CHARACTERIZATION OF AMINO ACIDS, PEPTIDES AND PROTEINS IN CYTOPLASMIC GRANULES FROM BOVINE POSTERIOR PITUITARY

A Thesis

Presented to the

University of Manitoba

In Partial Fulfillment

of the Requirements for the Degree

of Doctor of Philosophy

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by

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October, 1969

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ABSTRACT

A large number of peptides and amino acids, representing 30-40% of the total acid-extractable, ninhydrin-positive material of the tissue was associated with cytoplasmic granules (sedimenting at 3,000,000 g-min after preliminary removal of "nuclei and debris") isolated from bovine posterior pituitary glands. Acetic acid (0.2M) extracts of a purified neurosecretory granule fraction showed only slight differences in the pattern of peptides and amino acids from extracts of the total cell particulate fraction. Gel filtration of extracts on Sephadex G-25 yielded three major fractions: Fraction I consisting of peptide material of molecular weights $> 4,000$; Fraction II of molecular weights averaging about 3,000; and Fraction III of molecular weights $< 2,000$. Fraction III was further resolved by anion-exchange chromatography into 12 subfractions. Vasopressin and oxytocin were contained in subfractions 2 and 3, respectively. Each of these subfractions was in turn chromatographed on a cation-exchange resin and resolved into

a total for Fraction III of 22 major components: lysine, arginine, phenylalanine, ammonia, and 18 peptides. Three of the peptides contained only aspartic and glutamic acids in the ratios 8:1, 5:1, and 4:1. The sequences of 4 dipeptides were ascertained. Another peptide was not retarded by Dowex 50 and yielded glutamic acid upon acid hydrolysis. Still another peptide yielded tyrosine plus an unknown ninhydrin-positive component after hydrolysis. The amino acid compositions were determined for nine other peptides containing 3 - 9 residues. Additional peptides in Fraction III were detected in lesser or trace amounts. Isolated granule fractions from both bovine posterior pituitary and rat liver were dialyzed against isotonic sucrose or distilled water. The decrease of ninhydrin-positive material from the sample dialyzed against water indicated that a large proportion of the "free" amino acids and peptides of these tissues were contained within intracellular organelles.

The neurohypophysial carrier protein, neurophysin, to which the peptide hormones are bound within secretory granules exists as at least six polymorphs which are in equilibrium. Chromatography of neurophysins on Sephadex generally gives molecular weight estimates of approximately 8,000 to 20,000, but with 0.1M choline as the eluant components considerably smaller than 8,000 are produced. Dissimilar subunits are indicated, since

all six species differ in amino acid composition. Each of the polymorphs binds a maximum of 100 U of oxytocin per mg neurophysin. SDS-polyacrylamide gel electrophoresis of the total neurophysin resolved three components of differing molecular size.

ACKNOWLEDGEMENTS

The author is indebted to many persons for their help during the preparation of this thesis, especially to:

Dr. Frank S. LaBella, my director, for his patient guidance and able advice during all phases of this investigation.

Dr. Donald Thornhill, Dr. Melvin Krass, Dr. Jerry Wang, Mr. Fred Keeley and Mr. Stan Vivian for their helpful discussion of various parts of this work with me.

Miss Marsha Cohen for proof reading.

Mr. Gary Queen, Mr. Doug Stein, Mr. Larry Eddie and Mr. Roy Joseph for their technical assistance.

Mr. Roy Simpson for the preparation of the photographs.

To my wife

HEE SOOK

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STATEMENT OF THE PROBLEM

The posterior pituitary is composed primarily of neuron terminals whose cell bodies originate in the supraoptic and paraventricular nuclei of the hypothalamus. The neurosecretory granules are synthesized in the cell bodies and pass down the axons to the posterior pituitary gland where they are stored. Since van Dyke and co-workers extracted a homogeneous amorphous protein-hormone complex in 1942, many workers have attempted to isolate the hormone-binding protein, neurophysin. There are numerous apparent discrepancies in the reported molecular weight and number of components in purified neurophysin preparations, and the chemical properties and physiological role of neurophysin are, thus, not yet completely elucidated.

A remarkably high concentration of peptides is present in the posterior pituitary. Two peptide hormones, oxytocin and vasopressin are believed to be the major physiological products of the mammalian neurohypophysis. Several other peptides with no known physiological activity have been characterized chemically.

This investigation was designed:

(1) to systematically examine purified neurophysin preparations, since apparent discrepancies exist for the number of neurophysins, their molecular weights and their hormone-binding abilities.

(2) to determine the chemical nature of posterior pituitary peptides, other than oxytocin and vasopressin.

(3) to determine whether significant amounts of posterior pituitary peptides, in addition to oxytocin and vasopressin, are associated with the neurosecretory granules.

II. GENERAL INTRODUCTION

GENERAL INTRODUCTION

ANATOMY

The pituitary gland (hypophysis cerebri) is attached by a short stalk to the tuber cinereum region of the hypothalamus. It lies within the small cavity formed by the sella turcica of the sphenoid bone. The hypophysis is composed of two major parts, the anterior lobe and the posterior lobe. Embryonically, the pituitary has a dual origin. The adenohypophysis, which includes the pars distalis (anterior lobe), parts tuberalis and the pars intermedia is embryologically derived from the ectoderm in the roof of the stomodeum (primitive mouth) just in front of the buccopharyngeal membrane. The evagination called Rathke's pouch is extended to the anterior wall by means of pressure upon its posterior wall by nervous tissue growth. This connection of Rathke's pouch with stomodeum is eventually closed. This part of the hypophysis originating from the stomodeum becomes glandular tissue and is, therefore, called the adenohypophysis. The neurohypophysis of which the major part is posterior lobe, is subdivided into three parts: infundibular process (neural lobe), infundibular stem, and median eminence. The posterior lobe includes the pars intermedia and the infundibular process. The neurohypophysis is formed by downgrowth of the central nervous system from the floor of the third

ventricle between the optic chiasma and the mammillary body (Harris, 1967). The posterior lobe remains having the connection by neural stalk (infundibular stem and median eminence) to the brain.

BLOOD SUPPLY

In many mammals the blood supply of the hypophysis is provided from two different sources: the superior hypophysial artery, a branch of the internal carotid artery, and the inferior hypophysial artery originating from carotid rete. The anterior lobe is not provided with arterial blood but has portal vessels instead. The capillary loops in the neural stalk and median eminence which are supplied by the superior hypophysial arteries provide portal vessels, "long portal vessels" which account for a large portion of the anterior lobe blood supply (Bell et al., 1965). Houssay et al., (1935) observed these portal vessels in the South American toad and determined the direction of the blood flow in this species. They were the first to observe the living hypophysio-portal circulation. Fourteen years later Green and Harris (1949) demonstrated with living rats that the blood flow was observed to start from the median eminence and flow towards the anterior pituitary lobe. The flow showed no signs of pulsation and was always of a uniform character. Worthington (1955) confirmed these same results in the mouse and described that the fine intercon-

necting capillaries within and between groups of capillaries showed a rapid intermittence, opening and closing many times in a minute, and remaining closed for longer periods. This suggested that a very delicate mechanism controls the rates of blood flow through the portal vessels, and possibly this mechanism controls the rate at which substances in the portal blood may be introduced into the humoral environment of the secretory cells of the anterior lobe gland. This introduction system of neurohumoral transmitters may have a profound influence on the anterior lobe function. Furthermore, it is suggested that the neurovascular link between hypothalamic nuclei and the anterior lobe of the pituitary may include many pathways. Individual groups of anterior lobe cells of similar function could thus receive specific transmitters produced by particular groups of hypothalamic nerve cells (Adams et al., 1964).

The capillary bed of the neural lobe is drained by two different sets of vessels: the systemic veins and the portal vessels. The systemic drainage is provided chiefly by a vein on each side of the midline. This vein leaves the lobe on its caudal surface, and enters a transverse venous sinus. These veins drain the deeper part of the neural lobe's capillary bed. Capillaries near the surface of the dorsal and caudal aspects of the neural lobe form a first capillary bed.

These capillaries which are supplied from the inferior hypophysial arteries, constitute a set of numerous small portal vessels.

The portal vessels provide the anterior lobe with its blood supply. This second set of portal vessels lies in the region of the lower pituitary stalk and posterior pituitary lobe, and the portal system called "short portal vessels" supplies a portion of the anterior lobe adjacent to the lower infundibular stem (Daniel and Prichard, 1956, 1957a).

When the pituitary stalk is cut and a barrier is introduced an extensive necrosis is shown in the anterior lobe. The necrosis is due to the fact that the greater part of the anterior lobe is deprived of its blood supply. A small portion of anterior lobe survives due to the existence of the short portal vessels. The circulation of the short portal vessel is not interrupted by stalk section (Daniel and Prichard, 1957b; Rothballer and Skoryna, 1960; Goldman et al., 1962).

It was also found that the anastomoses, joining the vessels of the rostral part of the posterior lobe with the vessels of adjacent parts of the anterior lobe and pars tuberalis, form an additional vascular route in addition to "long" and "short portal vessels" (Jewel, 1956; Homes and Zuckerman, 1959).

NERVE SUPPLY

(a) Anterior pituitary

Histochemical studies (Rasmussen, 1938; Green, 1951, 1966) show no innervation of the anterior pituitary. However, there have been suggestions from time to time that the anterior pituitary may be innervated (Dandy, 1913; Croll, 1928; Hair, 1938; Brooks and Gersh, 1941; Truscot, 1944). The scanty nerve fibers in the anterior pituitary innervate the blood vessels. The function of these nerve fibers is not known, but it is suggested that the sympathetic fibers are not concerned with regulation of anterior pituitary hormonal output (Green, 1966). Complete removal of these sympathetic chains and ganglia does not prevent reflex ovulation and impregnancy (Brooks, 1940; Hinsey and Markee, 1933; Haterius, 1934).

(b) Posterior pituitary

Nerve fibers richly innervate the posterior pituitary and these nerve fibers originate from the hypothalamus. The hypothalamo-hypophysial nerve tract consists of the hypothalamic nerve. The fibers of the hypothalamo-hypophysial tract are derived from the cell bodies of supraoptic and paraventricular nuclei (Christ, 1966) of the hypothalamus. Early workers have traced the cell body by means of a specific retrograde degradation. In this technique about 80 per cent of the supraoptic

nucleuscells disappear with the complete removal of neural lobe and the cutting of the infundibular stem below the median eminence. These disappearances are not believed to be due to the shortage of blood supply since there is very little change in the interstitial tissue and no degradation is seen nearby (Magoun and Ranson, 1939; Rasmussen, 1940). There is a loss of about 35% of the paraventricular nucleus cells after complete hypophysectomy (Frykman, 1942). Greater losses are seen in supraoptic and paraventricular nuclei cells with higher lesions which involve the median eminence. These results based on retrograde degradation experiments seem to indicate that nearly all of the supraoptic and some of the paraventricular nuclei cells send fibers into the neural lobe (Rasmussen, 1940). Scharrer and Scharrer (1954) have traced the nerve tracts by staining neurosecretory materials.

There are also many fibers from other sources in addition to the paraventricular and supraoptic nucleus cells. For example, there are nerve fibers which come from the lateral tuberal nuclei and from the region of the median forebrain bundle (Green, 1951).

NEUROHYPOPHYSIAL HORMONES

(a) General concept

Oliver and Schäfer (1895) were the first to show that extracts of whole pituitary gland have potent

vasopressor action in mammals. Three years later this pressor effect was shown to be restricted to the posterior lobe (Howell, 1898). Since that time, other biological activities of posterior pituitary extracts have been found. Dale (1906, 1909) demonstrated that pituitary extract has a strong uterine contracting effect. He concluded that the effect on the uterus is a direct stimulation of involuntary muscle, not involving innervation. Milk ejecting effect of pituitary extract was demonstrated by Otto and Scott (1910). Subsequently, an arterial blood pressure lowering effect in birds, using the duck (Paton and Watson, 1912) and an antidiuretic effect (von den Velden, 1912; Motzfeldt, 1917) were found. Since the three major activities, oxytocic, vasopressor and antidiuretic activity were discovered, there have been disagreements among workers between a single component concept with multiple activities and multiple components concept with single activities.

The first concept is based on a purely isolated product which has all three activities and considers the fact that the proportion of the activities is same as they exist in the gland itself. Abel (1930) insisted on the single component concept for a long time even after oxytocic and vasopressor activities were separated. Abel and his coworkers claimed that the violent treatment could break up a hormone with several activities into

several fragments with one or more activities left (Abel et al., 1923). van Dyke and his coworkers supported the single component concept by isolation of a homogeneous biologically active protein (van Dyke et al., 1942). The purified protein possessed the three major biological activities. The ratio of biological activities was the same as U.S.P. reference standard powder. They have claimed that these three activities are chemically united with each other because of their behavior during extraction procedure, their homogeneity of solubility, their ultracentrifugation and electrophoresis behavior.

The second concept, the multiple component concept, is based on Dudley's work (1919). Dudley has shown that oxytocic and vasopressor principles show different chemical properties. An acidic water extract of posterior pituitary powder as treated with colloidal ferric hydroxide and the filtrate was extracted with butyl alcohol under reduced pressure. This butyl alcohol extract yields a crystalline residue which contains all the uterine activity, together with some of vasopressor principle and contaminant substances. Kamm and his coworkers critically supported the multiple component concept (Kamm et al., 1928). They extracted two active principles with 0.25 per cent acetic acid under a short period heat. A substantial separation of the two active principles had been achieved by the method of salting

out and by the use of solvent and precipitants.

The active principle in press juice of the glands can be sedimented by ultracentrifugation (Rosenfeld, 1940). If the pressed juice is treated with dilute acetic acid under heat, the active principle behaves as a low molecular weight (mol wt) substance. This suggests that the native hormone can be cleaved in part to yield small peptides with biological activity.

The argument between the two concepts was finally concluded by Acher et al. (1956). There is no such thing as a pure protein possessing all the biological activities but rather a non-covalent complex formed by an inactive protein, neurophysin (NP) and the neurohypophysial hormones, oxytocin (OT) and vasopressin (VP).

The neurohypophysial hormones, OT, arginine-VP, lysine-VP, vasotocin, isotocin, mesotocin, glumitocin, have been detected in various species of animals. They are all octapeptides with a ring of 20 atoms closed by S-S of two half cystines. The structural differences of the seven neurohypophysial hormones are due to different amino acids at positions 3, 4 and 8 according to the numbering system introduced by Konzett and Berde (1959) and by Bodanszky and du Vigneaud (1959). Their chemical structures are shown in Table I.

(b) Purification

Hormone purification methods can be classified

TABLE I

PHYLOGENETIC DISTRIBUTION OF NEUROHYPOPHYSIAL HORMONES

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

into two groups. The first is a classical separation in which the active principle is extracted by means of differing solubility and precipitating the components with various reagents such as organic solvents. The second group of methods includes the modern techniques of counter-current distribution, electrophoresis, ion exchange chromatography and gel filtration.

The most common method involves extraction of the hormone with hot 0.25 per cent acetic acid (Kamm et al., 1928) in which the hormones are stable. Kamm and his coworkers (1928) precipitated the hormone by saturating with ammonium sulfate, dissolving in glacial acetic acid, and reprecipitating the hormones by adding acetone, ether, and petroleum ether. The purified dry product possessed oxytocic activity of 160 U/mg and pressor activity of 61 U/mg.

Stehle and Fraser (1935) prepared a material by a modification of Kamm's process which has an oxytocic activity of 250 U/mg and a pressor activity of 200 U/mg. The modification is based on changing the precipitating organic solvent, such as methyl alcohol or ethyl alcohol with ethyl acetate. It is shown that both oxytocic and pressor preparations are polypeptides containing tyrosine, cystine and arginine plus other amino acids. The different ratio of oxytocic and pressor activity between the Kamm product and the Stehle and

Fraser product shows that the solubilities of the oxytocic and vasopressor principles differ and indicates that the two principles are chemically different entities.

Ion-exchange chromatography was introduced to purify the hormones by Potts and Gallaghter (1942, 1944). The oxytocic principle was not absorbed on the permutit column; the absorbed pressor substance was subsequently eluted with sodium chloride solution. They obtained highly purified amorphous powders of the hormones.

These authors showed that the strikingly low content of arginine in the oxytocic principle is in good contrast to the significant amount in the pressor component. Livermore and du Vigneaud (1949) applied counter-current distribution techniques to obtain pure OT and claimed that this OT possesses 865 U/mg of oxytocic activity. Three years later, the oxytocic potency was revised by the same group of workers (Pierce et al., 1952) to 490 U/mg. Pierce and du Vigneaud (1950) prepared pure OT by a counter-current distribution technique. This preparation contained leucine, isoleucine, tyrosine, proline, glutamic acid, glycine, ammonia and cystine in equimolar amounts and 3.46 moles of ammonia.

Condliffe (1955) proposed a method of partition chromatography on Hyflo Supercel to purify small amounts of neurohypophysial hormones. Carboxylic acid resins (Amberite IRC-50: Acher et al., 1958; Carboxymethyl

cellulose: Ward and Guillemin, 1957; Schally et al., 1959) were also introduced to separate OT and VP.

Among many interesting separation methods Frankland et al., (1966) have separated OT and VP by gel filtration on Sephadex G-25, eluting with 0.1 N formic acid. The retardation on Sephadex G-25 due to only one additional aromatic amino acid in the neurohypophysial hormone with mol wt about 1,000 is enough to separate VP from OT. Portanova and Sachs (1967) synthesized a cellulose-neurophysin resin from bromoacetyl cellulose and NP and demonstrated that the resin has specific affinity for VP. Elution of VP from the resin can occur with either 0.25 N acetic acid or 0.1 N ammonium acetate, pH 8.5.

(c) Chemical properties

(i) Vasopressin: VP is an octapeptide with mol wt of 1,084 and an isoelectric point of 10.9. The structure of the hormone was determined by du Vigneaud and coworkers (du Vigneaud, 1954-1955; Turner et al., 1951; Popenoe and du Vigneaud, 1953, 1954; du Vigneaud et al., 1953a), and by Acher and Chauvet (1953, 1954). The two groups have applied almost the same method. First they oxidized the cystine. A partial hydrolysis was then done with hydrochloric acid and an enzyme hydrolysis with trypsin and papain.

(ii) Oxytocin: OT is an octapeptide with mol wt, 1007 and an isoelectric point 7.7. The structure of the hormone was determined by du Vigneaud (1954-1955) and coworkers, and by Tuppy and Michl (1953). du Vigneaud et al. (1953c) applied the Edman degradation technique to oxidized OT and determined the sequence of the first four residues from the N-terminal end. The rest of the sequence was determined on partial acid hydrolysates with the Edman degradation technique.

The three amide groups of glutamic acid, aspartic acid and C-terminal glycine were deduced from the isoelectric point of OT. It was demonstrated that OT was an ampholyte by electrophoresis, ampholytic property being proof of the presence of both acidic and basic groups. Since there are no basic amino acids present in OT, it is assumed that basic group might be a free α-amino group. As for the acidic groups only carboxyl and phenolic groups occur in the component amino acids of OT. With an isoelectric point at 7.7, it is likely that phenolic hydrogen plays a role in the amphoteric character of OT. This means the carboxyl groups of the two acidic amino acids, glutamic acid, aspartic acid, and C-terminal amino acid glycine are not free (Kunkel et al., 1953).

Tuppy and Michl (1953) independently elucidated the structure of OT. Both acid and enzymes were used to

hydrolyze the oxidized OT and the sequence was deduced from the small peptide fragments.

du Vigneaud and his coworkers (1953b, 1954) have totally synthesized OT. The biologically active synthetic material has been purified by counter-current distribution and compared with natural OT as to potency, specific rotation, partition coefficients, amino acid composition, electrophoretic mobility, infrared pattern, mol wt, enzymatic and acid inactivation and chromatography on the resin, IRC-50. The synthetic and natural OT were also compared with respect to milk ejection and induction of labor in the human as well as rat uterus contraction in vitro. They have found that synthetic and natural OTs are identical in the physical, chemical and biological properties. Thus, du Vigneaud's work marked the first total chemical synthesis of a physiologically active peptide hormone.

(d) Formation

The supraoptic and paraventricular nuclei are connected by a bundle of nerve fibers with endings in the neural lobe of the pituitary. The neurohypophysial hormones are synthesized by the cells of the supraoptic and paraventricular nuclei and pass along the axons of the supraoptic hypophysial tract into the neural lobe. The hormones are stored in the small secretory granules in association with a stainable carrier protein, NP

(Bargmann and Scharrer, 1951; Scharrer and Scharrer, 1954; Bargmann, 1960).

A small amount of hormonal activity was detected in the median eminence and neural stalk (van Dyke, 1926; Sato, 1928). It was shown that an injury to the supra-opticohypophysial system results in diminution of hormone amount, and the decreased amount of hormone brings about a primary polyuria (Ranson et al., 1938). These workers believed that the diuretic processes are normally under control of the anterior pituitary and that polyuria is due to a disturbance of the normal equilibrium normally obtained between anterior and posterior pituitary. Sachs (1960) used ion-exchange chromatography to isolate labeled VP from the hypothalamus and posterior pituitary after ^{35}S-cystine infusion into dogs. After dogs were infused continuously with ^{35}S-cystine for 16, 24 and 36 hrs, the mean ratio of radioactive specific activity of VP in the hypothalamus to VP in the posterior pituitary were 2.9, 2.0 and 3.1 respectively. Sachs (1963b) also found in dog hypothalami that VP associated with hypothalamic neurosecretory granules (NSGs) had the lowest radioactive specific activity after continuous infusion of ^{35}S-cysteine 3-6 hrs into the dog third ventricle, but the protein in the NSG fraction possessed the highest specific activity. Highly labeled VP was associated with the hypothalamic nuclear fraction, a large granule fraction,

and a microsomal membranous component rather than with ribosomal fraction. On this experimental basis, Sachs (1963b) proposed a hypothesis that biosynthesis of VP occurs on ribosomal RNA but in a biologically inactive macromolecule, and is then incorporated into NSGs. They have further suggested that the release of newly formed VP from the macromolecule would take place during the formation and maturation of NSGs and then transportation along the axon. VP production is completed when the granules arrive at the posterior pituitary (Sachs and Takabatake, 1964). This hormone formation from the precursor molecule is not inhibited by puromycin *in vivo* or *in vitro* (Sachs and Takabatake, 1964). The hormones thus produced are believed to be stored in NSG bound to NP, since the distributions of NP and hormones in the posterior pituitary are similar (LaBella *et al.*, 1963; Ginsburg and Ireland, 1966).

The concept of VP activation from a macromolecule is supported by the fact that more VP per mg protein is present in posterior pituitary NSGs (Weinstein *et al.*, 1961) than in hypothalamic NSGs (Sachs, 1963a).

The hormones which are synthesized and carried along the axon, are normally stored in the posterior pituitary and are present in the tissue during *in vitro* incubation for several days. Along with the degradation of nerve fibers in tissue culture, the hormone and stain-

able material also disappeared. The culture does not produce the neurohypophysial hormones if they have been previously depleted in the posterior lobe, in situations such as the dehydration of animals (Hild, 1954).

Very little is known of the factors which regulate synthesis and transport of the hormones. Takabatake and Sachs (1964) were the first to show direct evidence of synthesis of neurohypophysial hormones. Median eminences from dehydrated guinea pigs were incubated with labelled amino acids in vitro and the label appeared in newly-synthesized VP. It was also shown that the infundibular stem and neural lobe could not synthesize VP, as no labelled VP appeared after incubation. Slices of median eminence from 4 day water-deprived guinea pigs incorporated two to four times more labelled amino acids into VP than slices of median eminence from water-supplied animals. However, it is not known whether the increased rate of synthesis is due to depletion of stored VP or due to direct stimulation of an enzyme system in the hypothalamus.

(e) Release

It is generally accepted that the neurohypophysial hormones are stored in NSGs and released from the posterior lobe. Most research work on release of neurohypophysial hormones has been done on VP. When water is not supplied (Lederis, 1962) or hypertonic salt

solution is injected into an animal (Gerschenfeld et al., 1960), most of the NSGs disappear and only empty membranes are observed in the endings. The granules persist in other regions of the axon. The relationship between the NSG and hormonal activity is clarified by isolation of pure NSGs (LaBella et al., 1963; LaBella, 1968).

Although the physiological releasing mechanism of neurohypophysial hormones is not yet clearly shown, it appears to be mediated by nervous factors. Physiological stimuli apparently do not directly act on the storage granules in nerve endings of the neural lobe but through cell bodies in the hypothalamus (Jewell and Verney, 1957; Douglas and Poisner, 1964; Daniel and Lederies, 1966).

Abrahams et al. (1957) have found true cholinesterase activity which was confined to three nuclei of the anterior hypothalamus, the supraoptic, paraventricular and suprachiasmatic, and they suggested that axons of the supraoptic neurosecretory cells may be cholinergic and that acetylcholine may initiate the release of hormones from the same axons. Electron microscopy has shown micro-vesicles in the nerve endings in the neurohypophysis of the toad. In acutely hydrated animals the number of micro-vesicles is decreased on the basis of electron microscopical observations (Gerschenfeld et al., 1960).

Later the presence of acetylcholine has been shown in the bovine posterior pituitary gland on the basis of 9 criteria of the active principle which is estimated to be about 1/5 to 1/10 activity of bovine brain cortex. Furthermore, subcellular localization in the posterior pituitary showed the highest activity in the neurosecretosome and micro-vesicle fractions, thus providing strong support for the hypothesis that acetylcholine is localized in the vesicles. Choline acetylase was also identified in the bovine posterior pituitary gland, estimated to contain about 1/5 the concentration of this enzyme as whole rat brain (LaBella, 1968). There may be a correlation between the released acetylcholine from the micro-vesicles and the release of hormones. Koelle (1961) proposed that acetylcholine within the vesicles of the posterior pituitary is released from the nerve endings and acts back on the same cells to promote VP and OT secretion; such a mechanism would be in accordance with a similar proposal made by him for the presynaptic action of acetylcholine release from peripheral cholinergic motor neurons.

Pickford (1939) showed that intravenous injection of acetylcholine into an anesthetized dog caused a temporary inhibition of diuresis during water diuresis, and this antidiuretic effect was not shown after the posterior lobe was removed. Pickford (1939) concluded from her ob-

servations that the acetylcholine effect on urine excretion is due to an action in the central nervous system. Pickford (1947) also demonstrated that direct injection of acetylcholine into the supraoptic region produces an antidiuretic action in the chloralose-anesthetized dog. The acetylcholine effect on antidiuresis was prolonged by physostigmine sulfate which by itself inhibited urine flow. This antidiuretic action of acetylcholine disappeared upon removal of the pituitary gland. An antidiuretic effect occurred when the supraoptic and paraventricular area were stimulated electrically. Atropine did not block this hypothalamically induced VP release, but n-ethyl-nortropin-benzhydrylether hydrobromide (Ethybenztrine) and dibenzyline did block (Fang et al., 1962).

However, this cholinergic mechanism was objected to because of a direct releasing mechanism by the demonstration that acetylcholine alone or with an anticholinesterase, physostigmine or DFP did not liberate the hormones from the isolated rat pituitary (Douglas and Poisner, 1964).

Douglas and Poisner (1964) demonstrated that the rate of release of the hormone from the isolated rat posterior lobe was greatly accelerated when the potassium concentration was increased or when electric stimulation was applied to the stalk. The secretory response to

electric stimulation was completely abolished when the calcium concentration in the incubation medium was lowered to 0.1 mM (Mitiken and Douglas, 1965). Potassium did not stimulate VP release when calcium was removed from the incubation medium but was more effective upon increasing calcium concentration (Douglas and Poisner, 1964). They suggested that VP release from the posterior lobe was regulated by nerve impulses through the hypothalamo-hypophysial tract and that the final release of VP from the bound protein is due to the entry of calcium ions into the cell following depolarization. This potassium and calcium effect was confirmed by Daniel and Lederis (1963), and Thorn (1965, 1966).

A calcium effect as a physiological hormone releasing mechanism is difficult to accept without demonstrating specific calcium entry to specific hormone storage sites by a given stimulus, since there is abundant evidence to indicate that there is some degree of independence between VP and OT with respect to secretion from the posterior pituitary (LaBella, 1968).

RELEASING FACTORS IN POSTERIOR PITUITARY

The hypothalamus and the anterior pituitary are closely related in physiological function but no significant nervous connection between the hypothalamus and anterior pituitary has been shown. There is no doubt that physiological communication between hypothalamus

and anterior pituitary is vascular. The relationship between the hypothalamus and the anterior pituitary is well established since Harris (1948) published his brilliant review article on hypothalamic control of anterior pituitary tropic hormone secretion.

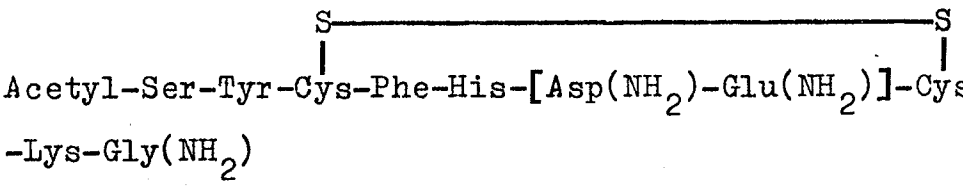
The neurohumoral transmitters, the so-called releasing factors, are synthesized in the hypothalamus and released into the long portal vessels running down the pituitary stalk which supply blood to the greater part of the anterior lobe. The releasing factors control the rate of release of anterior pituitary hormones (McCann and Dhariwal, 1966; Martini, 1966; Meites and Nicoll, 1966; Guillemin, 1967; McCann et al., 1968).

ACTH releasing factor has been identified by studying the effects of tissue extracts on corticoid production by isolated rat adrenals (Saffran and Schally, 1955a,b). Saffran et al. (1955) were able to isolate an active component from a commercially concentrated posterior pituitary extract by serial paper chromatography in four different solvent systems. The active component significantly stimulated the release of ACTH at doses as low as 1 μ mg. They have shown that this active component is a peptide but is different from the known neurohypophysial hormones, OT and VP as determined by paper chromatography.

They named this component "corticotropin

releasing factor" (CRF) and suggested that the posterior pituitary is an important link between hypothalamic nuclei and anterior pituitary for storage and secretion of a CRF as well as of VP and OT (Saffran and Schally, 1955a, b). Their purified CRF contained cystine, aspartic acid, glutamic acid, glycine, proline, lysine, phenylalanine, alanine, serine and histidine (Schally *et al.*, 1958).

Later Schally and Bowers (1964) and Schally and Guillemin (1963) applied counter-current distribution, ion exchange chromatography, electrophoresis and molecular sieving techniques to separate CRF and proposed the following partial structure with unknown sequences in parentheses.



The amino acid composition and chemical structure is closely related to VP. Guillemin *et al.* (1957) extracted the Kamm product of the hypothalamus and a commercial posterior pituitary preparation (Protopituitrin$^{(R)}$, Parke Davis) with 90% methanol and isolated a CRF from both posterior pituitary and hypothalamus by paper chromatography. The active component was shown to be a small peptide which is different from OT, VP and ACTH.

Schally et al. (1962) isolated from pig posterior pituitary gland another CRF which is different from the compound isolated by Guillemin et al. (1957), and Schally et al. (1958).

CRF became the common name for a family of compounds which have ACTH releasing activity and a nomenclature was proposed to distinguish these compounds (Schally et al., 1960). The compound containing methionine and which is related to α-MSH was named α-CRF and the releasing factor containing cystine and related to VP was designated β-CRF. On CMC column chromatography β-CRF emerged before lysine-VP and α-CRF before α-MSH. Another CRF (α_2-CRF) which was isolated by Schally et al. (1962) emerged just after α-MSH and its amino acid composition is related to α-MSH. The amino acid sequence of this CRF was said to be identical with that of α-MSH, the only difference being in the radical which blocks N-terminus. This radical of α-MSH is an acetyl group but is unknown for CRF.

The following partial chemical structure of the CRF is proposed with an unknown compound indicated as R on the N-terminus (Schally et al., 1962).

R-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Try-Gly-Lys-Pro-Val(NH_2)

This peptide has CRF activity, identical potency

as α-MSH on melanophore activity but a lower inherent adrenocroticotropic activity than α-MSH. α-MSH has no CRF activity although it has inherent minimal corticotropic and vasopressor activity (Guillemin, 1964). Guillemin et al. (1960) isolated another α-CRF which has all the amino acids of α-MSH plus threonine, alanine, leucine. The same compound was independently isolated by Privat de Garilhe et al. (1960). These two CRFs are related to α-MSH. To distinguish these two, α_1-CRF is designated for the one isolated by Guillemin et al. (1960) and α_2-CRF for another purified by Schally et al. (1962).

The early work on CRF was significant in drawing attention to the posterior pituitary as a possible source for the isolation of releasing factors in a pure form. More recently Franchimont and Legros (1969) demonstrated that the administration of total neurohypophysial extracts to man was followed by an increase of serum levels of trophic hormones (GH, FSH and prolactin), while the corresponding unitage of lysine-VP had no effect, and OT raised only FSH level. But they did not consider the possibility of the presence of other releasing factors in addition to the neurohypophysial hormones.

Since the discovery of CRF in posterior pituitary and hypothalamus, attempts were made to extract and purify many releasing factors and inhibiting factors from hypothalamus tissues. There are good possibilities that the

posterior lobe of the pituitary may contribute physiologically to the control of anterior pituitary hormone secretion due to the close relationship of median eminence and some connection of portal vessels between anterior and posterior lobe. So far, the posterior lobe has been virtually neglected as a source of releasing and inhibiting factors.

OTHER PEPTIDES IN POSTERIOR PITUITARY

It was demonstrated by chemical tests that the posterior pituitary gland contains high concentrations of "peptones" and "proteoses" as early as 1917 (Abel and Pincoff, 1917). Since research has been concentrated on biologically active material, characterization of inactive peptides has been neglected for many years.

Winnick et al. (1955) systematically studied amino acids and peptides in fresh beef and pork posterior pituitary. They fractionated peptides by charcoal adsorption and ionophoresis. Peptides were quantitated by the biuret reaction, and a paper chromatographic technique was used to analyze the amino acids. They showed by both biuret and ninhydrin reactions that beef and pork posterior pituitary glands contain high concentrations of peptides, several times greater than the concentration of the known peptide hormones.

Subsequently, Ramachandran and Winnick (1957) extracted acetone dried pork posterior pituitaries with

dilute acetic acid and the protein was removed by precipitation with trichloroacetic acid. The deproteinized solution was subjected to procedures involving organic solvents, adsorption on the charcoal, and dinitrophenylation. The crude peptide fractions were subsequently resolved by chromatography on celite, silicic acid, cellulose powder and paper. Some of the small peptide sequences were determined. Glutamic acid, glycine, alanine, aspartic acid, serine, threonine, valine and leucine were found to be the most abundant amino acids. The physiological significance of such a large number and quantity of peptides is unknown. However, it is possible that some of the peptides in posterior pituitary may be physiologically active substances or analogues of the known physiologically active material derived from the different tissues.

Lately, several peptides in the posterior pituitary which have unknown biological activity have been isolated (Witter et al., 1964; Preddie and Saffran, 1965; Preddie, 1965; Penders and Arens, 1966; Schally and Barrett, 1968).

NEUROPHYSIN

Rosenfeld (1940), using the pressed juice of fresh posterior pituitary lobes, demonstrated that the pressor and oxytocic principles exist in the form of either a single large molecule or mixture of two different

entities which are very similar in their sedimentation rates. The mol wt of this large active principle was estimated to be 20,000 to 30,000 by sedimentation rate. He also showed that the physiologically active low mol wt compounds are present, and indicated that the low mol wt compounds are cleavage products formed by boiling in 0.25 per cent acetic acid. Rosenfeld's so called native hormone was isolated as a homogeneous amorphous form two years later (van Dyke et al., 1942)

van Dyke and his coworkers (1942) isolated the protein (van Dyke protein) from fresh bovine posterior pituitaries by repeated sodium chloride precipitation. The isolated protein was claimed to be pure so far as shown by constant solubility and by the Schlieren optical pattern in the ultracentrifuge. The mol wt was estimated to be about 30,000. Three major biological activities, oxytocic, vasopressor and antidiuretic, are all present in the same ratio as those of the U.S.P. reference standard. The biological activities are abolished by reduction of cystine by thioglycollate. The van Dyke protein is composed of 16 amino acids, glycine, glutamic acid and cystine being the most abundant and contains no tryptophan and methionine (Block and van Dyke, 1950, 1952).

Acher et al. (1956) prepared the van Dyke protein by the method of van Dyke and coworkers (1942).

When the van Dyke protein was dialyzed against water, the biological activities remained inside of the dialyzing bag. However, when the same van Dyke protein was placed in the central compartment of a three compartment cell separated by a cellophane membrane and subjected to electrophoresis at 1000 V, the protein remained in the central compartment after 6 hrs, whereas the biological activites were found in the cathode compartment. In a counter-current distribution study using a solvent system of secondary butyl alcohol: 0.5% trichloroacetic acid, they could separate the three components, OT, VP and an inactive protein. After the protein was precipitated by trichloroacetic acid, 70 to 80 per cent of the OT and VP were recovered from the supernatant. The conditions which they had applied were too mild to cleave the covalent peptide bonds. Finally, they concluded that the van Dyke protein is a complex between two active peptides, OT and VP, and a physiologically inactive protein. This inactive protein was named neurophysin (NP) by them. The van Dyke protein was reconstituted from three components by Chauvet et al. (1960).

NP was purified by means of Sephadex G-25, carboxymethyl cellulose and Sephadex G-75 as described by Ginsburg and Ireland (1963b, 1965). They claimed that the purified protein with mol wt 25,000 has max-

imal OT binding capacity of 125 U/mg and 62 U/mg for arginine-VP equivalent to seven moles of OT and four moles of arginine-VP per mole of the protein respectively. Ginsburg and Ireland (1963a, 1964) have studied the pH effect on the binding ability of the protein using a Sephadex G-25 column. Maximum binding ability was found to occur in range of pH 5.2 to 5.8. The same results were obtained with a dialysing method. Pork NP has a maximum binding ability of 232 U OT/mg protein and 48 U lysine-VP/mg protein (Ginsburg et al., 1966).

Multiple components in bovine NP were demonstrated by Hope and Hollenberg (1966; Hollenberg and Hope, 1967), who isolated NP by the means of Sephadex gel filtration; five components were resolved by ion-exchange chromatography on carboxymethyl Sephadex C-50. One major component of mol wt 23,000 was very similar in amino acid composition to van Dyke protein. However, Hollenberg's NP has one residue of methionine but is entirely free of histidine. They proposed the name of neurophysin-M (NP-M) for this protein (Hope and Hollenberg, 1966; Hollenberg and Hope, 1967). They succeeded in crystallizing NP-M as a complex of neurohypophysial hormones (Hollenberg and Hope, 1966).

Hollenberg and Hope (1968) believed that cathepsins in acetone dried posterior pituitary may be active

during NP extraction but destroyed by 0.1 N hydrochloric acid pH 1.5. In later work, Hollenberg and Hope (1968) extracted acetone dried bovine posterior pituitary powder with 0.1 N hydrochloric acid and isolated NP (Hollenberg and Hope, 1967) with a slight modification of the buffer gradient used on carboxymethyl Sephadex C-50. The new system resolved two major components and the NP fractions are claimed to be nonhydrolyzed native proteins (Dean and Hope, 1966; Dean and Hope, 1967; Hollenberg and Hope, 1967; Dean et al., 1968; Dean and Hope, 1968). These "native" NPs are called neurophysin-I (NP-I) and neurophysin-II (NP-II), having similar amino acid composition, except that of NP-I with mol wt 19,000 has two histidine residues per mole, while NP-II with mol wt 21,000 is free of histidine. Later Rauch et al. (1968) isolated a third NP present in much smaller amounts which contains two residues of methionine, assuming a mol wt close to 20,000, and commented that small amounts of methionine in NP-I can be due to a third NP.

Breslow and Abrash (1966) supported the multi-neurophysin concept by resolving NP into four major components on diethylaminoethyl Sephadex A-50. Binding studies on one of the four components indicated that one mole of NP (assumed mol wt 25,000) bound 2 moles of OT.

NP was isolated from cod posterior pituitary by Sephadex G-25 and Sephadex G-75, and four major com-

ponents from the protein were subfractionated by ion exchange chromatography on diethylaminoethyl Sephadex A-50 (Pickering, 1968). One of the major components was named cod NP. Pickering (1968) also tried to purify the NP fraction derived from Sephadex G-75 by chromatography on carboxymethylcellulose under the conditions used by Ginsburg and Ireland (1965), but most of the NP was unretarded suggesting that cod NP might be more acidic than porcine NP. One mole of the cod NP bound 2.2 moles of OT, assuming a mol wt of 14,000 (Pickering, 1968). Wuu and Saffran (1969) isolated a NP mol wt 9170 from desiccated pig posterior pituitary using the methods of percolation, gel filtration on Sephadex G-25, Sephadex G-50, and diethylaminoethyl cellulose. This low mol wt polypeptide possesses a binding capacity for 0.87 mole OT and 1.03 mole lysine-VP per mole NP (mol wt 9170).

The subcellular distribution of NP and neurohypophysial hormones are closely related to each other (LaBella et al., 1962; Ginsburg and Ireland, 1966), and furthermore it is claimed that NP-I and OT are stored together in one population of neurosecretory granule and NP-II plus arginine-VP in another (Dean et al., 1968).

NP-like substances were demonstrated immunologically in pork kidney, uterus, mammary gland and serum (Ginsburg and Jayasena, 1968a) and the purified NP-like compounds claimed to have specificity for binding OT and

VP. Protein from uterus and mammary gland bound OT but not lysine-VP while proteins from kidney bound lysine-VP but not OT; serum protein bound both hormones. Proteins prepared in the same way from pork liver, spleen, skeletal muscle and brain, which had not shown immunological cross-reactivity, did not bind the hormones (Ginsburg and Jayasena, 1968b).

III. CHARACTERIZATION OF AMINO ACIDS AND LOW-MOLECULAR-WEIGHT PEPTIDES BOUND TO CYTOPLASMIC GRANULES FROM BOVINE POSTERIOR PITUITARY.

INTRODUCTION

The posterior pituitary gland is known to contain a remarkably high concentration of peptides relative to concentrations in other tissues (Lande et al., 1965; Upton et al.,1966; Winnick et al.,1955; Saffran et al.,1962). Winnick et al. (1955) estimated that peptides comprise 3-4% of the dry weight of porcine posterior pituitary. Two peptide hormones, vasopressin (VP) and oxytocin (OT) are thought to be the major physiological products of the mammalian neurohypophysis. Several other peptides have been characterized chemically by Preddie (1965), Preddie and Saffran (1965), Penders and Arens (1966) and Schally and Barrett (1968).

The posterior pituitary is composed primarily of terminals of neurons, the perikarya of which are situated in the supraoptic and paraventricular nuclei of the anterior hypothalamus. These terminals store OT and VP in vesicles of 100 - 300 mμ in diameter, the so-called neurosecretory granules, (NSGs) (Palay, 1957). NSG can be sedimented from homogenates of the posterior pituitary by centrifugation and contain both OT and VP (Schriebler, 1952; LaBella et al.,1962, 1963). It has been suggested that NSGs are packets of neurophysin (NP) to which the hormones are bound, since the van Dyke protein and the NSG protein are apparently identical (LaBella et

al.,1967). The peptide hormones are easily dissociated from and reassociated with the protein (Frankland et al., 1966; Ginsburg and Ireland, 1964). Because of the large number of peptides present in the gland, it seemed likely that peptides other than OT and VP might be bound to the NSG and perhaps to NP. In the present study the chemical composition of several isolated posterior pituitary peptides was determined as a prelude to investigation of possible physiological roles of additional granule-bound peptides. A low molecular weight (mol wt) ($< 2{,}000$) peptide fraction obtained by gel filtration of acetic acid extracts of bovine posterior pituitaries was examined. The chromatographic behavior and amino acid composition was determined for several of the peptides. In addition, it was found that the so-called "free" amino acids and peptides of tissues in general are to a large extent associated with, and apparently contained within, intracellular organelles.

MATERIALS AND METHODS

Bovine posterior pituitary glands

Glands were obtained from the slaughterhouse 30-40 min after death, placed in vessels surrounded by ice, and transported to the laboratory. The time elapsing between death of the animals and tissue homogenization was 2 hrs. About 100 posterior lobes (25 g) were used

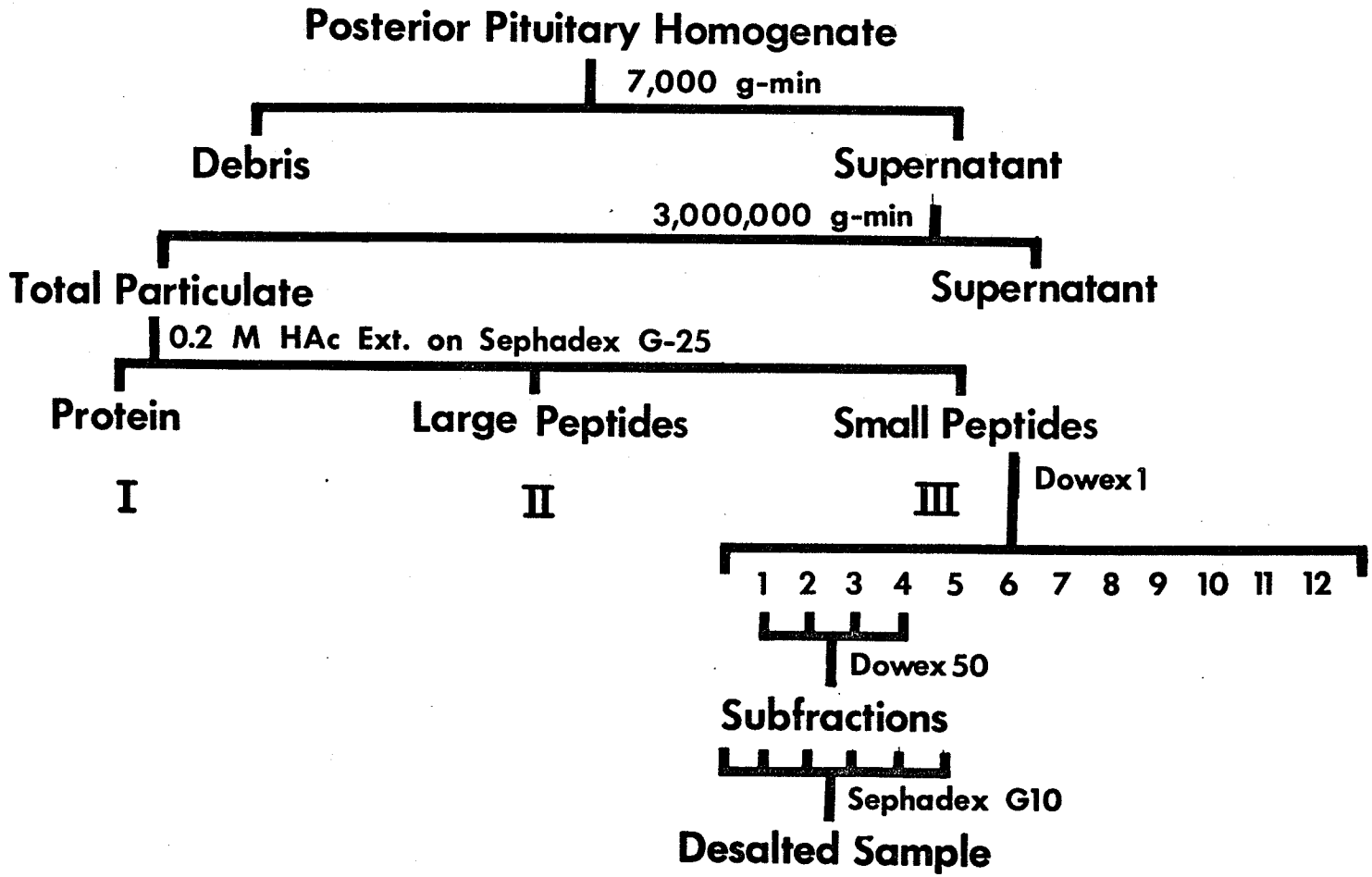


Figure 1. Flow sheet of purification of amino acid and peptides.

as starting material for a given extraction.

Isolation of subcellular granules

The tissue was minced with scissors and homogenized in 10% (w/v) sucrose in a glass tube with a motor-driven teflon pestle (LaBella et al.,1963). An initial centrifugation at 7,000 g-min (R_{max}) to remove nuclei and debris, was done in a Servall centrifuge. Subsequent centrifugation of the supernatant was done at 3,000,000 g-min (R_{max}) in an International Preparative Ultracentrifuge B-60 in order to prepare a total cytoplasmic granule fraction. This granule fraction would be expected to contain the subcellular components of the neurosecretory cell terminals, which make up the bulk of the posterior pituitary, together with a minority population derived from pituicytes, other supporting cells, and endothelial cells (Bindler et al.,1967). Where indicated, a purified NSG fraction (LaBella et al., 1967) was used as starting material for acid extraction.

Extraction of peptides

The isolated granules were stirred for 24 hrs at 4^{o} in 0.2M acetic acid. The precipitate was discarded and the supernatant was concentrated in a rotary evaporator at 40^{o} under reduced pressure from a water aspirator.

Gel filtration

Columns of Sephadex G-10, G-25 fine, and G-50

fine (Pharmacia-Uppsala, Sweden) were prepared essentially according to the manufacturer's recommendations. "Fines" were removed from 100 g of Sephadex by decantation and the sediment was suspended in 2 l of distilled water. After 12 hrs the water was replaced with an equal amount of 0.2M acetic acid, and 1 hr later with fresh 0.2M acetic acid. A small amount of 0.2M acetic acid was poured into the column, the outlet was closed, and the column was filled with the slurry. Column dimensions were 2.2 x 103 cm for G-10; 5.4 x 60 cm for G-25; and 2.8 x 59 cm for G-50. The samples were dissolved in a minimum amount of 0.2M acetic acid, applied to the Sephadex column without disturbing the gel, and eluted with 0.2M acetic acid. Flow rates for G-10 and G-50 were 40 ml/hr and for G-25, 75 ml/hr. Vitamin B_{12} and NaCl were used as markers on the G-25 column.

Ion-exchange chromatography

Dowex 1-X2 Cl^- (200-400 mesh) and Dowex 50W-X2 H^+ (200-400 mesh) were obtained from Bio-Rad Laboratories (Richmond, California). Packing and regeneration of the column were done according to the method of Schroeder et al. (1962). The 2.3 x 57 cm column for 1-X2 and the 1 x 57 cm column for 50W-X2 were maintained at 50°. The sample to be chromatographed on 1-X2 was dissolved in 2 ml of collidine-pyridine-acetic acid-water buffer (40:40:1.5:3918.5 by vol) pH 8.3 (Schroeder et al.,1962)

TABLE II

GRADIENT SYSTEMS FOR ION-EXCHANGE CHROMATOGRAPHY

Chamber No.	Dowex 1-X2 column					Dowex 50W-X2 column			
	collidine:pyridine: acetic acid:water (40:40:1.5:3918) pH 8.3	acetic acid (M) 0.1	0.5	1	2	0.2M NaAc pH3.10	2M NaAc pH 5.10	0.2M $NaHCO_3$ pH 8.30	H_2O
1	100	0	0	0	0	90	0	0	0
2	0	100	0	0	0	90	0	0	0
3	0	100	0	0	0	90	0	0	0
4	0	0	100	0	0	90	0	0	0
5	0	0	100	0	0	55	15	0	20
6	0	0	0	100	0	5	45	0	38
7	0	0	0	100	0	0	87	0	0
8	0	0	0	0	100	0	65	22	0
9	0	0	0	0	100	0	0	87	0

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

For details, see text under Methods.

and adjusted to pH 8.5; the samples to be chromatographed on 50W-X2 were dissolved in 2 ml of 0.2M sodium acetate, pH 3.1, and adjusted to pH 3.0.

The sample, dissolved in buffer solution, was washed into the column by gravity flow with a small portion of the same buffer. Elution from the 1-X2 column was carried out stepwise with the collidine-pyridine-acetic acid buffer followed by 0.1M, 0.5M, 1.0M, and 2.0M acetic acid (Table II). Elution from the 50W-X2 column was carried out stepwise with 0.2M sodium acetate, (pH 3.1), followed by 2M sodium acetate (pH 5.1), and 0.2M sodium bicarbonate (pH 8.3) (Table II). A Technicon Autograd with 9 chambers was used as a gradient device for both anion and cation ion-exchange chromatography. The flow rate for each column was 40 ml/hr. Fractions were collected and portions were reacted with ninhydrin (Cadavid and Paladini, 1964) or the standard protein reagent (Lowry et al., 1951) in a Technicon Autoanalyzer.

Bioassay

Oxytocic activity of chromatographic fractions was determined on the isolated rat uterus (Holton, 1948) and vasopressor activity was determined by a modification (Bindler et al., 1967) of the method of Landgrebe et al., (1946).

Amino acid analysis

Purified peptides were hydrolyzed for 24 hrs

in 6 N HCl at 110° under nitrogen in sealed glass vials. Analyses of amino acids were carried out on a Technicon Instrument as previously described (LaBella et al., 1967). The values for amino acids were not corrected for destruction during hydrolysis. For estimations of tryptophan, samples were hydrolyzed in 1 ml of 5 N NaOH for 24 hrs at 110° in Corning No. 7280, alkali-resistant glass tubes; the cooled hydrolysates were acidified with 1.5 ml of 5 N H_2SO_4, an aliquot was rendered alkaline with 1M $NaHCO_3$, and fluorescence of tryptophan was determined with an Aminco-Bowman fluorometer.

Thin layer chromatography and electrophoresis

To determine homogeneity, each purified peptide or amino acid fraction was examined by ascending thin layer chromatography (TLC) on MN-Polygram cel 300/UV_{254} (Macherey-Nagel & Co., Dueren, Germany) in n-butanol-acetic acid-water (4:1:5 by vol.), and by electrophoresis in pyridine-acetic acid-water (1:10:289 by vol.), pH 3.7 (Katz et al., 1959), at 3,000 V/10 min. Ninhydrin aerosol spray NIN-3 (Sigma Chemical Co., St. Louis, Mo.) was used to visualize the spots. Silica gel TLC on plastic sheet, MN-Polygram Sil S-HR/UV_{254} (5 x 20 cm) (Macherey-Nagel & Co., Dueren, Germany) was used for detecting DNP-amino acids using chloroform-benzyl alcohol-acetic acid (70:30:3 by vol.) as developing solvent. DNP-amino acids (Mann Research Labor-

atories, New York; Kit No. 418) were used as standards.

Dinitrophenylation

For determinations of amino-terminal amino acids peptide dinitrophenylation was done according to Fraenkel-Conrat et al. (1955).

Dialysis of cytoplasmic granules in hypotonic solution

100 ml portions of the total granule fraction isolated from posterior pituitary were placed in cellophane bags (seamless 1-1/8" diameter; Fisher Scientific Co., Pittsburg, Pa.) and dialyzed at 4^{o} (with stirring) against 1 liter of 0.005M pyridine acetate (pH 6.0) in distilled water or in 0.29M sucrose. Portions (1 ml) were removed from the exterior volume and reacted with ninhydrin. The portions were diluted with 1 ml of 0.29M sucrose in the case of the distilled water medium or with 1 ml of water in the case of the sucrose-containing medium.

RESULTS

Three major, ninhydrin-positive fractions were obtained when an acid extract of the total cytoplasmic-granule fraction from 25 g of tissue was subjected to gel filtration on Sephadex G-25 (Fig. 2A). Fraction I (40 mg of nitrogen) was contained in the excluded volume and included components of estimated molecular weights $>4,000$. There were several components in this fraction as judged by chromatography on G-50 and anion-exchanger. Fraction II (10 mg of nitrogen) included components of estimated molecular weight $<4,000$, and Fraction III (13 mg of nitrogen) contained components of molecular weight $<2,000$. Fraction II was rechromatographed on Sephadex G-25 and the principal component was concentrated and chromatographed by anion-exchange to yield at least 9 ninhydrin-positive fractions. No further characterization of Fractions I and II was carried out.

This report deals with more extensive fractionation and characterization of the low-molecular-weight components of Fraction III only (Fig. 2, Table III). The general scheme of the peptides and amino acid fractionation is shown in Fig. 1. Fraction III obtained from chromatography on Sephadex G-25 yielded upon anion-exchange chromatography 12 ninhydrin-positive subfractions (Fig. 2B). Four of the principal subfractions were sub-

jected to cation-exchange chromatography and resolved into several additional components (Figs. 2C-2F). Each of these subfractions was desalted by gel filtration on Sephadex G-10, a process which, in some instances, promoted further fractionation. For example, Fraction A4-B2 gave rise to 3 components during passage through G-10. Homogeneity was established for 20 of the components on the basis of (a) ion-exchange chromatography, (b) gel filtration on Sephadex, (c) TLC, and (d) high voltage electrophoresis.

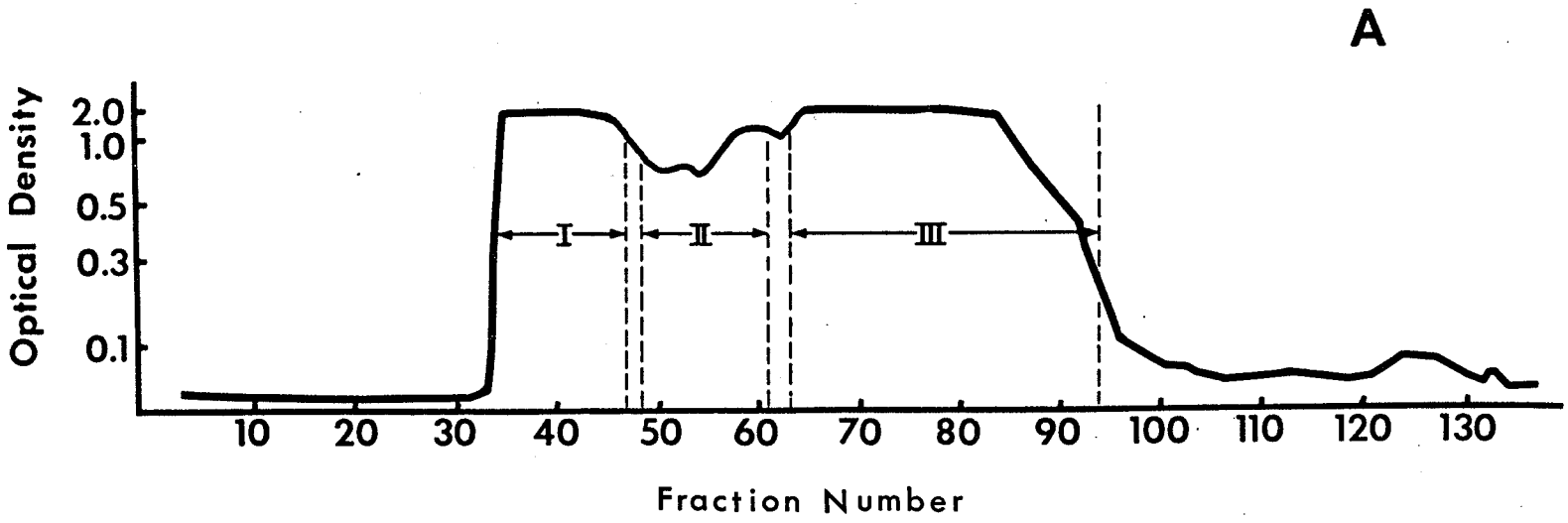
From anion-exchange chromatography, the first fraction (A1) was resolved by cation-exchange chromatography into ammonia, free lysine and arginine, a dipeptide (alanyl-arginine) and a fifth minor component (Fig. 2C). VP was identified in Fraction A2 (fraction number 19-21)and OT in A3 (fraction number 35-37) by means of bioassays for their biological activities. A2 and A3 each consisted of mixtures of several peptides, as shown by subsequent cation-exchange chromatography (Figs. 2D and 2E).

Subfraction A4 also consisted of a complex mixture of peptides (Fig. 2F, Table III). Free phenylalanine was identified in this fraction. A4-B1 yielded residues of eight identifiable amino acids after acid hydrolysis plus two unidentified ninhydrin-positive components. One of the unknown substances was not re-

Figure 2. (A) Elution profile from Sephadex G-25 (at 25°) of a 0.2M acetic-acid extract of the total granule fraction from the posterior pituitary. Flow rate was 75 ml/hr and 14.4 ml fractions were collected and measured after reaction with ninhydrin.

(B) Elution profile of fraction III of (A) on Dowex 1-X2 (at 50°), developed with collidine:pyridine:acetic acid (pH 8.3) and acetic acid. The flow rate was 40 ml/hr and 6.3 ml fractions were collected and measured after reaction with ninhydrin.

Subfractionation of fractions A1, A2, A3, and A4, respectively, on Dowex 50W-X2 (at 50°) developed with sodium acetate: sodium bicarbonate buffer (Table II). The flow rate was 40 ml/hr and 9.5, 11.1, 12.5, and 10.6 ml fractions, respectively, were collected and measured after reaction with ninhydrin (C, D, E, F).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1.0
0.5
0.3
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II
III
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20
30
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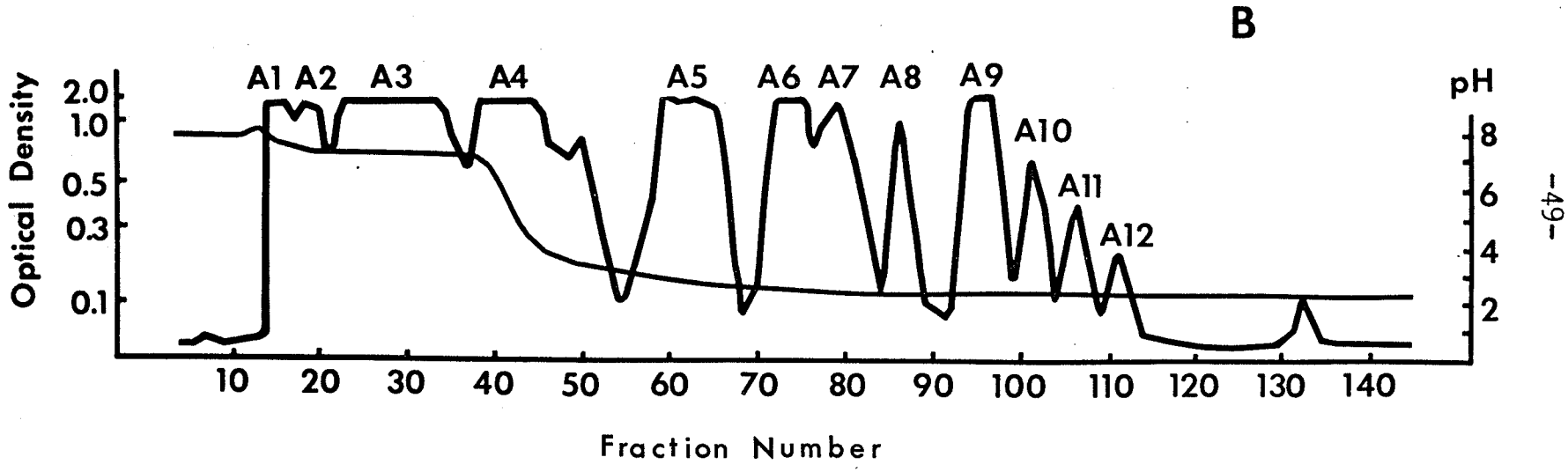
60
70
80
90
100
110
120
130
Fraction Number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1.0
0.5
0.3
0.1
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pH
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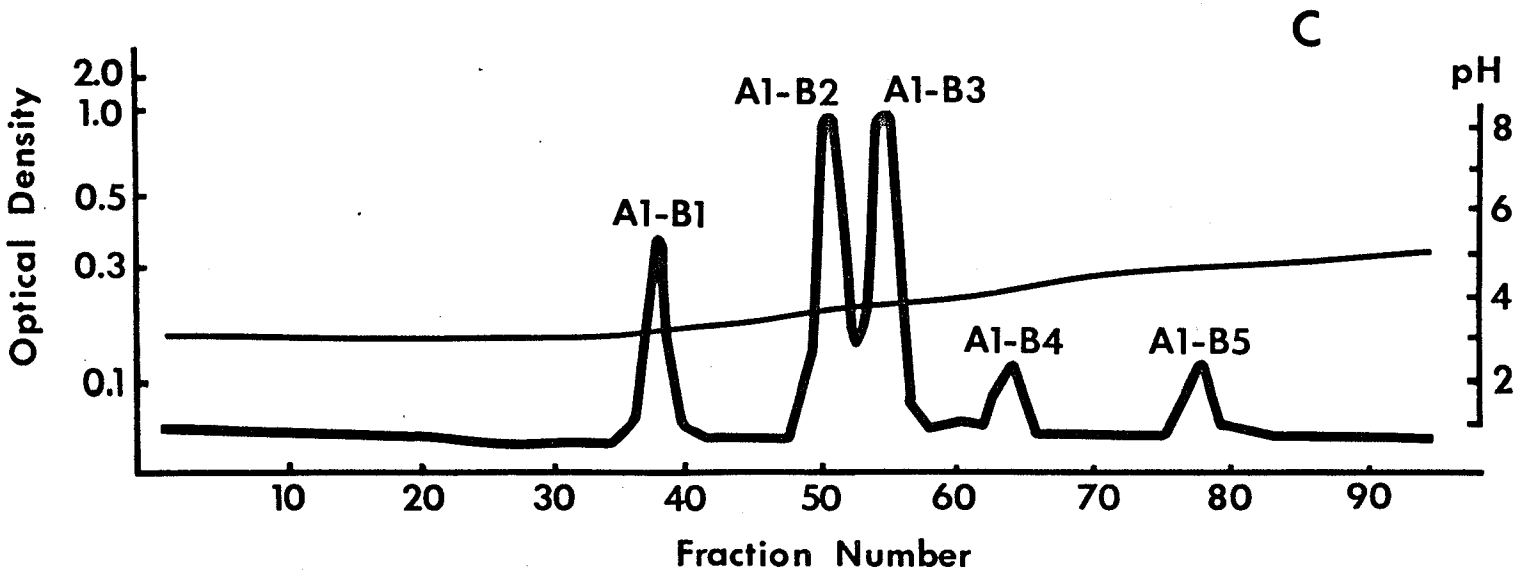
6
4
2
10 20 30 40 50 60 70 80 90 100 110 120 130 140
Fraction Number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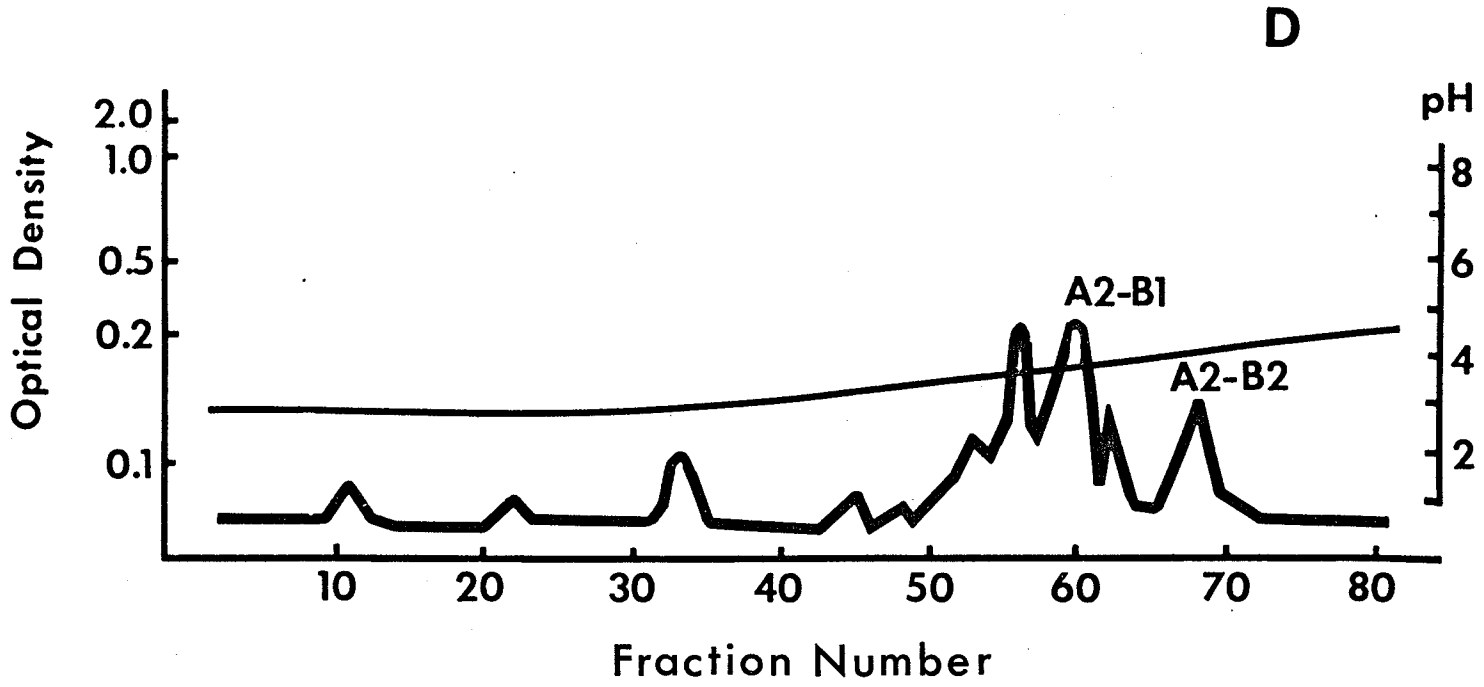
Fraction Number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70
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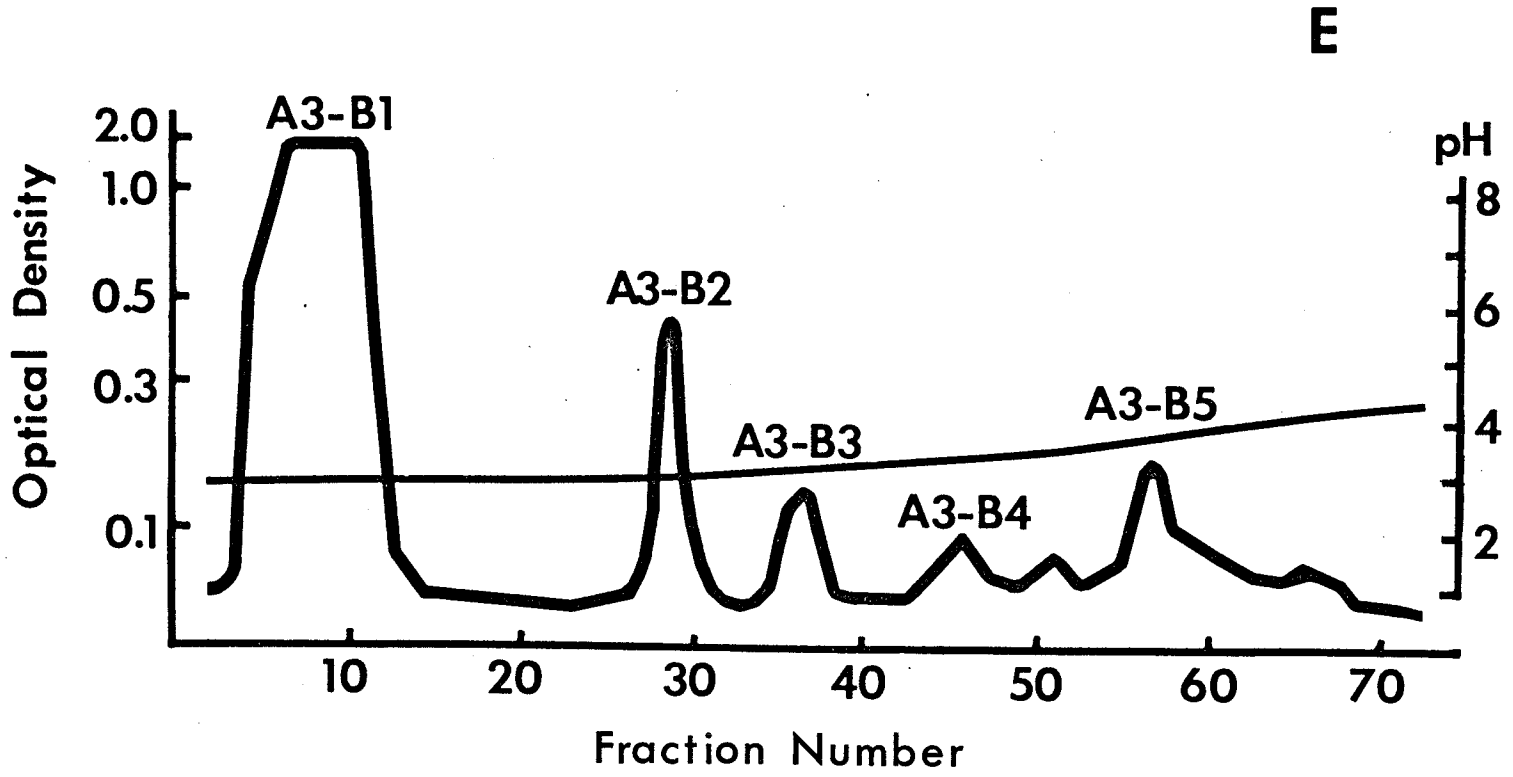
Fraction Number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A3-B2
A3-B3
A3-B4
A3-B5
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pH
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6
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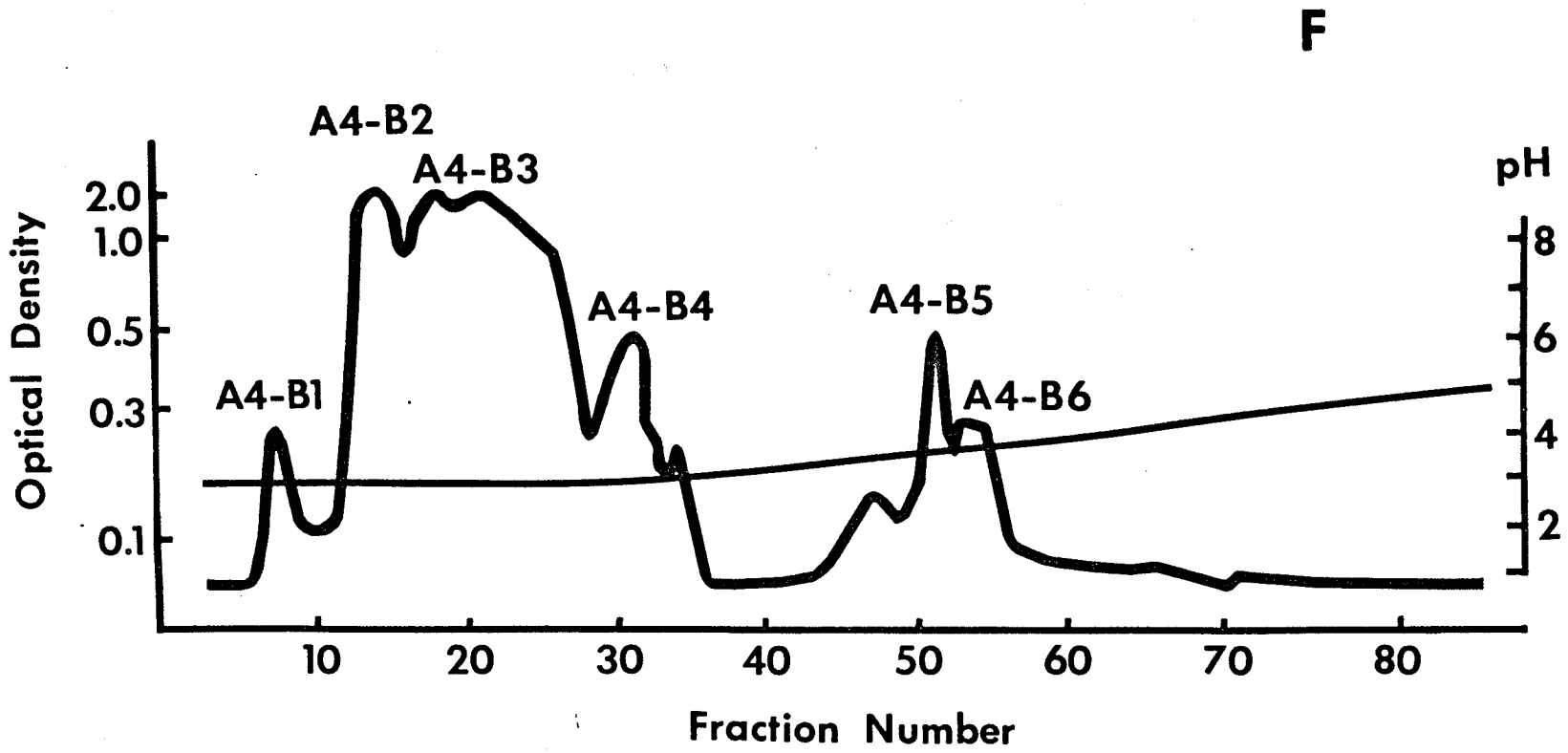
50
60
70
Fraction Number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A4-B5
A4-B1
A4-B6
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0.3
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pH
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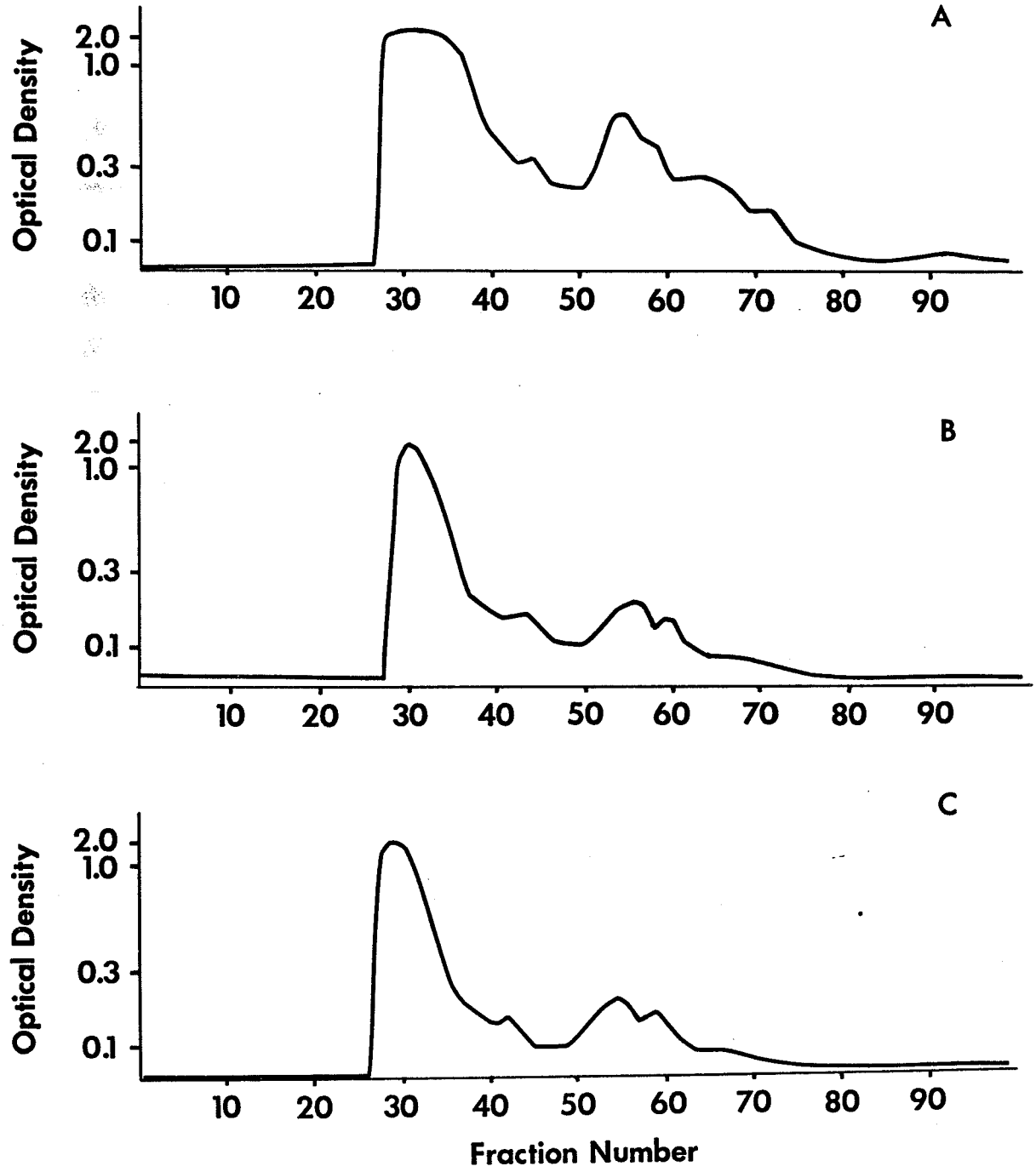
50
60
70
80
Fraction Number

tarded by the Dowex 50 column and the other emerged in the basic region of the chromatogram. A4-B2c was strongly retarded when subjected to Sephadex G-10, probably because of its high tyrosine content; acid hydrolysis of this peptide yielded 4 residues of tyrosine, 2 of lysine, and apparently 1 residue of a component whose position on elution corresponded to hydroxylysine. No further attempts were made to identify the latter substance.

Subfraction A5 (Fig. 2B; Table III) was resolved into ten ninhydrin-positive components by TLC. No further characterization of A5 was carried out. Subfraction A6 consisted of a single component according to 4 criteria mentioned above and the compound was not retarded by the Dowex 50 column used for separation of amino acids, neither by Dowex 50W-X2 column chromatography. After hydrolysis the amount of ninhydrin-positive material was decreased and only glutamic acid was detected in the chromatogram. Dinitrophenylation and subsequent hydrolysis of the peptide yielded DNP-glutamic acid. Subfractions A7, A8, A9, A10 and A12 were found to be relatively pure preparations of peptides composed exclusively of aspartate and glutamate in the ratios 1:4, 1:1, 8:1, and 5:1, respectively (Fig. 2B; Table III). A8 was characterized as aspartyl-glutamate and A10 as glutamyl-aspartate. A-11 appeared to be a


A
B
C
Optical Density
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0.3
0.1
10
20
30
40
50
60
70
80
90
Fraction Number

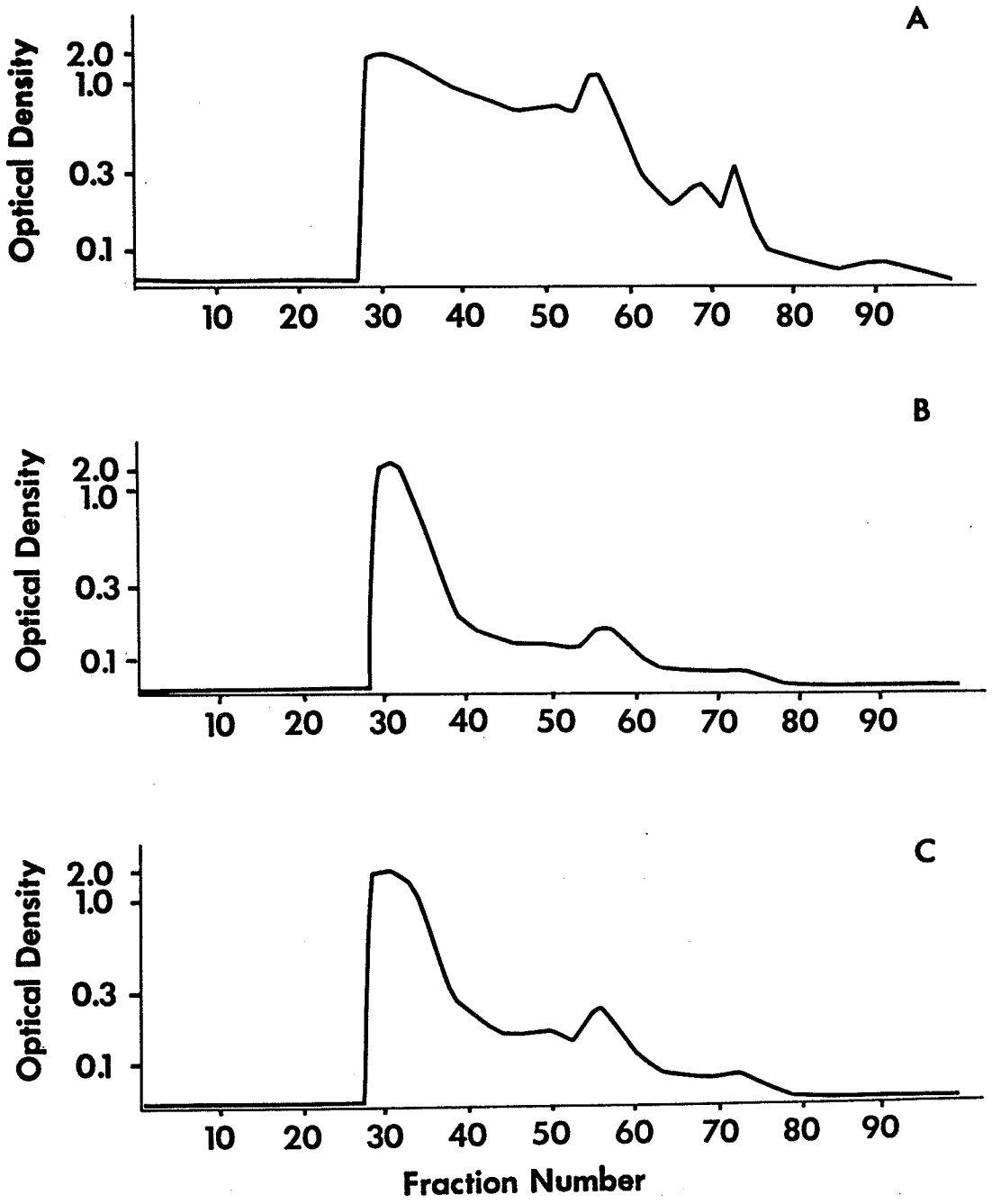


Figure 4. Elution profiles as in Figure 3, except that rat liver was used as the source of homogenate and granules.

tripeptide consisting of 2 aspartic residues and 1 leucine residue.

The elution profiles shown in Figs. 2-4 were reproduced almost identically with several different batches of posterior pituitary glands. An estimate was made of the proportion of acid-extractable, ninhydrin-positive material associated with the total granule fraction. In the case of the pituitary and, for comparison, in liver, approximately 40% of the total was sedimentable. In both tissues, no differences were noted in the distribution of ninhydrin-positive material between granule and supernatant fractions, when prepared from homogenates at either pH 7.4 or pH 6.0. This observation suggested that the binding of amino acids and of peptides to the granule was relatively insensitive to changes of pH or that they were contained within the granules (Figs. 3 and 4). In order to obtain information on the nature of the association between cytoplasmic granules and amino acids and peptides, rupture of these intracellular organelles by hypotonicity was resorted to. Isolated granules were suspended and in water or in 0.29M sucrose. Loss of ninhydrin-positive material was greater from granules suspended in water (Fig. 5), as would be expected if a significant proportion of the amino acids and peptides were contained within the granules.

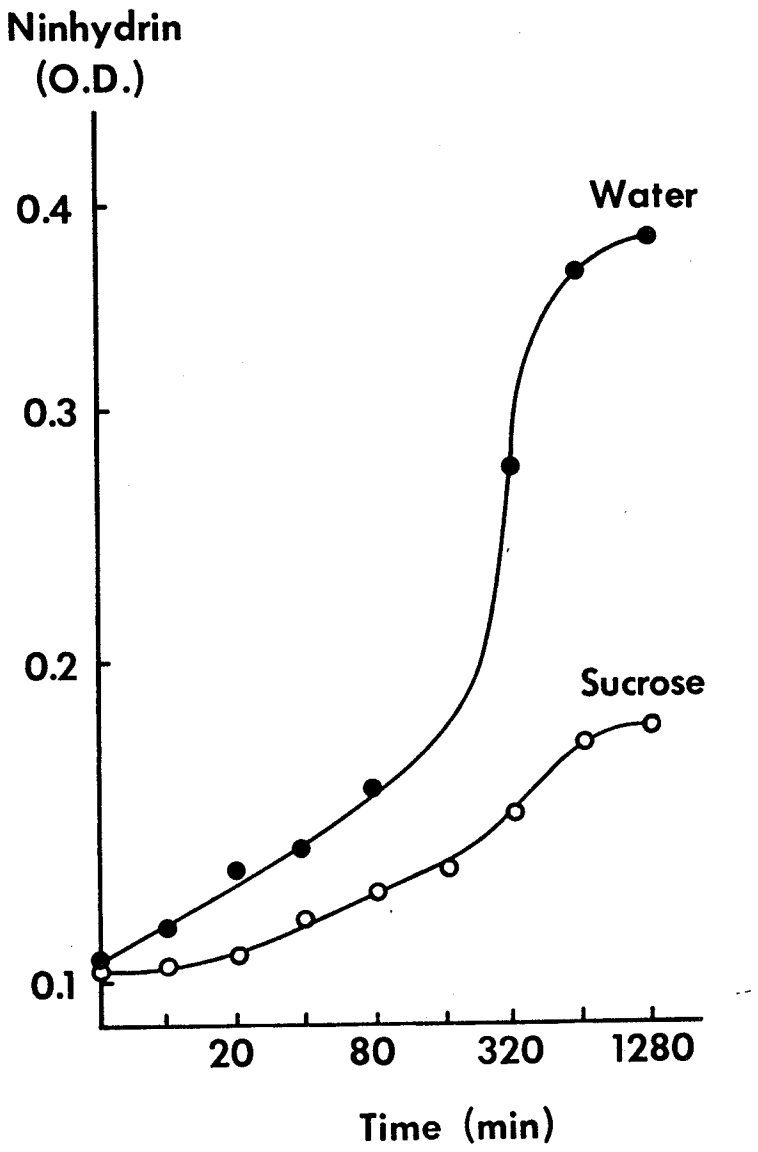


Figure 5. Portions of the total granule fraction from posterior pituitary were dialyzed against 0.005M pyridine-acetate buffer (pH 6.0) in (a) distilled water and (b) 0.29M sucrose. At intervals, portions were removed from the solution surrounding the dialysis bag and reacted with ninhydrin.

DISCUSSION

The number and quantity of peptides found in the bovine posterior pituitary confirms previous observations for this gland (Winnick et al., 1955). The present investigation has emphasized the association of a large proportion of the total glandular pool of peptides and amino acids with the sedimentable fraction of the tissue homogenate. Approximately the same proportion was associated with the particulate fraction of rat liver. The enhanced release of diffusible material from osmotically shocked granules was a further indication that the amino acids and peptides were contained within cytoplasmic structures. Preparation of a highly purified fraction of NSGs (LaBella et al., 1967) and its subsequent extraction resulted in a pattern of peptides which differed only slightly from that found in extracts of the total granule fraction. Although some peptides in the NSG fraction may be derived from contaminating organelles, the latter should be present in only small amounts. Since the pattern of peptides from purified NSG is almost identical to that from the total granule fraction, it appears that the bulk of the sedimentable peptides and amino acids were contained in the NSGs. We have additional support for this postulate, namely, the association of several peptides, besides VP and OT, with the purified carrier protein known to be present in the NSG.

It had been generally assumed that acid-extractable peptides and amino acids in tissues are "free" in the cellular cytoplasm, whereas in fact, these tissue components are in part associated with cellular organelles and membranes.

In this study our original aim was to examine the possibility that peptides other than VP and OT were bound to the carrier protein contained within the NSGs of the neurohypophysis. In view of the large number of peptides existing in the posterior pituitary, it appeared likely to us that other peptides would be found in association with the carrier protein for the established hormones. Previously it was shown in our laboratory that VP was located in a denser and OT in a lighter NSG fraction (LaBella et al.,1962; LaBella et al.,1963), and subsequently in denser and lighter nerve ending particles (Bindler et al.,1967). Recently, Dean et al. (1968) have reported that the dense NSGs contain VP in association with one species of carrier protein, "NP-II", and OT with another, "NP-I". Witter et al. (1964) reported that the van Dyke protein (a complex of protein and VP plus OT)contained other peptides and amino acids. The extraction procedure for isolating the van Dyke protein is relatively prolonged and involves several steps; one could argue that these procedures could promote artifactual binding to the granule-derived protein

of substances, which are ordinarily restricted to an extragranular compartment. Our observations on the binding of peptides and amino acids to subcellular particles isolated by centrifugation of sucrose homogenates would seem to be less subject to this criticism, but it must be acknowledged that the 2 hrs delay between the death of animals at the slaughterhouse and the subsequent extraction of tissue may augment the peptide pool by autolytic processes. We are ascertaining the extent of possible autolysis by examining quick-frozen glands from freshly-killed animals in order to establish whether or not these multiple components are granule-bound in situ. An argument against a significant role of autolysis as a source of posterior pituitary peptides is the fact that the presence of an abundant and diverse population of peptides in this tissue has been confirmed by many workers who have worked with tissues removed from various species at varying intervals after death. Also, rat liver which was homogenized in the cold immediately after sacrifice gave a complex pattern of peptides. It seems quite possible that additional peptides (of presently unknown hormonal functions) are synthesized, stored, and secreted in a manner similar to that of the two established peptide hormones. Such hypothetical peptides could be contained within the same neurons as VP or OT or in separate neurons.

NSGs, composed of the peptide hormones bound to carrier protein constitute the major portion of protein in the posterior pituitary. The abundance of amino acids and peptides in the gland may result from enzymatic degradation of the carrier protein. Ingestion of NSGs by lysosomes within the nerve endings can be observed frequently electronmicroscopically (submitted for publication). Autophagy of cytoplasmic granules has been observed in a number of tissues and is probably one of the continuous, dynamic cellular processes concerned with maintenance of a constant granule population. In addition to the proteolysis of granule protein by lysosomal enzymes in the nerve terminals, pituicytes and other connective tissue cells may also degrade proteins that possibly could be released from the neurons. There is no morphological evidence to indicate that the NSG are secreted <u>in toto</u> from the cell, as is apparently the case for other glandular cells that contain and secrete products from proteinaceous storage granules. Therefore, it is generally believed that neurohypophysial hormones diffuse away from the NSG and are secreted. Disposal of the residual carrier protein by enzymatic degradation may be the reason for the large peptide pool characteristic of the posterior pituitary gland.

Ramachandran and Winnick (1957) have determined the amino acid composition of several peptides isolated

from porcine posterior pituitaries. Their initial peptide fraction was further fractionated by precipitation in organic solvents, adsorption on charcoal, and dinitrophenylation with subsequent chromatography. The fraction they characterized represented only about one-third of the total peptides, and the members ranged in size from dipeptides to one containing possibly as many as 87 residues. The data on compositions of amino acids indicated very little similarity of their isolated low-molecular-weight components to those in our fraction which contained nonapeptides as the largest members.

In the low-molecular-weight population in the present study several unusual peptides were identified which contained only glutamic and aspartic acids in ratios of 1:1, 1:5, 1:8 and 4:1, respectively. Glutamic acid was found in 10 of the 19 peptides and comprised 4 of the 8 residues of peptide A5 and was linked with an unidentified component in A6. Aspartic acid was present in 11 of the peptides. Aspartic and glutamic acids are among the most abundant of the amino acids found in NP, the carrier protein (LaBella et al.,1967). Histidine constituted 5 of the 8 residues in A3-B3 and tyrosine 4 of the 7 residues of A4-B2C.

TABLE III

AMINO ACID COMPOSITION OF ISOLATED PEPTIDES

Peptide fraction	Major components after acid hydrolysis
A1-B1*	NH_3
A1-B2*	lysine (free)
A1-B3*	arginine (free)
A1-B4*	alanyl-arginine
A2-B1	arg_2, gly_1, leu_1, val_1
A2-B2*	leu_4, his_2, gly_1
A3-B2*	NH_3
A3-B3	his_5, gly_2, ser_1
A3-B4	lys_2, ala_1, arg_1, asp_1, glu_1, gly_1, phe_1, ser_1
A3-B5*	lys_3, phe_3, asp_1, pro_1
A4-B1*	glu_2, ala_1, asp_1, gly_1, pro_1, ser_1, thr_1, X_1, Y_1
A4-B2a*	ala_2, met_2, asp_1, ser_1
A4-B2b*	phenylalanine (free)
A4-B2c*	tyr_4, lys_2, $hylys(?)_1$
A4-B4a*	phe_3, ala_1, gly_1, tyr_1

continued...

TABLE III Continued

Peptide fraction	Major components after acid hydrolysis
A4-B4b*	phe_2, ala_1, gly_1, tyr_1
A4-B5*	lysyl-glutamic
A5	ten spots on TLC
A6*	glu, X, (free NH_2 of glu)
A7	glu_4, asp_1
A8*	aspartyl-glutamic
A9*	asp_8, glu_1
A10*	glutamyl-aspartic
A11*	asp_2, leu_1
A12*	asp_5, glu_1

* The fractions noted by an asterisk are assumed to be homogeneous on the basis of ion-exchange chromatography, gel filtration, TLC, and high voltage electrophoresis.

IV. MULTIPLE FORMS OF BOVINE NEUROPHYSIN RESULTING FROM ASSOCIATION AND DISSOCIATION OF SUBUNITS

INTRODUCTION

It is well established that the posterior pituitary gland contains a remarkably large number and quantity of peptides (Winnick et al.,1955; Ramachandran and Winnick, 1957). Several peptides in addition to the known neurohypophysial hormones have been characterized (Witter et al.,1964; Preddie and Saffran, 1957; Preddie, 1965; Penders and Arens, 1966; Schally and Barret, 1968).

Since van Dyke et al. (1942) isolated a homogeneous amorphous protein, many workers have isolated the hormone-binding protein, neurophysin (NP) (Acher et al., 1956; Ginsburg and Ireland, 1965; Hollenberg and Hope, 1968). Some workers report that NP is a single entity (Acher et al., 1956; Ginsburg and Ireland, 1968), while others claim that it is composed of several hormone-binding protein fractions (Breslow and Abrash, 1966; Hollenberg and Hope, 1967, 1968). The NPs differ in reported molecular sizes and hormone binding ability according to various workers. Moreover, Ginsburg and Jayasena (1968b) found several "extra-neurohypophysial NPs", in tissues other than the neurohypophysis, but which bind OT and VP.

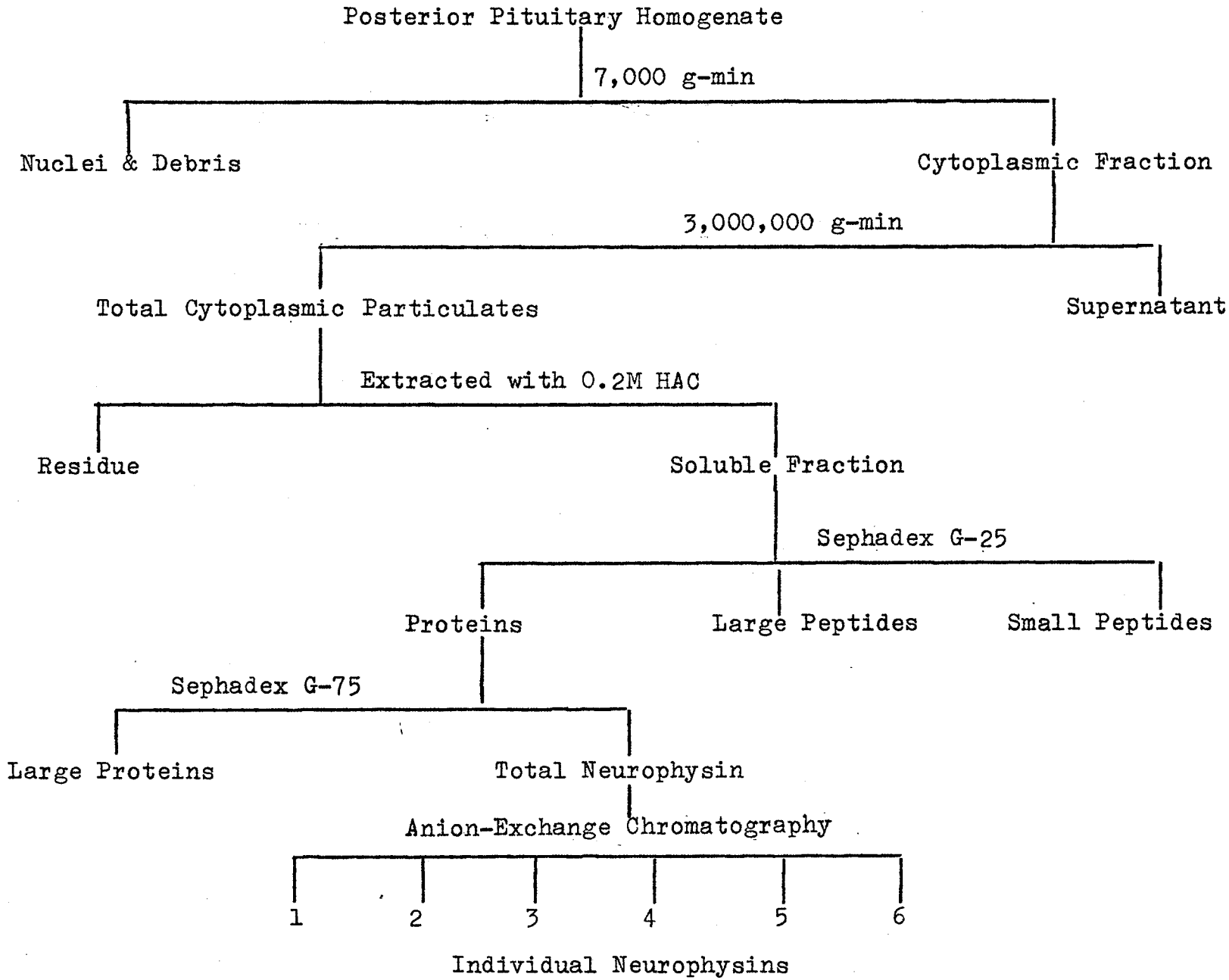
Large cells in the supraoptic and paraventricular nuclei synthesize neurosecretory material. The

neurosecretory granules (NSGs) pass along the axons to the posterior pituitary and are deposited there to release neurohypophysial hormones into blood vessels upon appropriate stimulation. It was shown in our laboratory that a denser NSG contains vasopressin (VP) and a lighter fraction oxytocin (OT) (LaBella et al., 1963). Dean et al. (1968) showed that two different NPs were concentrated in different granule populations, each specific for one of the neurohypophysial hormones.

Neurohypophysial hormones can be rapidly released into the circulation in response to various kinds of stimuli with a concomitant decrease in the number of dense granules of 100-300 mμ in diameter (Pickford, 1947; Fang et al., 1962; Hild, 1954). This suggests that the granule-bound NP may have disappeared by diffusion or by disintegration.

In the present study we have prepared total NP from cytoplasmic granules in order to minimize contamination by other cytoplasmic components. We have found that the hormone-binding protein fraction, NP, consists of a family of proteins in equilibrium with one another apparently due to association and dissociation among dissimilar subunits. We feel that these findings readily account for the apparent discrepancies in the literature concerning the number of NP and their molecular weights (mol wt).

Figure 6. Flow sheet of purification and fractionation of NP from fresh bovine posterior pituitary.


Posterior Pituitary Homogenate
7,000 g-min
Nuclei & Debris
Cytoplasmic Fraction
3,000,000 g-min
Total Cytoplasmic Particulates
Supernatant
Extracted with 0.2M HAC
Residue
Soluble Fraction
Sephadex G-25
Proteins
Large Peptides
Small Peptides
Sephadex G-75
Large Proteins
Total Neurophysin
Anion-Exchange Chromatography
1
2
3
4
5
6
Individual Neurophysins

MATERIALS AND METHODS

Bovine posterior pituitary glands

The glands were obtained at a nearby slaughterhouse approximately 30-40 min after death of animals. The tissues were placed in vessels surrounded by ice and transported to the laboratory.

Isolation of subcellular granules

The tissue was minced with scissors and homogenized in 0.29M sucrose with a motor-driven teflon pestle-glass vessel homogenizer (LaBella et al., 1963), and centrifuged at 7,000 g-min (R_{max}) to remove nuclei and cell debris which were discarded. A subsequent centrifugation of the supernatant was done at 3,000,000 g-min (R_{max}) in the International Preparative Ultracentrifuge B-60 in order to prepare a total cytoplasmic granule fraction (Fig. 6).

Extraction of neurophysin

The cytoplasmic granules were extracted at 4^{o} in 0.2M acetic acid with stirring for 24 hrs. The mixture was centrifuged and the supernatant concentrated at 40^{o} under reduced pressure. This extract was used to prepare NP by subsequent gel filtration on Sephadex G-25 and Sephadex G-75 as described by Hollenberg and Hope (1967).

Gel filtration

Columns of Sephadex were prepared essentially according to the manufacturer's recommendation. Column dimensions and flow rates for G-25, G-50, analytical G-50 and G-75, were 5.4 x 60 cm and 75 ml/hr, 2.8 x 59 cm and 30 ml/hr, 1.2 x 110 cm and 20 ml/hr, and 3.1 x 58 cm and 20 ml/hr, respectively.

Concentration and storage of neurophysin

Total NP prepared by gel filtration of individual NP fractions isolated by anion exchange chromatography were concentrated to dryness with a rotary evaporator. The concentration procedure was repeated after adding distilled water until volatile buffers were no longer detectable. The dry samples were stored at 4^{o}.

Electrophoresis on cellulose acetate membrane

Cellulose acetate membrane (Seprophore III, Gelman Instrument Co.) electrophoresis was carried out with Michaelis buffer, pH 8.6, 0.2M pyridine acetate buffer, pH 5.8, or 0.2M acetic acid buffer, pH 2.8. The developed strips were stained with a saturated solution of Amido Black 10 B in methanol:glacial acetic acid (9:1). The excess and background stain were removed by rinsing in methanol:acetic acid.

Polyacrylamide gel electrophoresis

About 100 μg of sample was dissolved in 100 μl of 0.1M sodium phosphate buffer, pH 7.1 with or without

0.1% sodium dodecyl sulfate (SDS). The gel was modified from Maizel (1966) and consisted of 20% acrylamide, 0.26% N, N, bismethyleneacrylamide, 0.2% N, N, N', N', tetramethylenediamine and 0.075% ammonium persulfate in 0.1M sodium phosphate buffer, pH 7.2, and 0.1% SDS. Electrophoresis was carried out on a 6 x 110 mm acrylamide gel at 8 volt/cm for 3 hrs. The gel was fixed with sulfosalicylic acid for 16 hrs, stained with 0.25% Coomassie Brilliant Blue R 250 for 5 hrs and destained with 7% acetic acid as described by Shapiro et al. (1967). Five per cent gel without SDS was used to test homogeneity of samples.

Ion-exchange chromatography

A column of Dowex 1-X2 (200-400 mesh) in chloride form (Bio-Rad Laboratories) was packed and regenerated according to Schroeder et al. (1962). The sample to be chromatographed was dissolved in 2 ml of collidine-pyridine-acetic acid buffer, pH 8.3 (Schroeder et al., 1962) and the pH readjusted if necessary. Subsequently, a pH gradient with acetic acid was applied (Table II). The 2.3 x 59 cm column was maintained at 35° and the flow rate was 40 ml/hr. 7.5 ml fractions were collected and aliquots analysed for Lowry protein (Lowry et al., 1951) using a Technicon Autoanalyzer.

Amino acid analysis

Protein was hydrolyzed for 24 hrs in 6 N HCl

at 110° under nitrogen in sealed glass vials. Amino acid estimation was carried out on a Technicon Instrument as previously described (LaBella et al., 1967).

Bioassay

Oxytocin assay on chromatographic fractions was carried out on the isolated rat uterus (Holton, 1948).

Binding of oxytocin to neurophysin

Maximum binding of NP with OT was estimated by equilibrium dialysis (Craig et al., 1957; Ginsburg and Ireland, 1964) against 0.2M pyridine acetate buffer, pH 5.8 and using 1 x 8 cm micro-dialyzing tubes (Oxford Laboratories, Calif.). One ml samples containing OT plus NP were dialyzed against 25 ml of the buffer, and the dialyzing unit shaken at a rate of 120 cycles per minute. Binding was also studied by gel filtration: a Sephadex G-50 column (2.8 x 60 cm) was equilibrated with 0.2M pyridine-acetate, pH 5.8. One ml of the buffer containing 20-30 mg of NP and 30 U of OT was placed on a Sephadex G-50 column and eluted with the buffer. Oxytocic activity of each fraction was determined.

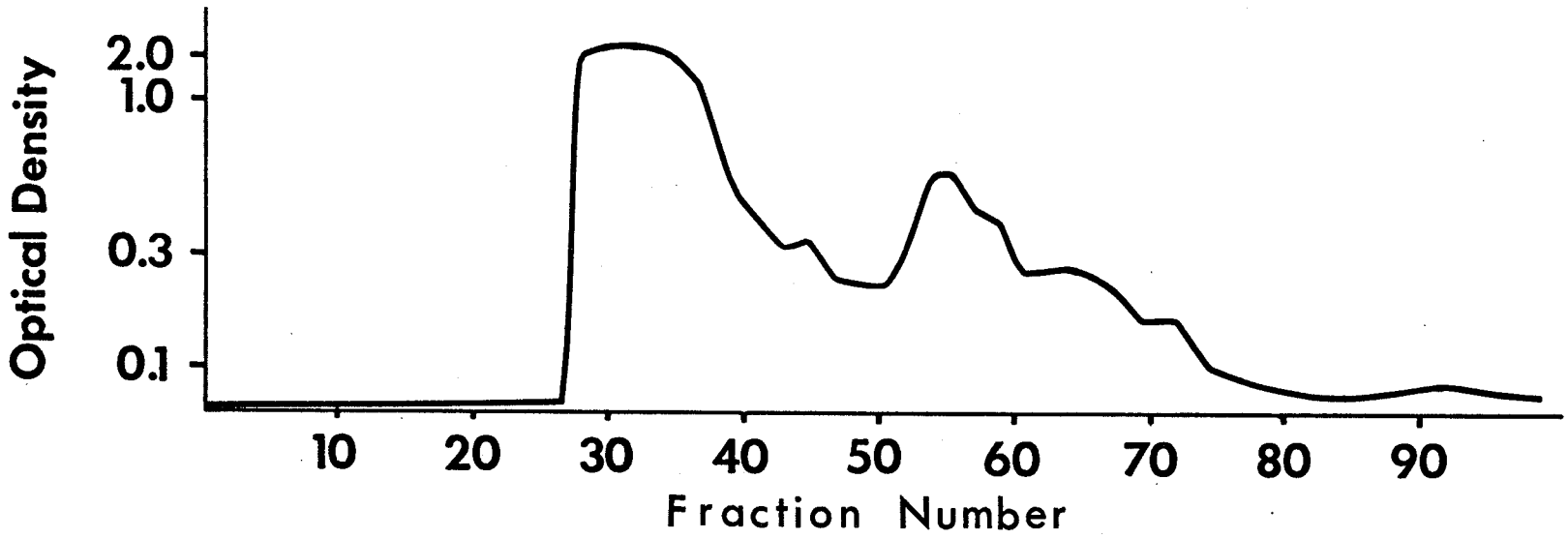
RESULTS

Extraction and fractionation of the total protein

Thirty grams wet weight of fresh bovine posterior pituitary glands yielded a creamy subcellular particulate fraction containing 1,600 mg protein. Extraction of the particulate fraction with 0.2M acetic acid resulted in the solubilization of 500 mg Lowry protein. The extract was fractionated into three peaks by gel filtration on Sephadex G-25 with 0.2M acetic acid (Fig.7). The first peak, which was not retarded on Sephadex G-25 contained 54%, 270 mg, of the soluble protein and was further fractionated into two major components on Sephadex G-75 (Fig. 8). A major portion of the protein (230 mg) was strongly retarded and constituted the "total NP" fraction (Hollenberg and Hope, 1967). The general scheme of the NP fractionation is shown in Fig. 6. The "total NP" peak was resolved into two bands by electrophoresis on cellulose acetate in three different buffers or on polyacrylamide gel.

Evidence for a subunit structure of neurophysin

Since we found evidence for association-dissociation phenomena of NP on Dowex 1-X2 column chromatography, several reagents, 6M urea, 0.2M calcium chloride, 0.2M EDTA, 0.1M dithiothreitol, as well as variation of pH from 2 to 8.3, were used in conjunction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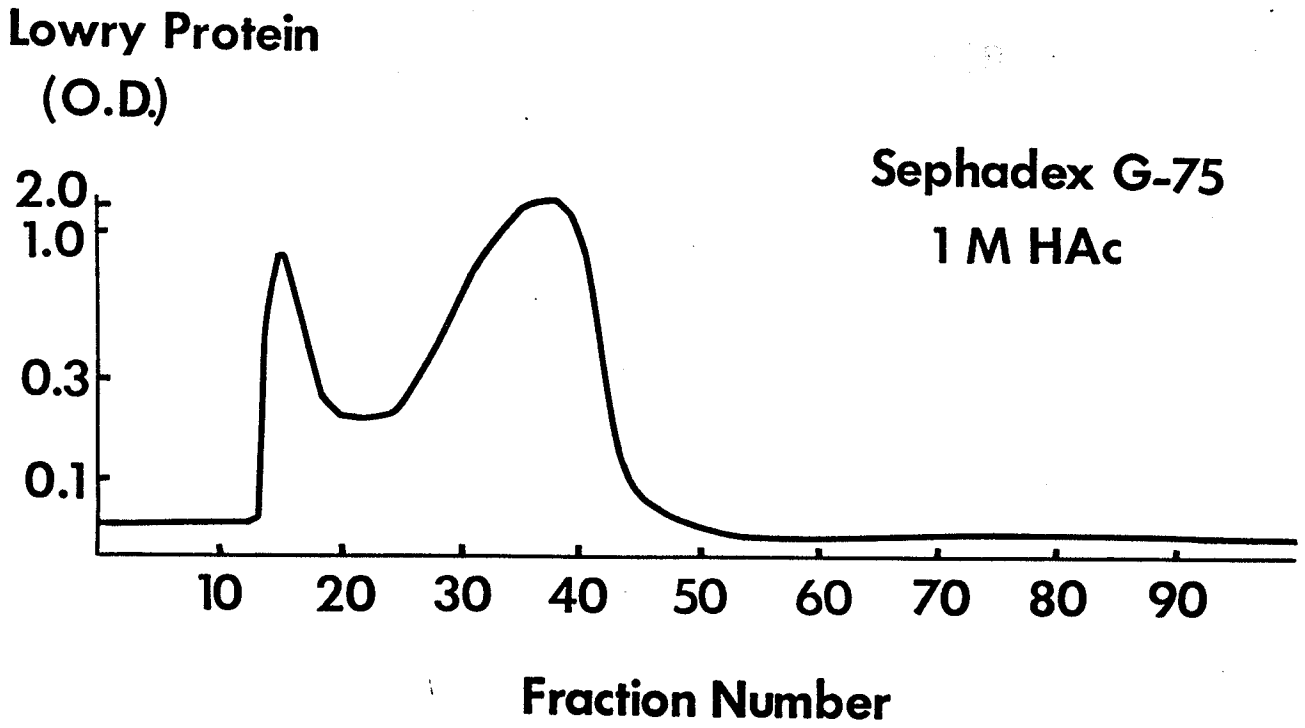
0.1
10
20
30
40
50
60
70
80
90
Fraction Number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(O.D.)
2.0
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0.3
0.1
Sephadex G-75
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10
20
30
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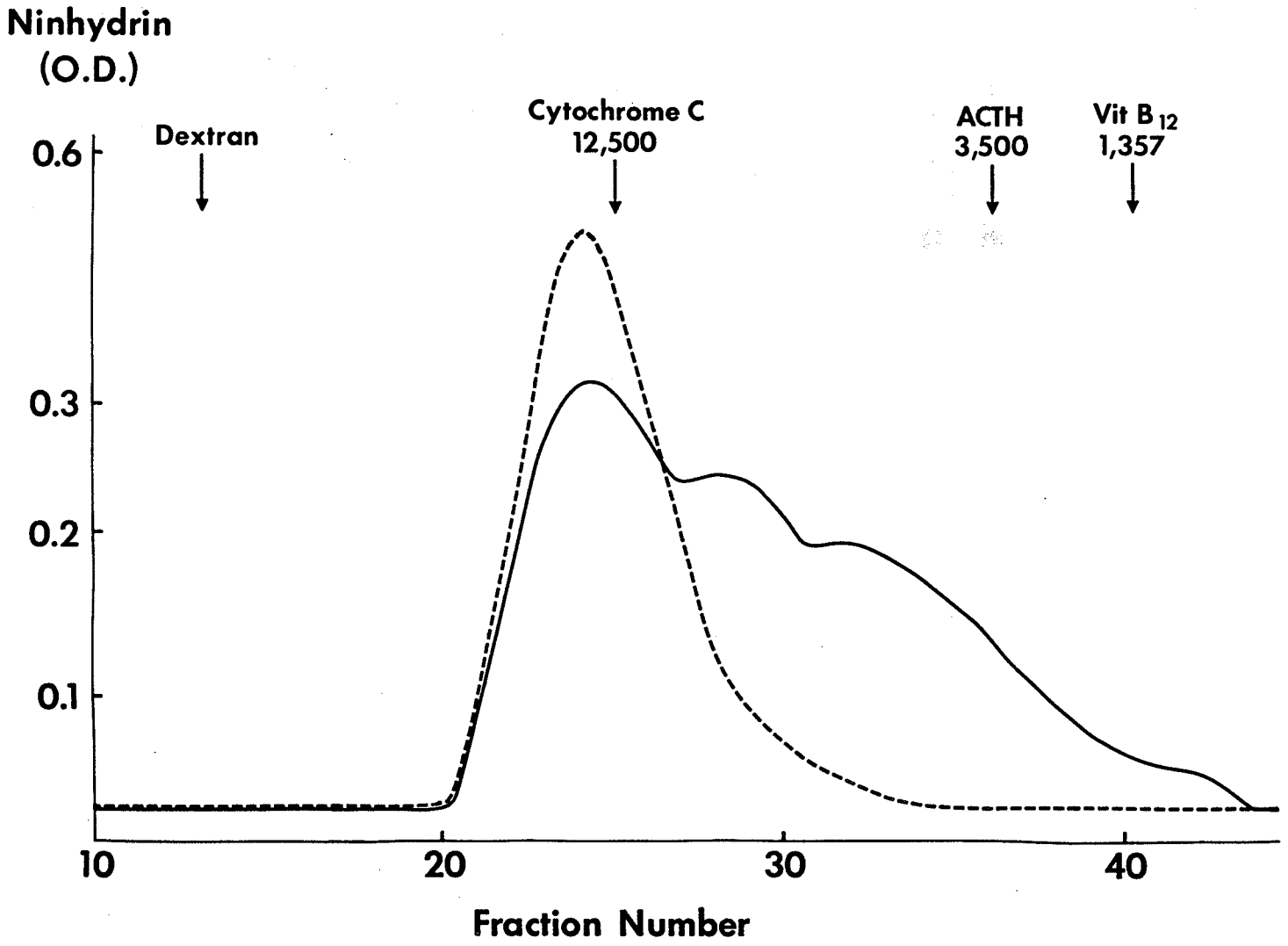
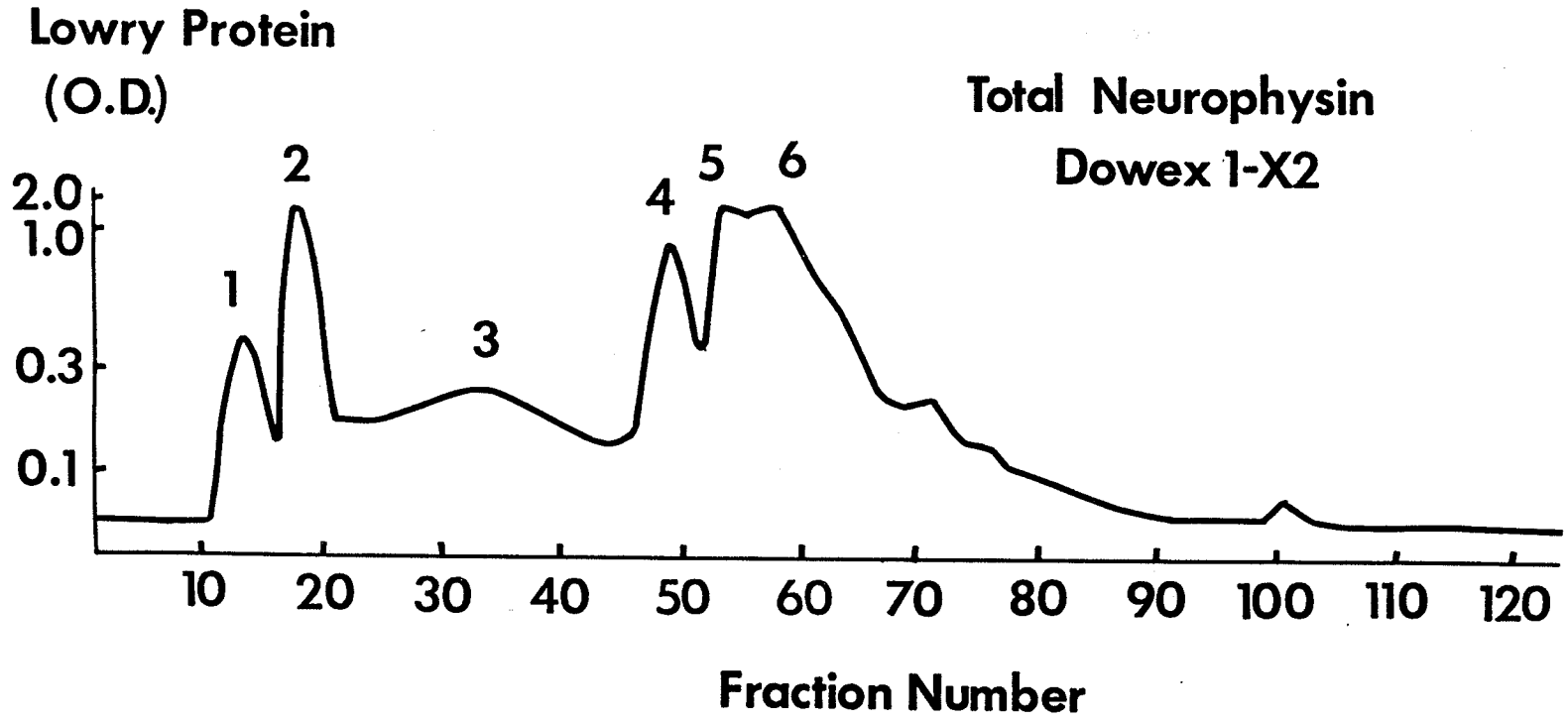
50
60
70
80
90
Fraction Number


Ninhydrin
(O.D.)
Dextran
Cytochrome C
12,500
ACTH
3,500
Vit B 12
1,357
0.6
0.3
0.2
0.1
10
20
30
40
Fraction Number

with filtration on Sephadex G-50 but failed to dissociate "total NP" into subunits. 0.1M SDS was also tried in conjunction with filtration on Sephadex G-50, but it was difficult to assess any possible change of mol wt because the void volume was unusually increased with this reagent. Choline, a quaternary ammonium compound, was tested as a dissociating reagent (Fig. 9), since Dowex 1-X2, containing quaternary ammonium groups, induced association-dissociation of NP preparations.

(a) Anion-exchange chromatography

A total NP fraction (240 mg) was evaporated to dryness under reduced pressure at 40° and was resolved into six fractions by chromatography on Dowex 1-X2. The chromatogram of a freshly prepared total NP fraction shows 6 peaks, each clearly separate except for NP-5 and -6, and was very reproducible with respect to shape, position and relative proportions of the various components, (Fig. 10). However, when a lower quantity of "total NP" was chromatographed, even NP-5 and -6 were more completely resolved. The fractions representing a given peak were pooled and concentrated. The relative proportions of the six NPs gradually changes during aging of the total NP solution. NP stored at 4° for 1 month produced much larger amounts of NP-6 with a proportional decrease in the others. Each NP was rechromatographed on Dowex 1-X2, only a very narrow band of the peak being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Total Neurophysin
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1.0
0.3
0.1
1
2
3
4
5
6
10
20
30
40
50
60
70
80
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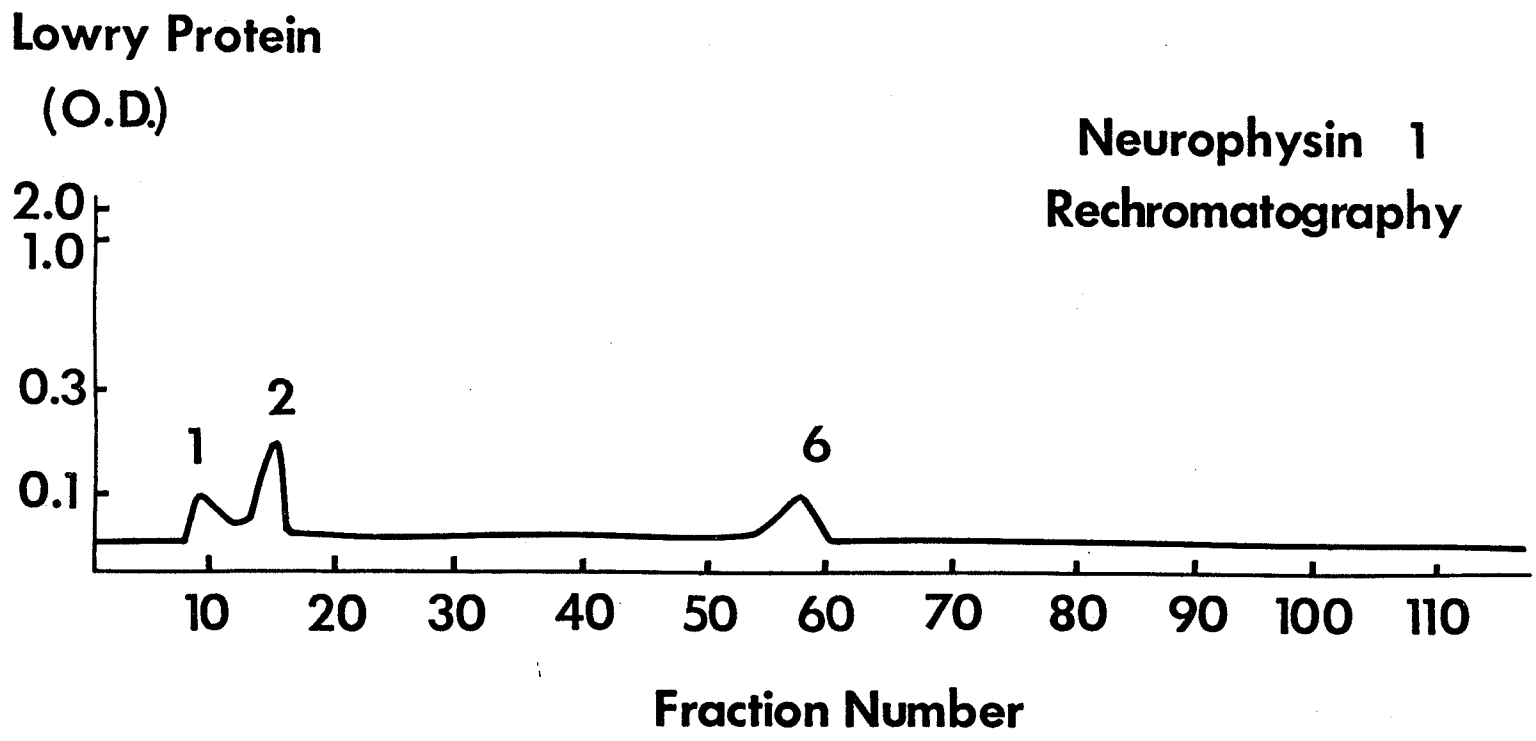
100
110
120
Fraction Number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Neurophysin 1
Rechromatography
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1
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60
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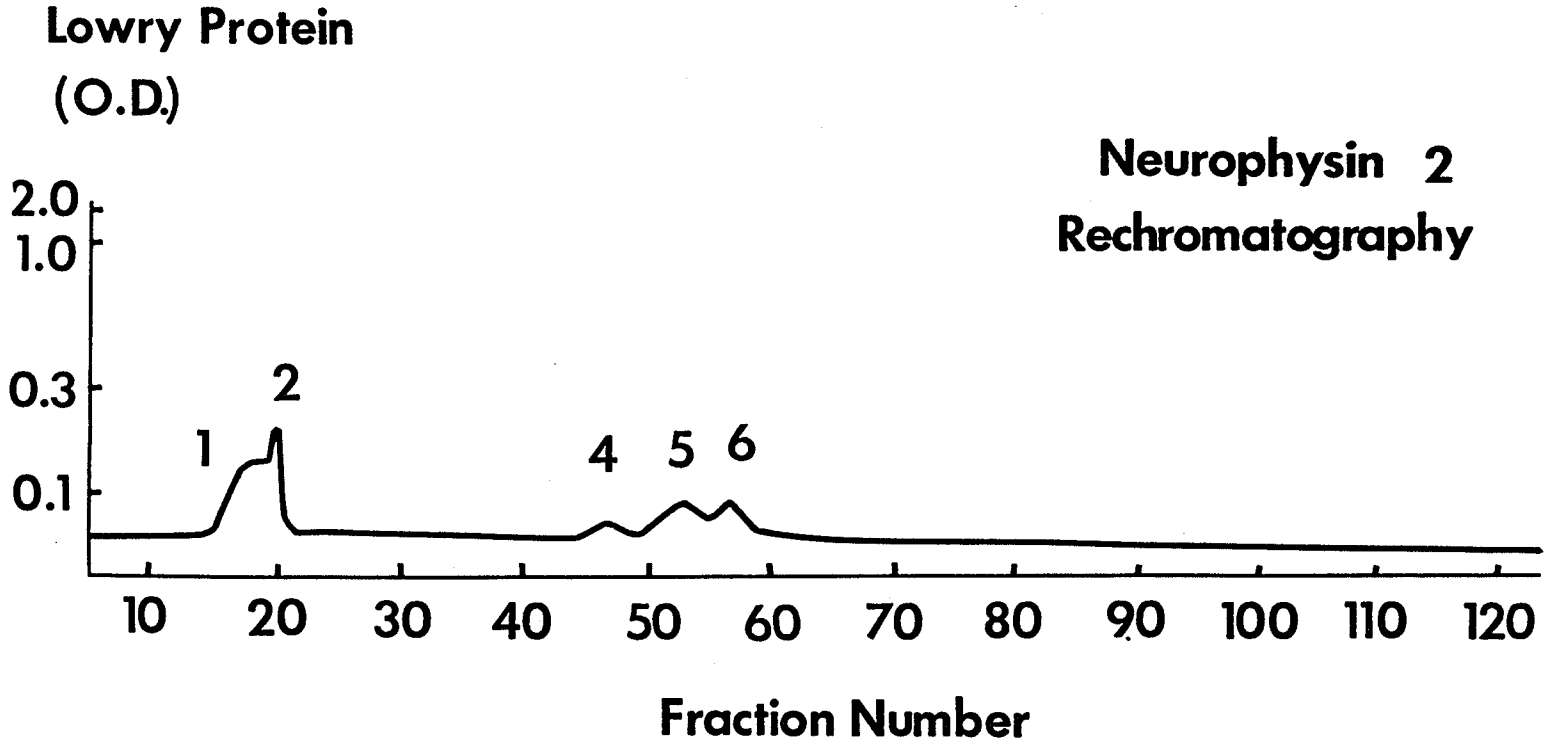
100
110
Fraction Number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(O.D.)
Neurophysin 2
Rechromatography
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1.0
0.3
0.1
1
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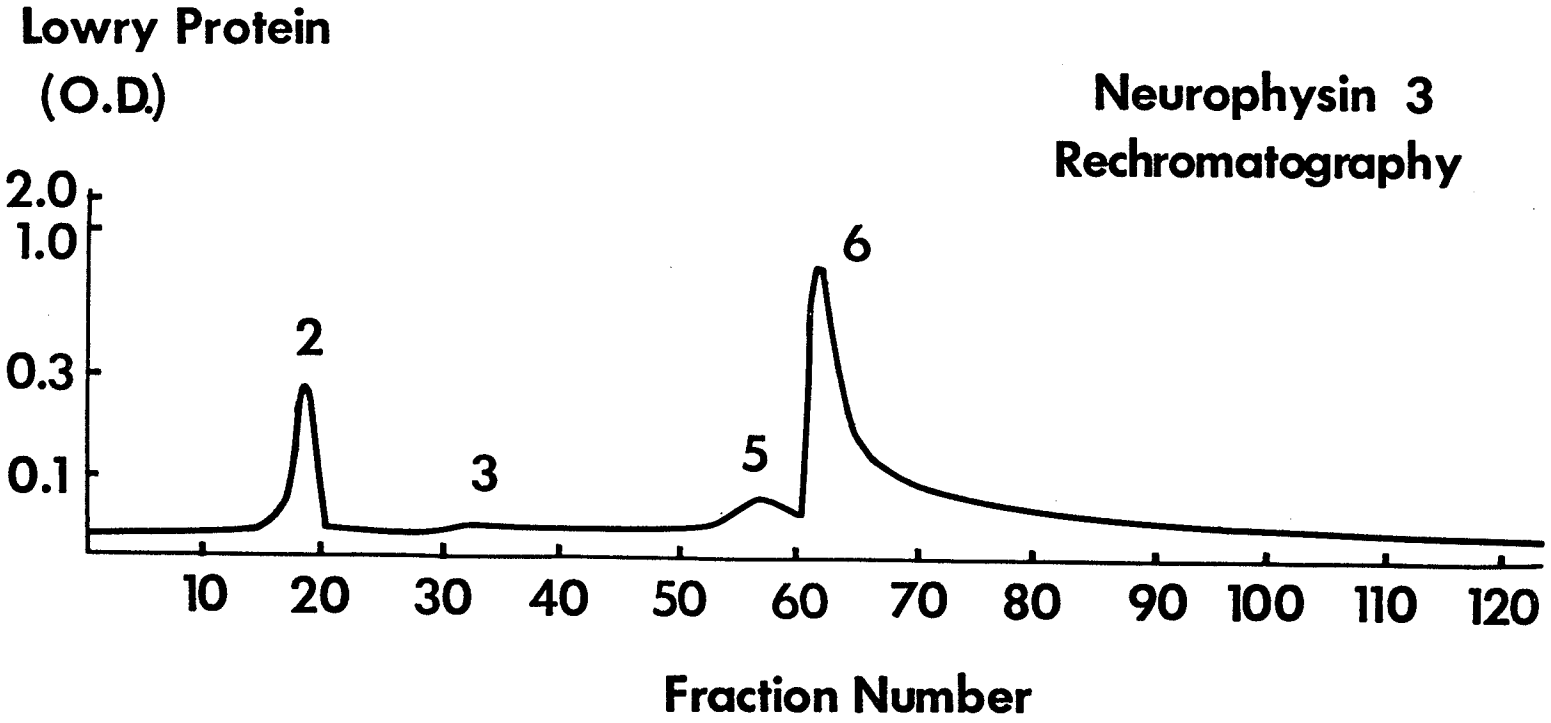
Fraction Number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(O.D.)
Neurophysin 3
Rechromatography
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1.0
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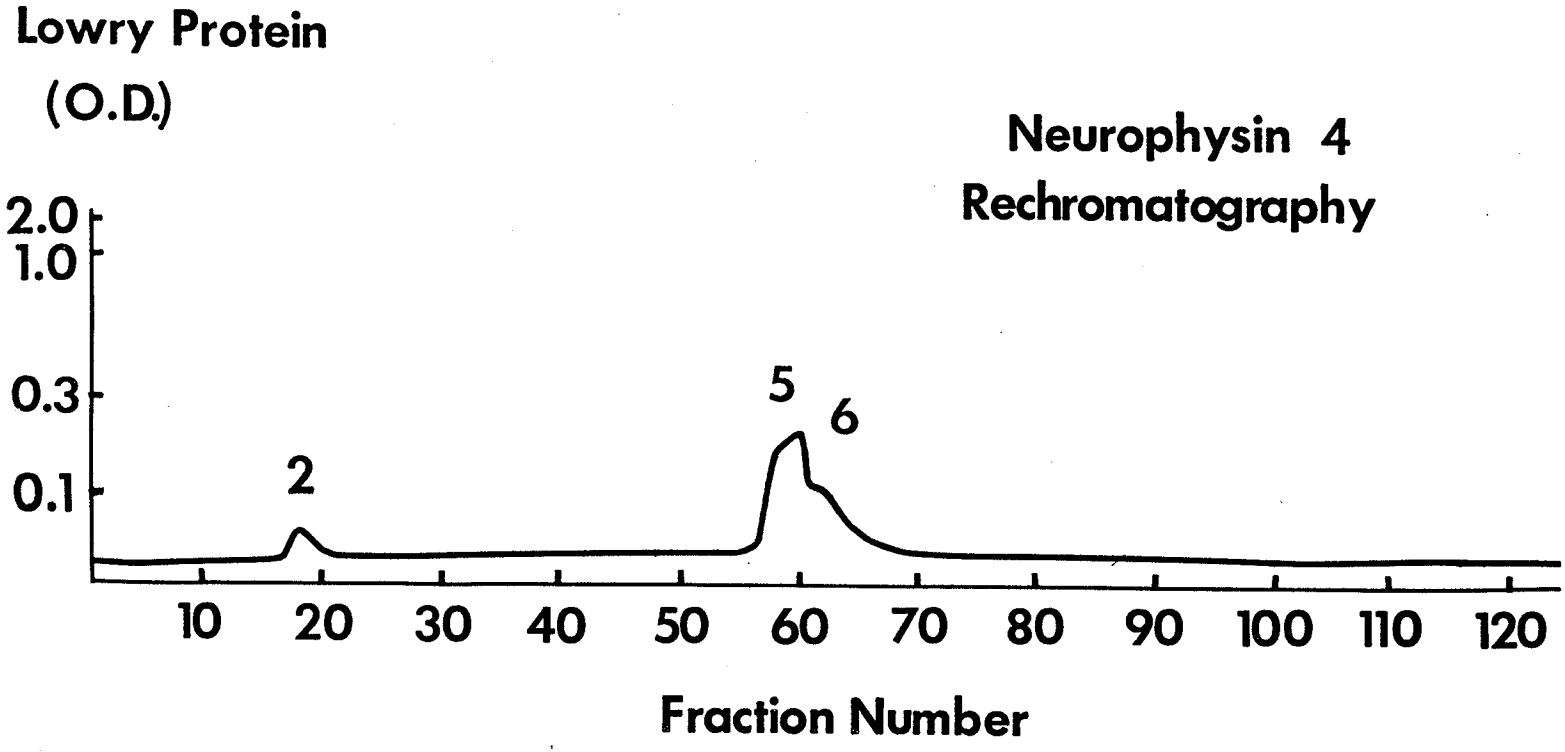
Fraction Number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(O.D.)
Neurophysin 4
Rechromatography
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1.0
0.3
0.1
2
5
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10
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30
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50
60
70
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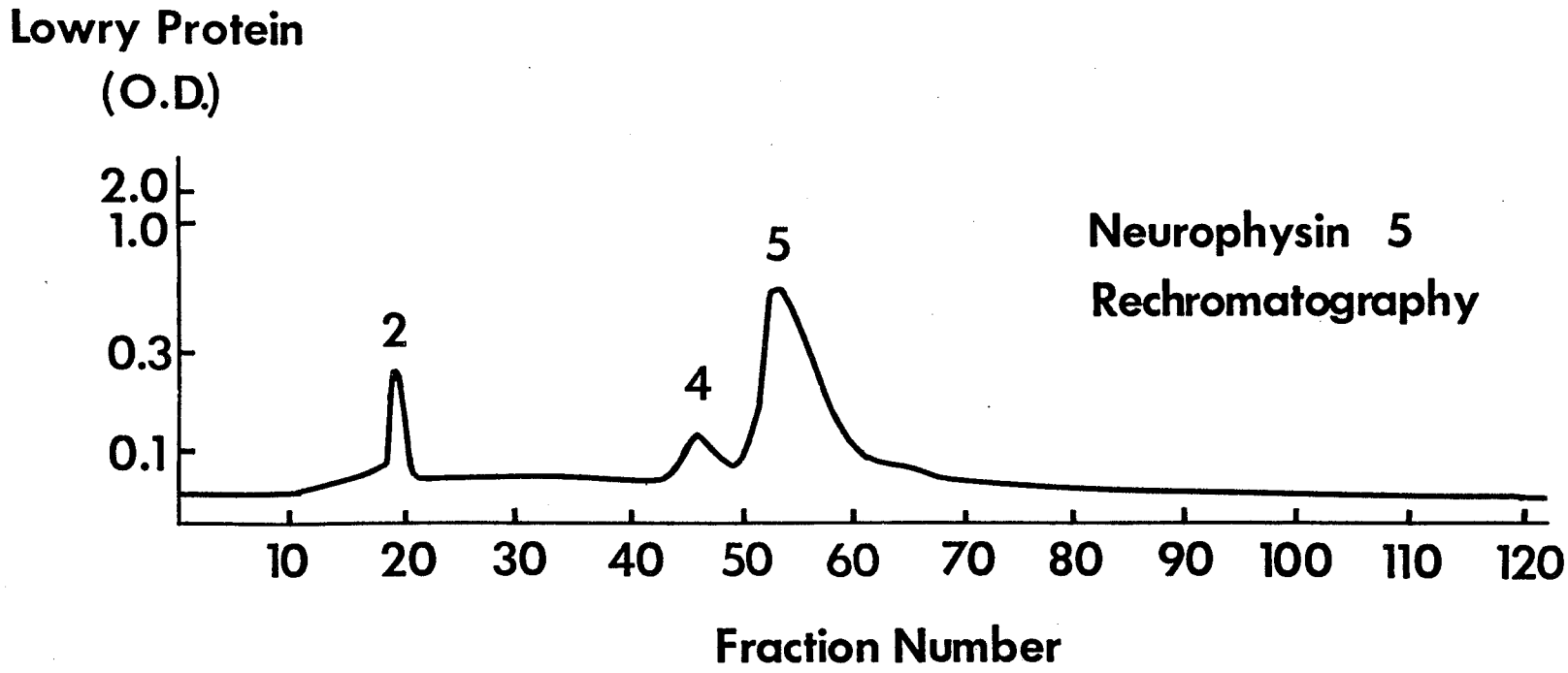
90
100
110
120
Fraction Number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(O.D.)
2.0
1.0
0.3
0.1
2
4
5
Neurophysin 5
Rechromatography
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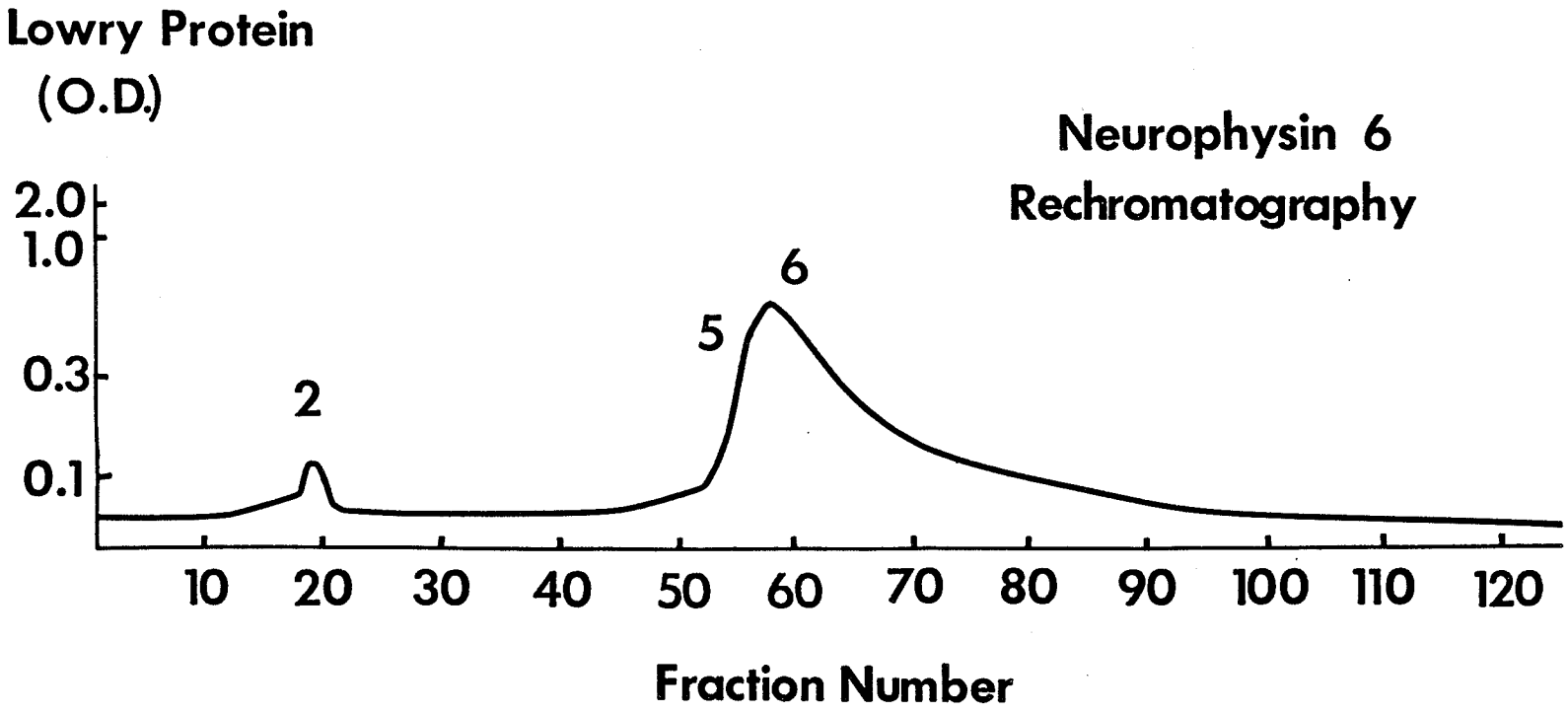
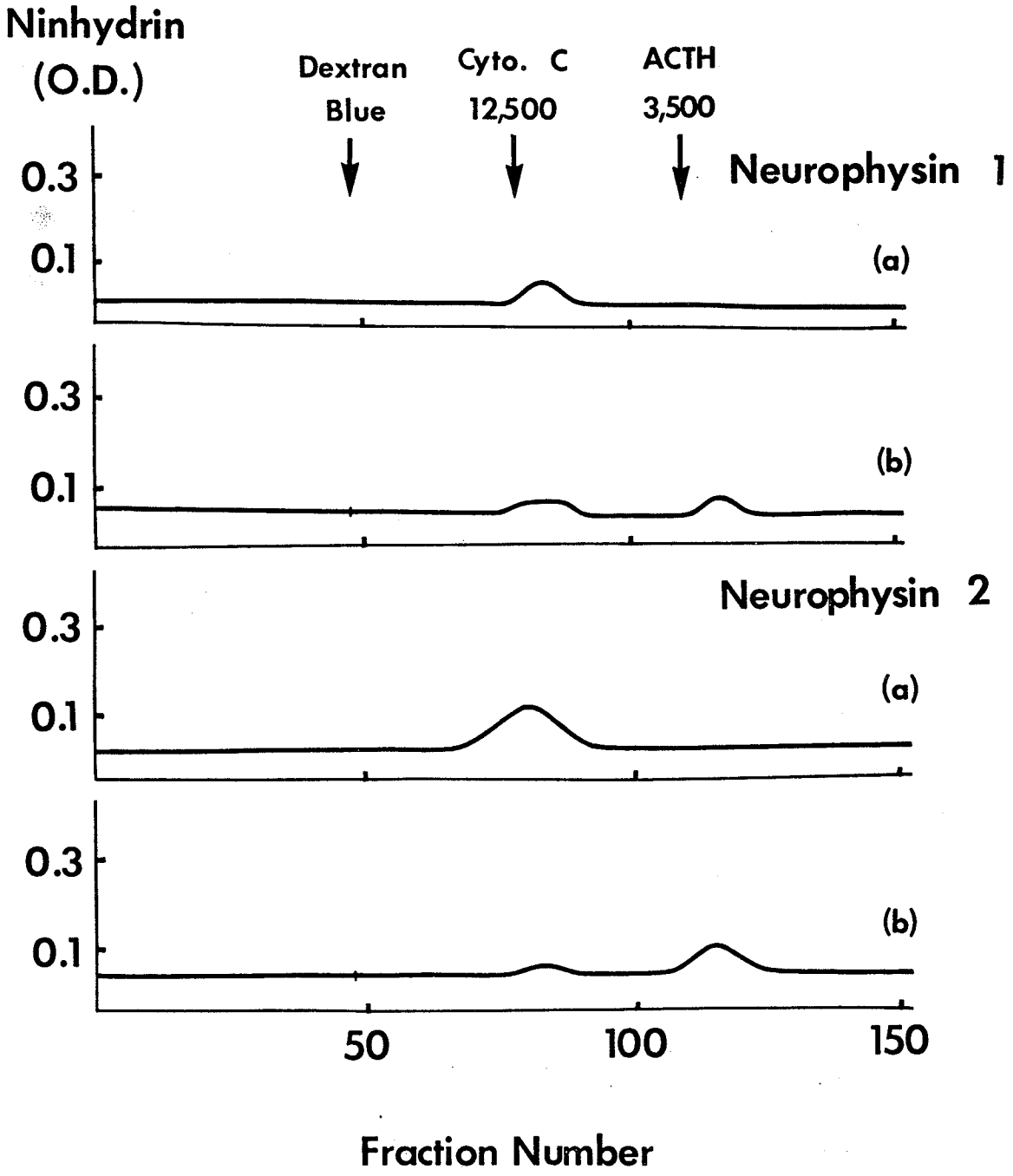
40
50
60
70
80
90
100
110
120
Fraction Number

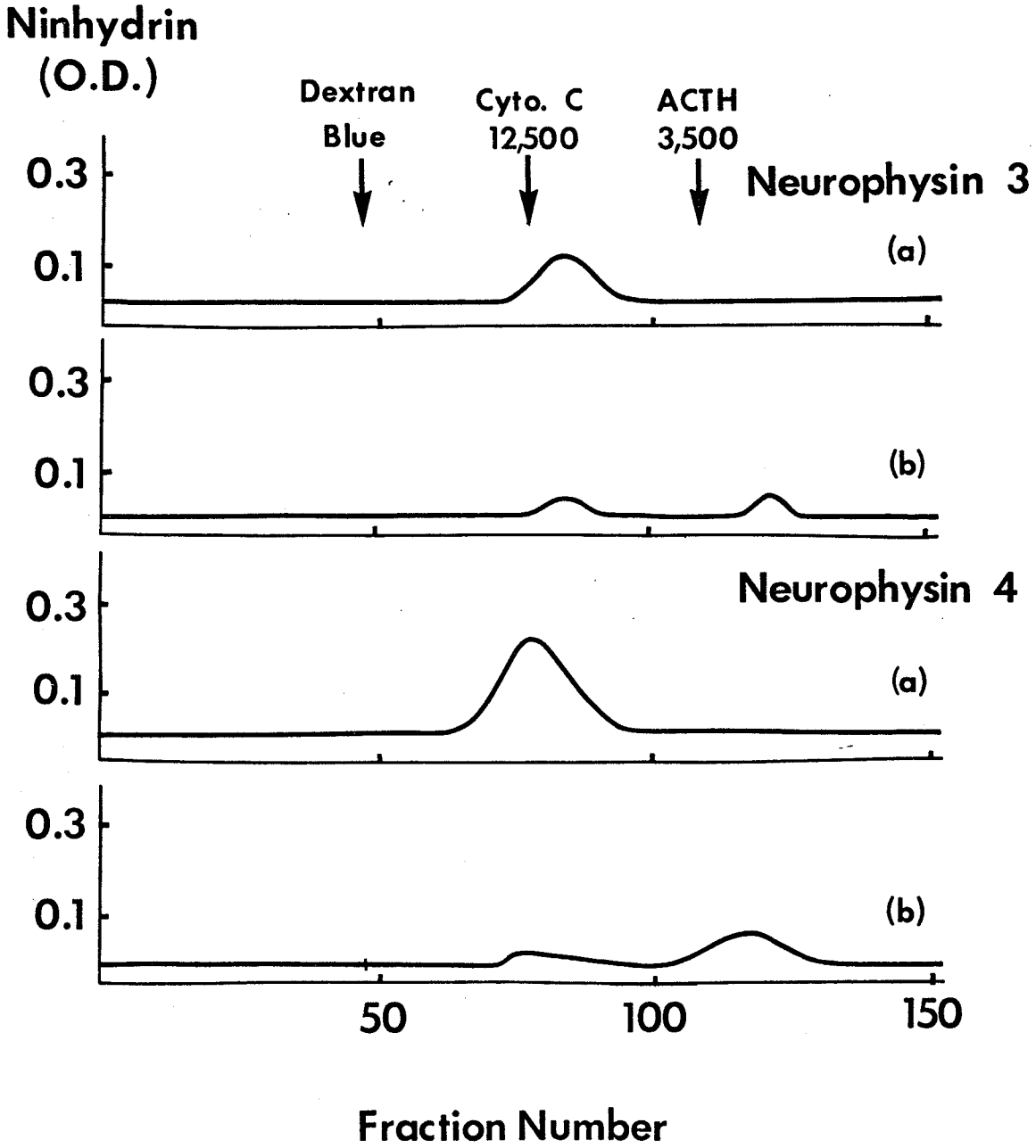

Lowry Protein
(O.D.)
Neurophysin 6
Rechromatography
2.0
1.0
0.3
0.1
2
5
6
10
20
30
40
50
60
70
80
90
100
110
120
Fraction Number

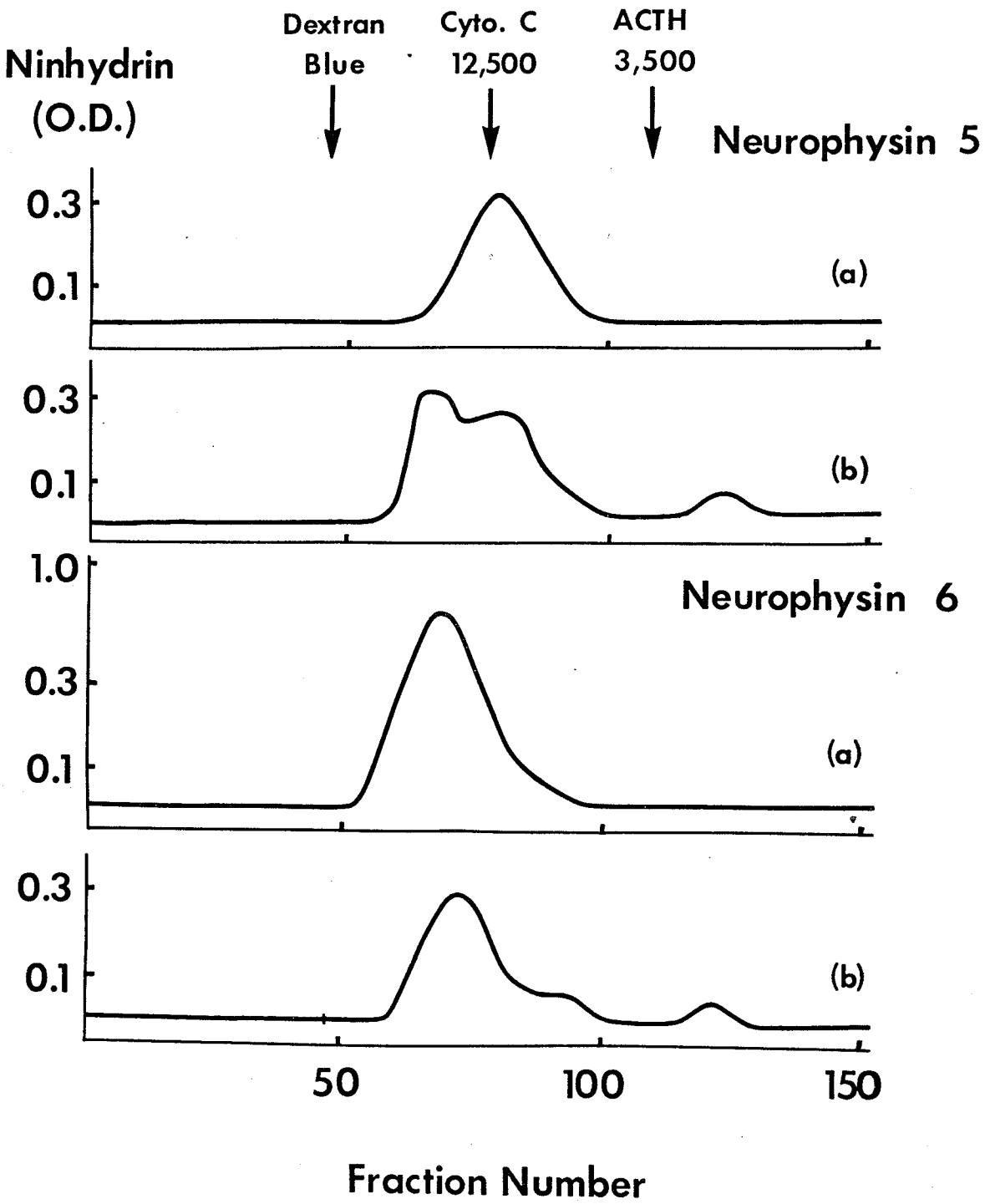
taken to avoid contamination from adjacent peaks. There was no significant cross contamination among the fractions, except for NP-5 and -6. Each individual NP produced several of the others (Fig. 11). NP-2, for example, produced NPs-1, -4, -5 and -6. When a small amount of "total NP" was chromatographed on Dowex 1-X2, a broad small peak, or sometimes an undetectable peak, of NP-3, resulted. NP-3 which was far removed from NP-2 and -4, produced NP-2, -5 and -6, and itself had disappeared upon rechromatography on Dowex 1-X2 (Fig. 11). NP-1, -2, -3, -4, -5, and -6 gave mol wt of 8,500, 9,500, 8,500, 10,500, 9,500 and 15,000, respectively, as determined by gel filtration. The mol wts for the NPs were not changed by concentration to dryness or by other mild treatment such as changing of pH.

(b) Dissociation by choline

"Total NP" was eluted as one symmetrical peak on Sephadex G-50 gel filtration with 0.2M acetic acid but was resolved into 4 apparent, incompletely resolved peaks when subsequently equilibrated and eluted with acetic acid containing 0.1M choline (Fig. 9). The mol wt of the first peak was estimated to be 15,000 and the more retarded peaks were successively smaller and ranged approximately from 15,000 to less than 4,000 mol wt. Individual NPs were also eluted with 0.1M choline on Sephadex G-50 column. NP-1, NP-2, NP-3, NP-4 were


Ninhydrin
(O.D.)
Dextran
Blue
Cyto. C
12,500
ACTH
3,500
Neurophysin 1
0.3
0.1
(a)
0.3
0.1
(b)
Neurophysin 2
0.3
0.1
(a)
0.3
0.1
(b)
50
100
150
Fraction Number


Ninhydrin
(O.D.)
Dextran
Blue
Cyto. C
12,500
ACTH
3,500
Neurophysin 3
0.3
0.1
(a)
0.3
0.1
(b)
Neurophysin 4
0.3
0.1
(a)
0.3
0.1
(b)
50
100
150
Fraction Number


Dextran Blue
Cyto. C 12,500
ACTH 3,500
Ninhydrin (O.D.)
Neurophysin 5
0.3
0.1
(a)
0.3
0.1
(b)
1.0
Neurophysin 6
0.3
0.1
(a)
0.3
0.1
(b)
50
100
150
Fraction Number

dissociated into two components, protein and small peptides. NP-5 and NP-6 showed two protein and one small peptide components. Dissociations of individual NPs are shown in Fig. 12.

(c) Effect of sodium dodecylsulfate

Electrophoresis in SDS-polyacrylamide gel was carried out on 20 per cent gel. The NP sample was pre-incubated in the electrophoresis buffer for 24 hrs before application. The middle band of 3 bands was estimated to be of molecular weight 12,000 and the faster migrating component of lower mol wt (Fig. 13) according to the criteria of Shapiro et al. (1967). However, it was difficult to estimate molecular size of the smallest component, since standard proteins of mol wt as low as 3,500 would not migrate further than the fastest migrating NP fractions.

(d) Amino acid composition

The amino acid compositions of the various individual NPs are shown in Table IV. The amino acid compositions of individual NPs differed from each other, and in general the content of acidic amino acid increased and of basic amino acids decreased progressively from NP-1, to NP-6. Gly, glu, asp and cys were the most abundant amino acids (Table IV).

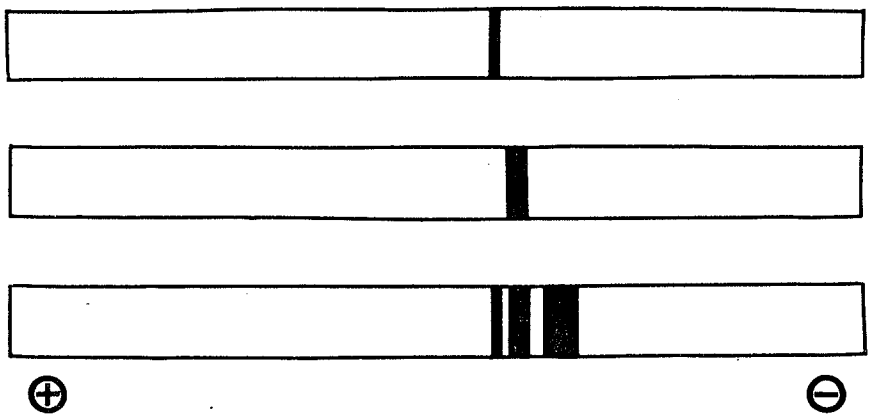


Figure 13. SDS-polyacrylamide gel electrophoresis of 100 µg of total NP previously treated for 24 hrs with SDS.

TABLE IV

	NEUROPHYSINS					
	1	2	3	4	5	6
	residues/1000 residues					
ASP	80	90	101	178	74	71
GLU	86	84	94	32	146	146
GLY	143	123	117	108	141	142
ALA	95	94	88	127	68	96
VAL	49	55	50	28	41	29
LEU	38	51	21	5	66	66
ILE	15	21	70	104	19	18
PRO	77	52	59	50	84	97
LYS	63	69	60	25	28	23
HIS	10	13	12	17	5	7
ARG	77	56	65	52	70	54
CYS*	79	89	75	105	123	145
SER	101	96	92	103	72	61
THR	37	54	51	48	27	23
TYR	16	16	13	6	10	10
PHE	33	39	34	14	28	28

*Probably underestimates in these acid hydrolysates

Hormone-binding ability of the neurophysins

(a) Qualitative assay

A qualitative test for OT binding to NP was carried out on Sephadex G-50 with 0.2M pyridine-acetate buffer, pH 5.8. NP-2, -5 and -6 were examined. Two discrete regions of oxytocic activity were evident in the effluent for all 3 NPs, one coincided with the protein peak and the other was more retarded. These results showed that most OT was in a protein bound form with NP and a trace amount of OT was free.

(b) Quantitative assay

Quantitative estimation of binding was carried out by equilibrium dialysis. In our system the half-equilibrium time was 130 min (Fig. 14). The maximum binding capacity for each of the 6 NPs was estimated to be about 100 U/mg protein (Table V).

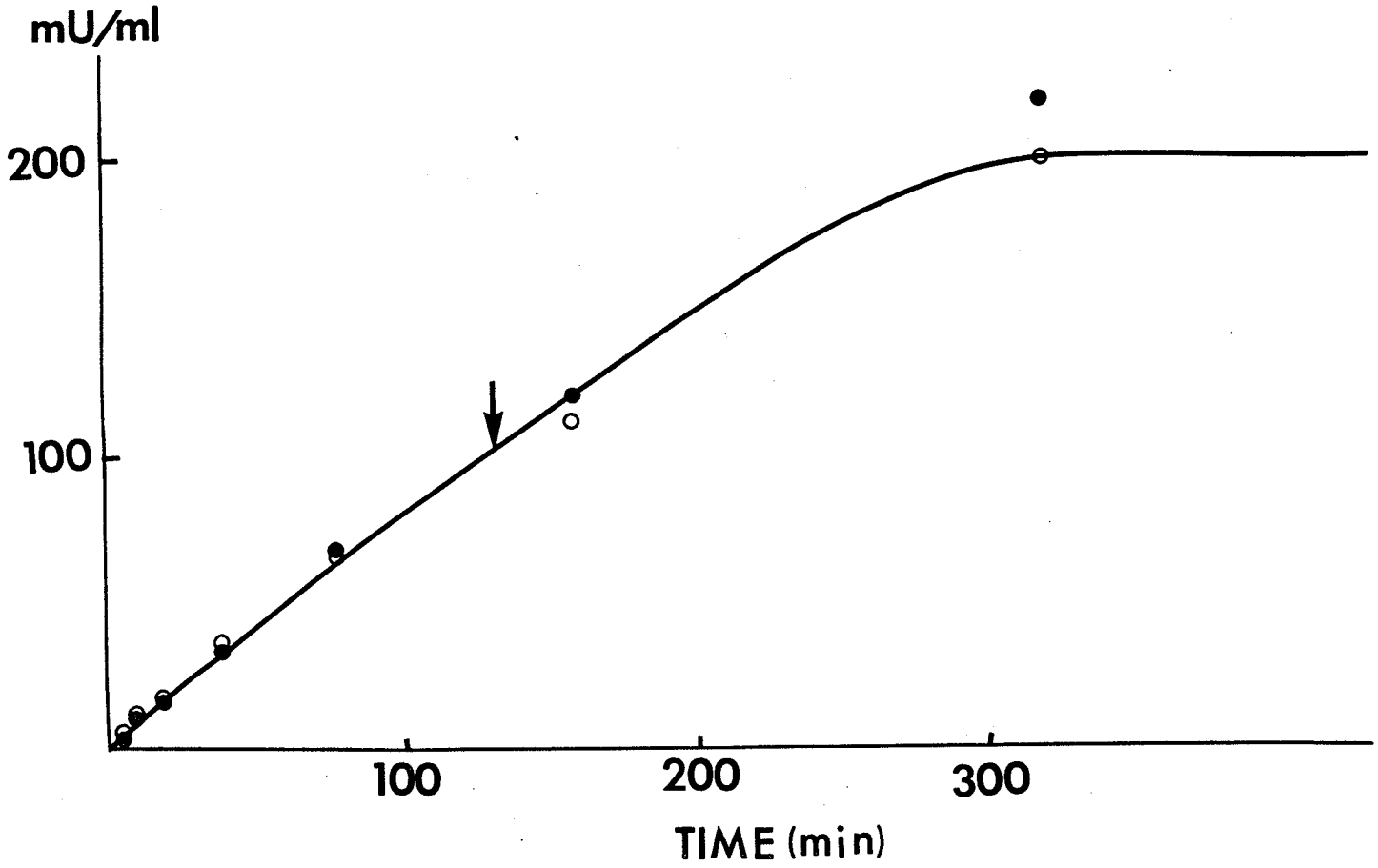

mU/ml
200
100
100
200
300
TIME (min)

TABLE V

BINDING OF OXYTOCIN (OT) TO NEUROPHYSINS (NP)
AS DETERMINED BY EQUILIBRIUM DIALYSIS

		Neurophysin					
		1	2	3	4	5	6
µg NP	U OT	OT (mU/ml)*					
10	0.25	2.6	2.3	2.2	2.6	2.6	1.0
10	0.50	5.0	4.0	4.0	4.0	5.0	1.6
10	1.00	10.0	8.0	11.0	10.0	8.0	4.0
100	2.5	26	22	23	25	20	15
100	5.0	52	30	50	50	40	40
100	10.0	80	80	100	80	80	80
Calculated Maximum binding (U of OT/mg NP)		111	111	98	105	111	109

*Values are the concentrations of OT in the diffusate at half-equilibrium

DISCUSSION

A remarkably large concentration and variety of peptides are present in the posterior pituitary (Winnick et al., 1955), much of them associated with subcellular particulates. The amino acid composition of NSGs is very similar to that of the van Dyke protein and it has been proposed that the NSG is a package of van Dyke protein (LaBella et al., 1967). It was also suggested that OT and VP are stored in separate granules and separate neurons. (LaBella et al., 1963; Bindler et al., 1967). Furthermore, Dean et al. (1968) suggested that one, of what they believed to be the major NPs, NP-I and OT are stored together in one NSG and, another, NP-II, and arginine-VP in another NSG.

The number of components previously reported from neurophysin preparations.

The name neurophysin originally designated what was believed to be a single protein (Acher et al., 1956; Chauvet et al., 1960) whose only known physiological role is to bind the neurohypophysial hormones. Later, several different NPs, as far as mol wt was concerned, were reported and, thus, NP became a common name for a family of related proteins. Ginsburg and coworkers believed the mol wt of bovine and porcine NP to be 25,000 (Ginsburg and Ireland, 1965; Ginsburg et al. 1966). Also, NP-M with mol wt 23,000 (Hope and Hollen-

berg, 1966), and a later revised mol wt of 20,000 (Hollenberg and Hope, 1967), was isolated. A year later Hollenberg and Hope (1968) isolated NP-I (mol wt, 19,000) and NP-II (mol wt, 21,000) which were supposedly not attacked by cathepsin, since they had extracted acetone-dried posterior pituitary powder with 0.1 N hydrochloric acid with presumably destroyed activity of cathepsin. These presumed native NPs (Dean and Hope, 1966; Dean and Hope, 1967; Dean et al., 1967; Hollenberg and Hope, 1968; Dean and Hope, 1968) are somewhat smaller than NP-M. Pickering (1968) quoted Thomas' unpublished observation of a mol wt of 13,500, suggesting that the mol wt of 25,000 for Ginsburg's porcine NP could be a result of the aggregation of two molecules of mol wt around 13,500. He further stated a mol wt of 14,000 for cod NP. The lowest mol wt reported for a NP is 9170 for a peptide from porcine pituitary powder that has ability to bind VP and OT (Wuu and Saffran, 1969).

Since the van Dyke protein appears to be homogeneous with respect to van Dyke's criteria of constant solubility, sedimentation, electrophoretic mobility and consistent biological activity, NP originating from van Dyke protein should be the same homogeneous protein molecule. NP varies not only in reported mol wt, but several authors have also shown by ion-exchange chromato-

graphy the protein to consist of several components which bind neurohypophysial hormones (Breslow and Abrash, 1966; Hollenberg and Hope, 1967; Pickering, 1968; Hollenberg and Hope, 1968; Rauch et al., 1968).

Evidence for subunit structure of neurophysin

We have used subcellular granules of posterior pituitary as a source of NP to minimize co-extraction of other proteins and to avoid contact with enzymes. Since the extraction of NP from subcellular granules was carried out at 4^{o}, the covalent peptide bonds of our "total NP" are not believed to be attacked by cellular proteinases. On electrophoresis, "total NP" produced 2 major bands, which may be identical to the "native" NPs, NP-I and -II isolated by Hollenberg and Hope (1968). "Total NP" was fractionated into 6 components by Dowex 1-X2 and rechromatography of one component produced several of the others. Since the quaternary ammonium group of Dowex 1-X2 was believed to be responsible for dissociation of subunits, a solution containing choline, a quaternary ammonium compound, was used in an attempt to dissociate NP subunits during gel filtration. The homogeneous "total NP" in mol wt was resolved into 4 peaks, ranging in mol wts from 15,000 to less than 4,000. Indications of a subunit system were further demonstrated using SDS-acrylamide gel electrophoresis which showed three components, presumably

of different molecular size, from "total NP", homogeneous in molecular size. The NP subunit system is not believed to be a simple monomer-dimer or monomer-polymer relationship, since all 6 NPs differed in amino acid composition, and mol wt differences of associated and dissociated components are not single multiples of one another.

Significance of multiple neurophysins

Our results strongly suggest that several dissimilar subunits are present, because all 6 NPs have different amino acid compositions. The wide variety of mol wt (8,500-15,000) of the various NP isolated by anion exchange chromatography led us to propose that these dissimilar subunits aggregate, dissociate, and form more physicochemically stable NPs. Among our six NPs, NP-3 was the most unstable form, since it is not produced from the other NPs and is not detected if only a small amount of the original "total NP" is applied to the anion-exchange column. Furthermore, NP-3 had almost completely disappeared when it was rechromatographed. On the other hand, NP-6 was the most stable or firmly aggregated form; it was most readily produced from others, and was more abundant the longer the total NP was stored. We have interpreted previous apparently discrepant findings of others concerning the number and mol wts of NPs on the basis of protein subunit interaction leading to association and dissociation.

There are at least two different kinds of NSGs as determined by density-gradient centrifugation (LaBella et al., 1963) and the major component of the granules is NP. It was also shown that at least two different native NPs exist in the different granules (Dean et al., 1968). Neurohypophysial hormones are believed to be a cleavage product of a precursor protein, and coincidentally NP molecule will be produced (Sachs and Takabatake, 1964), to which the hormones are bound non-covalently. Since NP-1 and NP-2 bind different hormones (Dean et al., 1968), other unknown peptide hormones in posterior pituitary might be stored with the other forms of NP. Because a given stimulus selectively releases one of NP bound hormones, it is difficult to explain selectivity only on the basis of the effect of essential role of calcium ion, (Douglas and Poisner, 1964) but rearrangement of NP subunit system by quaternary ammonium compound, acetylcholine in situ might explain the selective release of hormone from its bound state with NP.

Binding of oxytocin and vasopressin by various neurophysins

The original van Dyke protein (mol wt about 30,000) is composed of one mole each of OT, arginine-VP, and NP (van Dyke et al., 1942). Ginsburg and Ireland (1965) calculated that one mole of NP (mol wt 25,000) binds 7 moles of OT or 4 moles of arginine-VP. However, Breslow and Abrash (1966) claimed the maximum binding to

be 2 moles of OT on the basis of a mol wt of 25,000 for NP and that the two binding sites on NP competed for NP and OT. If the affinities of OT and VP for binding sites are equal, the ratio of neurohypophysial hormones and NP would agree well with van Dyke protein. Three binding sites on NP-I (mol wt 19,000) and NP-II (mol wt 21,000) (Hollenberg and Hope, 1968) were reported. Furthermore, these 3 binding sites of NP-I are said to have greater affinity for OT. NP-II bound only 2 moles of either OT or VP, but, in the presence of both hormones, NP-II formed a complex with two moles of OT plus one mole of VP. Hollenberg and Hope (1968) also suggested that each of the two binding sites was specific for one hormone and that the third site could bind either hormone but had more affinity for OT.

Porcine NP (mol wt 25,000) showed a marked species difference due to its ability to bind 11-12 moles of OT according to Ginsburg et al. (1966). Cod NP (mol wt 14,000) has a slightly larger binding ability than Hollenberg's "native" NPs on protein weight basis (Pickering, 1968). Our binding experiments give values higher than those reported by Hollenberg and Hope (1968) for NP-I and NP-II and much lower than Ginsburg's NP. There are large mol wt differences in our 6 NPs, from 8,500 to 15,000, but no significant differences of OT binding among individual NPs and "total NP" on protein weight

basis. OT binding ability of our NPs is estimated to be 1 mole OT per 5,000 g NP.

V. SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Fresh bovine posterior pituitary glands were homogenized and the total particulates isolated by differential centrifugation. The subcellular particulates were used as starting material for extraction with 0.2M acetic acid and subsequent fractionation of the extract.

1). A large proportion of the total glandular pool of peptides and amino acids was associated with the sedimentable fraction of the bovine posterior pituitary homogenate. Approximately the same proportion of total liver peptides and amino acids was associated with the particulate fraction. In the pH range 6.0 to 7.4 the binding of peptides and amino acids to subcellular particulates was constant.

2). Ion-exchange chromatography on Dowex 1-X2 and 50W-X2, successively, resolved the small peptide fraction, molecular weight (mol wt) <2000, into 22 major components: lysine, arginine, phenylalanine, ammonia, and 18 peptides. Three of the peptides contained only aspartic and glutamic acids in the ratios 8:1, 5:1 and 4:1. The sequence of 4 dipeptides were ascertained as alanyl-arginine, lysyl-glutamate, aspartyl-glutamate and glutamyl-aspartate. Another component was not retarded by Dowex 50 and yielded only glutamic acid upon acid hydrolysis. Still another peptide yielded tyrosine

plus an unknown ninhydrin positive component after acid hydrolysis. Each peptide fraction was assayed for homogeneity and amino acid composition, and many were shown to be essentially pure.

3). A neurophysin (NP) preparation was chromatographed on Sephadex G-25 with 0.2M acetic acid. The excluded fraction was chromatographed on Sephadex G-75 with 1M acetic acid, and gave a retarded symmetrical peak of estimated average mol wt of 15,000. This was the "total NP" fraction. In the presence of 0.1M choline, total NP was resolved into 4 peaks on Sephadex G-50 ranging in mol wt from 15,000 to less than 4,000. Indications of protein subunits in total NP were further provided by using SDS-polyacrylamide gel electrophoresis which showed three components of differing molecular size.

4). Freshly prepared NP was resolved by ion-exchange chromatography on Dowex 1-X2 into six fractions with mol wts of 8,500, 9,500, 8,500, 10,500, 9,500 and 15,000, as determined by gel filtration. The amino acid compositions of individual NPs differed from each other, and the content of acidic amino acids increased and of basic amino acids decreased progressively from NP-1 to NP-6. Glu, gly, asp and cys were the most abundant amino acids.

5). Each individual NP produced several of the others upon rechromatography on Dowex 1-X2. NP-1, for example, produced NP-2 and -6. Among the six NPs, NP-3 was the most unstable form, since it was not produced in significant amounts from the other NPs and was not detected if only a small amount of the original total NP was applied to the Dowex 1-X2 column. Furthermore, NP-3 almost completely disappeared, giving size to other NPs, when rechromatographed. On the other hand, NP-6 was the most stable and apparently the most firmly aggregated form, since it was most readily produced from the other NPs and was more abundant the longer the total NP was stored.

6). A qualitative test for oxytocin binding to NP was carried out on gel filtration. Two discrete regions of oxytocic activity were evident in the effluent for the 3 individual NPs tested, with most of the oxytocin bound to NP. Equilibrium dialysis was used to quantitate binding of oxytocin to NP, and the maximum binding capacity of each of the six NPs was estimated to be about 100 U of oxytocin/mg protein.

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