

that is detected in small quantities, is a contamination, or is a true constituent of the peptide in question. In situations where the investigator must work with only limited quantities of material, the problem is magnified.

In the analysis of the amino acid composition of peptide B2b2, the amino acids leucine, lysine, and histidine were detectable in small concentrations. The amino acid composition shown in TABLE III was calculated assuming that these amino acids (lys, leu, his) were true constituents of a single B2b2 peptide. However, the minimal concentrations of the amino acids leucine, lysine and histidine that were detected, indicate the distinct possibility that these amino acids represent contamination. This interpretation is questionable, as it is not unreasonable to assume that the presence of these amino acids in the B2b2 fraction, prior to the determination of amino acid composition, would have been detected by ion-exchange chromatography, thin layer chromatography, or paper electrophoresis. However, the possibility remains that contamination of peptide B2b2 was introduced during the actual process of amino acid determination.

Alternate to the consideration that contamination is responsible for the observed discrepant results which preclude a single definite estimation of mol wt, is the suggestion that the amino acids lysine, leucine and histidine are contained by only a proportion of the peptides in the peptide fraction B2b2. The peptide fraction B2b2 would be composed of a single species of peptide, and a proportion of that peptide species would contain in addition, or as substitutions the amino acids leucine, lysine and/or histidine. Such addition and/or substitution

would not necessarily alter the chromatographic or electrophoretic behaviour of the peptide. Heterogeneity in a species of peptide may be produced by processess involved with peptide activation, cleavage of peptide from a precursor, or peptide synthesis. For example, until an attempt was made to determine the amino acid sequence of the enzyme carboxypeptidase, it was not appreciated that this enzyme exists in several forms within a given animal species (Bargetzi et al., 1964; Cox et al., 1964; Kumar et al., 1964 a,b, 1964). These enzymes, differing only slightly in length at the N-terminal are produced by unequal cleavage during activation from the precursor procarboxypeptidase. In addition, the sequence studies revealed that genetic differences within one animal were responsible for the production of heterogeneous forms of carboxypeptidase (heteroallelomorphism). Any preparation of natural carboxypeptidase is proportioned into forms of the enzyme in which the antipenultimate carboxy-terminal residue is either leucine or valine. This conservative substitution of amino acid residues doe not modify biological activity and has little apparent effect on the physical properties of the enzyme. While the mol wt of carboxypeptidase exceeds that of peptide B2b2, the enzyme serves to illustrate the natural occurrence of the above described process.

Confirmation of the existence of heteroallelomorphic forms of peptide B2b2 would require an extensive study involving amino acid sequence determination and peptide synthesis. Inspection of the amino acid composition of B2b2, nevertheless, provides at least an indication as to the feasibility of such an occurrence. In those peptides where conservative substitution of amino acids has been detected or accomplished synthetically, the conservation of the biological and physical properties

of the peptide has been attributed to the fact that, the amino acids which act as conservative substitutes possess a common property. This property is either, essential to the functional activity of the peptide, or does not distort the conformation of the peptide. The amino acid composition of peptide B2b2 provides ample margin wherein the amino acids leucine, lysine and histidine conceivably could act as conservative substitutes. For example, the amino acids leucine, and valine are both neutral hydrophobic amino acids and may be exchanged on this basis. The properties required of a particular residue may be highly specific, or of a general nature. The function served by an amino acid residue in the peptide sequence is not readily predictable. Therefore, the above examples do not attempt to exhaust the possibilities for the conservative substitution of amino acid residues in peptide B2b2.

The determination of end-group amino acids could provide decisive evidence as to the true solution to the above discussed problem. At this time, however, limited quantities of purified material prevent such a determination.

Recalculation of amino acid composition.

Recalculation of the amino acid composition of peptide B2b2 is justified in consideration of the possibility that the amino acids lysine, histidine and leucine are contained only in a minor proportion of the peptide B2b2, or represent contamination of the B2b2 peptide fraction. The two calculated amino acid compositions for B2b2 are shown in TABLE IV.

Comparison of B2b2 (41) and B2b2 (10)

To facilitate the discussion that is to follow, the two possible amino acid compositions of B2b2 will be referred to as "B2b2(41)" and "B2b2(10)." B2b2(41) denotes the peptide for which the amino acid composition was derived on the basis of the inclusion of the amino acids

TABLE IV

AMINO ACID COMPOSITIONS OF : B2b2(41), B2b2(10), Oxytocin, Vasopressin, Angiotensin, Bradykinin, and Substance P.***

Amino acid	B2b2(41)	B2b2(10)	Oxytocin	Vasopressin	Angiotensin	Bradykinin	Substance P
			(residues per minimum mol wt)				
ASP	4	1	1	1	1	---	1
GLU	8	2	1	1	---	---	1
GLY	8	2	1	1	---	1	1
ALA	4	1	---	---	---	---	1
VAL	3	1	---	---	1	---	1
LEU	1	---	1	---	---	---	1
ILE	---	---	1	---	1	---	1
PRO	---	---	1	1	1	3	1
LYS	1	---	---	1(porcine)	---	---	1
HIS	1	---	---	---	1	---	---
ARG	---	---	---	1(bovine)	1	2	1
CYS*	---*	---*	2	2	---	---	---
SER	9	2 (or 3)	---	---	---	1	1
THR	3	1	---	---	---	---	1
TYR	---	---	1	1	1	---	---
PHE	---	---	---	1	1	2	1
total	42	10	9	9	8	9	13
mol wt	4636	1012	1007	1084	1171	1204	1650 ± 250**

*determined from absence of cysteic acid.

**Volgler et al.,1963; Meinardi and Craig,1966.

***see table III for raw data.

leu, lys and his as true constituents in the structure of B2b2. B2b2(10) denotes the peptide for which the amino acid composition was derived in consideration of leu, lys and his as contaminants, or as present in a small proportion of B2b2. The numbers in parenthesis (41 and 10) refer to the number of amino acid residues contained in B2b2(41) and B2b2(10), respectively. B2b2(41) contains forty-one amino acid residues and has an approximate mol wt of 4500 (TABLE IV). B2b2(10) contains ten amino acids and the mol wt of B2b2(10) as estimated from the amino acid composition (TABLE IV) is approximately 1,000. In contrast to B2b2(41), the mol wt of B2b2(10) (1,000), as calculated from its amino acid composition, is consistent with the mol wt of peptide B2b2 (700-1200) as estimated by gel filtration.

The recalculation of amino acid composition effects a marked change in mol wt estimation of peptide B2b2. Much of the following discussion is valid in terms of both B2b2(10) and B2b2(41). As evidence does not permit a clear choice as to which amino acid composition is representative of the true situation, the remaining discussion will be in terms of B2b2(10), with appropriate distinction being made as regards B2b2(41). Where discussion is pertinent to both, the peptide will be referred to as B2b2.

Peptide B2b2 as distinct from oxytocin, vasopressin, angiotensin, bardykinin and substance P.

Peptide B2b2 considered as either B2b2(41) or B2b2(10) is distinct from the hormones oxytocin, vasopressin, angiotensin, bradykinin and substance P. The estimated mol wt of B2b2(41) (4500) exceeds that of B2b2(10) (1000) by a factor of four. Although the mol wt of peptide

B2b2(10) is similar to that of the above hormones, amino acid composition differentiates both B2b2(10) and B2b2(41) from these hormones as is shown in TABLE IV.

It must be noted at this point, that the absence of the amino acid cysteine from B2b2 was assumed in consideration of the absence of a detectable cysteic acid component in the acid hydrolysate of peptide B2b2. The production of cysteic acid occurs during acid hydrolysis of peptide, prior to the determination of amino acid composition. Cysteine residues contained in the peptide are oxidized (non-quantiatively) to form cysteic acid.

The uterus-stimulating activity of the peptide fraction is attributed to peptide B2b2 on the assumption that the procedures involved in the purification of B2b2 eliminate the possibility that, oxytocin and/or vasopressin, present in undetectable quantities, are responsible for the observed uterus-contracting activity. Synthetic vasopressin is eluted from Sephadex G-10 well in advance of the fraction containing peptide B2b2, and is clearly separated from peptide B2b2 (Fig. 6, Results). In as much as the mol wt of oxytocin is almost identical to that of vasopressin, and oxytocin contains fewer aromatic amino acid residues than does vasopressin, one would expect the elution volume of oxytocin on Sephadex G-10 to be the same as, if not less than that of vasopressin. Separation of peptide B2b2 from vasopressin therefore implies at least as clear a separation of the peptide fraction from the hormone oxytocin. If the assumption is made that oxytocin or vasopressin contamination is responsible for the observed uterus-stimulating activity of peptide B2b2, then at the concentration indicated by the rat-uterus bioassay, these hormones would be detected by ninhydrin

reaction. The results of thin layer chromatography and paper electrophoresis did not indicate the presence of more than one ninhydrin positive substance. Furthermore, amino acid residues of the hormones oxytocin and vasopressin were not contained in amino acid composition of B2b2 (TABLE IV). It was, therefore, assumed that the observed uterus-stimulating activity of peptide B2b2 could not be attributed to contamination by these hormones.

Peptide B2b2 as compared to other oxytocic peptides isolated from the posterior pituitary

Oxytocic substances have been isolated from the posterior pituitary and hypothalamus by other investigators (Smith and Rosenfeld, 1962; Preddie and Saffran, 1965 a,b; Hawker et al., 1969; Hope and Watkins, 1969). To date, however, the properties of these oxytocic substances published in the literature, indicate little similarity to the oxytocic peptide discussed in the present report.

Smith and Rosenfeld (1962) reported the presence of an oxytocic component in press juice prepared from bovine posterior pituitary. This oxytocic substance was differentiated from both free and neurophysin bound oxytocin. The molecular size of the oxytocic substance was estimated to be intermediate between 1000 and 13,700 mol wt. The mol wt of peptide B2b2(10) (1012) certainly fits into this range, however, Smith and Rosenfeld subsequent to their initial publication, have provided no further information on their oxytocic substance.

A large polypeptide (mol wt 5500) isolated from bovine posterior pituitary by Preddie and Saffran (1965) is worthy of some discussion. Structurally their peptide consists of forty-eight amino acids and "the carboxyl-terminal portion is characterized by a cyclic

octapeptide resembling in form the structure of oxytocin". Preddie suggests that this cyclic structure, and the minimal detectable oxytocic activity of the large polypeptide may indicate that the large peptide is a precursor to a smaller oxytocin-like substance. The amino acid compositions of peptide B2b2(10) and that of the carboxyl-terminal octapeptide of Preddie's large polypeptide have some features in common. Both peptides contain the amino acids alanine, valine, glycine, serine, glutamic acid and aspartic acid. However, peptide B2b2(10) contains the amino acid threonine, and does not contain the cystine and leucine present in Preddie's carboxyl-terminal peptide. Another dissimilarity between the two peptides is that while both contain serine and glutamic acid, peptide B2b2(10) contains two each of serine and glutamic acid and the carboxyl-terminal octapeptide contains only one of each amino acid. Perhaps the greatest dissimilarity between the two peptides is the apparent absence of the cystine disulfide bridge in peptide B2b2.

Less similarity is found between B2b2(41) and the large polypeptide isolated by Preddie. Preddie's polypeptide contains the amino acids, agrinine, proline, isoleucine, phenylalanine and cysteine. These five amino acids, not contained in the amino acid composition of B2b2(41), account for a total difference of fifteen amino acid residues between the two peptides.

In terms of biological activity, the "minimal oxytocic activity" (Quantitation of activity not provided) of Preddie's large polypeptide is in contrast to that of peptide B2b2.

Hawker et al. (1969) and Hope and Watkins (1969) have isolated a peptide with oxytocic activity from bovine hypothalamus and posterior pituitary, respectively. While Hawker et al. (1969) have not as yet

accumulated sufficient material for chemical characterization of their peptide, Hope and Watkins (1969) have pointed out similarities between the peptide studied by Hawker et al. (1969) and that peptide isolated in Hope's own laboratory. A preliminary amino acid analysis estimated the molecular weight of Hope's oxytocic peptide to 3,000 (Hope and Watkins, 1969). The amino acid composition (on which the mol wt estimation was based) was not published with the mol wt estimation. On the basis of mol wt alone, it would seem unlikely that Hope's oxytocic peptide and peptides B2b2(10) and B2b2(41) are similar.

Significance of B2b2 in the neurohypophysis

The production of artifactual proteins and peptides in tissue preparations as a result of the actions of the catheptic enzymes which are contained in the tissues is of great concern. Dean, Hollenberg and Hope (1967) provided evidence that extraction of bovine neurophysin with 0.1 N HCl irreversibly destroyed catheptic activity in neurohypophyseal homogenates. LaBella (1971) has shown that the properties and multiple components of bovine neurophysin were essentially identical for neurohypophyseal extracts prepared with either o.2 M acetic acid or 0.1 N HCl. Nevertheless, in order to minimize the possibility of an artifactual production of peptide B2b2, neurophysin was prepared (for the present study) from a neurohypophyseal homogenate which was extracted at $4^{o}C$ with 0.1 N HCl. Separation of a fraction containing peptide B2b2, from neurophysin prepared as above, was effected by gel filtration of the neurophysin on Sephadex G-50 at pH 9.6. Rechromatography of the neurophysin preparation on the basic Sephadex column did not result in a further yield of fractions containing the peptide B2b2. Chromatography of the original neurophysin preparation on the Sephadex G-50 column at

pH 2.3 did not afford a separation of the fraction containing peptide B2b2 from neurophysin, as had occurred when gel filtration was carried out at pH 9.6. Once achieved, the dissociation of peptide B2b2 from neurophysin could be reversed by recombination of the fractions collected from the basic (pH 9.6) Sephadex column, followed by readjustment of the pH of these pooled fractions from pH 9.6 to pH 2.3.

The association of peptide B2b2 with the extracted neurophysin implies a form of storage of peptide B2b2 within the neurohypophyseal neurosecretory granule not unlike that of the hormones oxytocin and vasopressin. It is worthy of note, that peptide B2b2 in its association with neurophysin differs from oxytocin and vasopressin. The association between peptide B2b2 and neurophysin is subject to disruption in basic medium while oxytocin and vasopressin are dissociated from neurophysin in more acidic conditions. While limited quantities of material have excluded extensive study of the binding of peptide B2b2 with neurophysin, the observation of a pH labile bond indicates a loose association of peptide B2b2 with neurophysin consistent with current theories of neurohypophyseal hormone storage and release. It is not unreasonable to consider that such an association of neurophysin and peptide B2b2 could be formed during the extraction of neurophysin from the tissue homogenate. More conclusive evidence for storage of peptide B2b2 within the neurosecretory granule requires isolation of the peptide from a pure neurosecretory granule preparation.

Regardless of the storage form of B2b2 in the posterior pituitary, the presence of the peptide in that gland must be explained in terms of function and physiological significance. The storage or non-storage of a physiologically active peptide in the neurosecretory

granule neither confirms, nor negates a hormonal role for the peptide in question. The associations formed with neurophysin by peptide B2b2 and the neurohypophyseal hormones are similar in that they do not appear to involve covalent bonding, yet they differ in that the associations are disrupted under completely opposite pH conditions. The present state of knowledge of the mechanisms governing the release of the neurohypophyseal hormones would allow little more than imaginative speculation as to the interpretation of the observation that, under conditions which are not duplicated physiologically in the neurohypophysis, the associations formed between the protein neurophysin and neurohypophyseal peptides may be reversibly disrupted.

Uterus-stimulating activity and an apparent ability to form reversible complexes with the protein neurophysin are properties possessed by the peptide B2b2 and are shared by the known neurohypophyseal hormones oxytocin and vasopressin. Consideration of the properties common to peptide B2b2 and the neurohypophyseal hormones strongly suggest the possibility of a hormonal role of peptide B2b2 in the posterior pituitary. However, such evidence considered alone is purely circumstantial, and acceptance of B2b2 in a hormonal capacity on that basis would be scientifically unfounded and premature. A more comprehensive examination of the peptide in terms of its physical and chemical properties as well as its distribution in the pituitary gland is required to establish conclusively peptide B2b2 as a native component of the neurohypophysis.

V. SUMMARY

SUMMARY

1. Whole bovine posterior pituitary glands were homogenized and extracted with 0.1 N HCl. The protein component of the extract, neurophysin, was isolated by gel filtration successively on Sephadex G-25 and G-75.

2. Chromatography of neurophysin on Sephadex G-50 at pH 9.6, resolved the protein into three subfractions of estimated mol wt > 4,000, < 4,000, and < 2,000, respectively. Only one component of estimated mol wt 13,000 was resolved at pH 2.3. A component, contained in the lowest mol wt subfraction induced contraction of the isolated rat uterus.

3. The active component was further purified by gel filtration on Sephadex G-10, paper chromatography, and ion-exchange chromatography on carboxy-methyl cellulose and QAE-Sephadex.

4. The active material is presumed to be a peptide on the basis of reaction of the purified fraction with ninhydrin, and is estimated to be approximately one-third as potent as synthetic oxytocin in producing contraction of the rat uterus.

5. Amino acid analysis indicated that the peptide contained aspartic acid, glutamic acid, glycine, alanine, valine, serine, threonine, as well as low concentrations of leucine, lycine, and histidine. The mol wt estimated from this composition was 4,500, as opposed to the estimate by gel filtration on Sephadex G-10 of 700-1200.

6. Homogeneity of the peptide was indicated by thin layer chromatography, high voltage paper electrophoresis, and ion-exchange chromatography, and thus did not favor consideration of the active fraction as a peptide mixture.

7. A second calculation of the amino acid composition of the peptide was made in consideration of the discrepancy in mol wt estimations and the low concentrations of leucine, lysine, and histidine detected in the amino acid analysis. The calculated mol wt of 1,012, estimated from the recalculated amino acid composition, was consistent with that of 700-1,200 mol wt, estimated by gel filtration.

8. The two possible amino acid compositions of the active material differ only in mol wt, i.e. 4,500 and 1,012, and there is little variation in terms of constituent amino acid residues.

9. In terms of chromatographic characteristics and amino acid composition, the active substance is apparently distinct from oxytocin, vasopressin, angiotensin, bradykinin, substance P, and from oxytocic peptides isolated from the hypothalamus and posterior pituitary by other workers.

10. Physiological significance and a possible hormonal role of the peptide has been discussed at length in this thesis. Positive conclusions in this respect, must await a more comprehensive examination of the peptide in terms of its physical and chemical properties, as well as, distribution in the neurohypophysis, biosynthesis, and secretion.

VI. BIBLIOGRAPHY

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