PEPTIDES OF THE HYPOTHALAMUS

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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Montreal, Quebec.

March, 1965

ACKNOWLEDGEMENTS

I wish to express my deepest gratitude to Dr. M. Saffran for his able direction and continued advice and encouragement during the entire course of these investigations.

I am indebted to Dr. C.F. MacPherson and Dr. L. Wolfe for stimulating discussions on certain aspects of this study.

Thanks are due to Dr. B. Rose for the use of facilities of his laboratory in running the ultracentrifuge experiment.

The valuable technical assistance of Mrs. B.U. Caplan and Mr. H. Pitt is gratefully acknowledged.

I would like to thank my friends for criticizing the manuscript. I would also like to thank all the members of the Research Laboratory for their help and co-operation.

Thanks are due to Miss Ann Mylchreest for her excellent typing of the thesis.

This Research Project was supported by grants from the National Institute of Health (NB 3380) and the Medical Research Council (MA 1322) to Dr. M. Saffran.

The author is indebted to the Faculty of Medicine for an award of a Charles Hubert Kinch Fellowship during the session 1963-64.

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INTRODUCTION

1. The Hypothalamus

(a) Anatomy of the hypothalamus

The hypothalamus is that part of the forebrain which forms the floor of the third ventricle in immediate topographical relation with the pituitary gland (1). The limiting boundary of the hypothalamus is ill-defined. It extends, without any sharp line of demarcation, into the parolfactory region of the telencephalon anteriorly and into the tegmental part of the mid-brain posteriorly. Laterally, the hypothalamus is directly continuous with the subthalamus (1).

For the purpose of description, the mammalian hypothalamus may be conveniently subdivided into three regions: the pars supra-optica, the tuber cinereum and the pars mamillaris. Each of these regions contains groups of nerve cell bodies, the nuclei. These nuclei are defined by their spatial positions and by their relation to fiber tracts which connect them. Among the multitude of hypothalamic nuclei mention can be made of the following: 1. Supraoptic nucleus, 2. Paraventricular nucleus, 3. Dorso-medial- and Ventro-medial hypothalamic nuclei, 4. Posterior hypothalamic nucleus, and 5. Lateral hypothalamic nucleus. Most of the hypothalamic nuclei receive nerve fibers from the medial forebrain bundle which runs in an anterio-posterior direction (1). Apart from interconnections among the nuclei, the hypothalamus has extensive connections with other parts of

the brain and with all sensory afferent pathways (2,3,4).

(b) Physiological functions of the hypothalamus

The hypothalamus is involved in complex functions of integrating somatomotor and visceromotor activities for the maintenance of a constant internal environment. In this respect, the physiological functions of the hypothalamus are of "autonomic" type. The hypothalamus is known to control such activities as vasodilation (5), vasoconstriction (6), heart rate (7), blood pressure (8), piloerection (9), shivering (10), sweat secretion (11), panting (12), gastro-intestinal tone (13), bladder tone (14), micturition (15), water metabolism (16), appetite (17), sleep-wakefulness (18) and respiration (19). Besides homeostatic functions, the hypothalamus has enormous influences over the secretion of endocrine glands, particularly the pituitary gland. The possible involvement of the hypothalamus in regulating aldosterone secretion has been indicated (20). The secretion of epinephrine and norepinephrine from the adrenals has also been shown to be controlled by this part of the brain (21,22).

2. The Posterior Pituitary Gland

(a) Anatomy of the neurohypophysis

The neurohypophysis or the posterior lobe of the pituitary gland is developed as an outgrowth from the floor of the third ventricle (1). The pituitary stalk is attached to the base of the hypothalamus in the region of the tuber cinereum. The actual

connections between the hypophysis and the hypothalamus are (a) nervous pathways, (b) vascular channels, and (c) non-vascular channels (23). The nerve-fibers passing into the posterior pituitary arise from the following nuclei: a) Supraoptic nucleus, b) Paraventricular nucleus, c) Suprachiasmatic nucleus, and d) Lateral hypothalamic nucleus (23). The neurohypophysis is thus abundant in nerve-fibers and nerve-endings (24). Perhaps the best description of the neurohypophysis in mammals includes (a) a group of hypothalamic specialized nuclei, (b) the median eminence, (c) the neurohypophysial tract, and (c) the neural lobe proper (25,26).

(b) The posterior pituitary hormones

Kamm et al. (27) separated posterior pituitary extracts into two fractions - pitocin and pitressin, containing oxytocic and pressor activities respectively. Du Vigneaud and his colleagues isolated oxytocin (28) and vasopressin (29) in the pure form. Both oxytocin and vasopressin are cystine-containing octapeptide amides(30).

(c) Neurosecretion in relation to the posterior pituitary hormones

The origin of the posterior pituitary hormones was first ascribed to the pituicytes (31). It soon became evident that the abundant nerve-fibers were responsible for the secretory activities of the posterior lobe. Bargmann (32) demonstrated granular inclusions in the hypothalamic nuclei and all over the neurosecretory neurons, from their cell bodies in the hypothalamus down to the axon endings in

the posterior lobe. He was able to show a continuous process from the supraoptic and paraventricular nuclei to the posterior pituitary, known as the supraoptico-hypophysial tract. Hild (33) observed that the neurosecretory granules originating in the perikarya were transported by means of axoplasmic flow via the supraoptico-hypophysial tract into the posterior pituitary.

The neurosecretory granules have been found closely associated with the posterior pituitary hormones. The hormone content of the supraoptic and paraventricular nuclei, the supraoptico-hypophysial tract and the neurohypophysis parallels, in general, the amount of stainable granules (34). The neurosecretory granules specifically stain for cystine (35), which also suggests their correlation with the cystine-containing hormones of the posterior pituitary. Although the neurosecretory granules are stained by the Gomori method, the pure hormones are not (36). It is likely that the stain colors the protein to which the hormone peptides are bound. The stainable carrier substances can be dissociated from the active peptides by extraction with alcohol or acetone, in which the granules are easily dissolved (34). According to the neurosecretory theory, the posterior lobe hormones are produced in the supraoptic and paraventricular nuclei of the hypothalamus and are carried down with the granules to the posterior pituitary gland for storage and release (37).

(d) Chemical nature of the neurosecretory granules

Histochemical studies showed the neurosecretory material to

be a carbohydrate-lipid-protein complex (38). A different opinion was observed by Howe et al. (39), suggesting that the neurosecretory substance is a protein or polypeptide and contains no polysaccharide, lipid or nucleic acid components. In 1942, Van Dyke and his associates (40) reported the isolation of a protein from the posterior pituitary gland, which had equivalent amounts of oxytocic and pressor activities. This protein was later called neurophysine by Acher et al. (41), and shown to be a complex of one molecule each of oxytocin and vasopressin per molecule of the protein, held together by adsorption or by electrostatic forces. The hormone molecules can be dissociated from the biologically inactive protein by relatively mild treatments. Neurophysine gives the same staining reaction, even with the hormones removed, as the neurosecretory material and is probably the substance constituting the Gomori-positive granules (42). Recently, Sachs (43) isolated labeled vasopressin from the hypothalamo-median eminence complex of the dog, after intraventricular infusion of labeled cysteine. The hormone was found to be associated with the neurosecretory particles. It appears, therefore, that the posterior pituitary hormones are produced in the hypothalamus in close association with the neurosecretory particles. Furthermore, there is evidence (44) that the paraventricular nucleus is chiefly engaged in synthesizing oxytocin.

3. The Anterior Pituitary Gland

(a) Anatomy of the adenohypophysis

The adenohypophysis is anatomically divided into three parts:

the pars tuberalis, the pars intermedia and the pars distalis (45). The pars tuberalis is in the immediate vicinity of the hypothalamus. This is the highly vascular part of the adenohypophysis and receives nerve fibers from the hypothalamo-hypophysial tract which are in close association with the blood-vessels. The pars distalis receives very few, if any, nerve fibers (45). While the hypothalamus is directly connected to the neurohypophysis by well-defined nerve fibers, there appear to be no such nervous connections to the adenohypophysis (45). But there exists a network of fine blood vessels in the hypothalamic area which constitutes the hypothalamo-hypophysial portal system, first described by Popa and Fielding (46). Blood collected in the hypothalamic region flows down to the adenohypophysis through this portal circulation.

(b) The anterior pituitary hormones

The adenohypophysis elaborates a number of hormones. At least six well-defined hormones have been isolated from this gland (47). They include 1. adrenocorticotropic hormone (ACTH), 2. thyrotropic hormone (TSH), 3. follicle stimulating hormone (FSH), 4. luteinizing hormone (LH) or interstitial cell stimulating hormone (ICSH), 5. lactogenic or luteotropic hormone (LTH, prolactin) and 6. growth hormone or somatotropic hormone (STH). The pars intermedia elaborates intermedin or the melanocyte-stimulating hormone (MSH). All of the adenohypophysial hormones are proteins or polypeptides. Some of them have been further characterized as glycoproteins. Prolactin, growth

hormone and ACTH are known to be simple proteins, whereas FSH, LH and TSH fall into the category of glycoproteins. Only in the cases of ACTH and MSH are the complete structures known (48).

4. Hypothalamic Control of Release of Adenohypophysial Hormones

Electrical stimulations of the hypothalamus caused an increased discharge of gonadotropic hormones (49,50) and ACTH (51), but not by stimulations of the pituitary gland directly. The effect of stress on the release of ACTH could be eliminated by sectioning of the pituitary stalk (52). Transplantation of the anterior pituitary to a site situated at a distance from the sella turcica resulted in a marked diminution in the anterior pituitary activities (53). The above facts and several others led Harris (52,45) to put forward the theory of neural control of the anterior pituitary. The great paucity of nerve fibers in the pars distalis and the lack of evidence for a nervous link between the hypothalamus and the adenohypophysis made it seem unlikely that the hypothalamic nerve control was mediated by a direct nerve supply. On the other hand, the hypothalamus is connected to the adenohypophysis by a portal system of blood vessels. According to Harris, the hypothalamus controls the anterior pituitary by a humoral mediator which is carried to the gland by the portal circulation.

Studies involving electrical stimulation or lesion in the hypothalamic area, or stalk section have definitely proved that the secretion of gonadotropins (49,50,54,55), TSH (56,57) and ACTH (51,58,

59,60,61) is regulated by hypothalamic humors. There is also evidence that the hypothalamus is in some way involved in the secretion of growth hormone (45). In fact, it appears that each adenohypophysial hormone is presumably controlled by specific hypothalamic humor, elaborated in response to proper nervous stimuli (62).

5. Biologically Active Substances of the Hypothalamus Other than the Adenohypophysial Hormone Releasing Factors

In view of its important role in diverse physiological functions, the hypothalamus has been searched for the presence of known physiologically active compounds. Acetylcholine (63), adrenalin and noradrenalin (64), histamine (65), serotonin (66) and substance P (67) have been found in higher concentrations in the hypothalamus than in any other parts of the brain. Vogt (68) demonstrated the presence of pressor, antidiuretic and oxytocic activities in ethanol-HCl extracts of the hypothalamus. The existence of oxytocin and vasopressin in the hypothalamus is now well established (34,43,44).

Acetylcholine, adrenalin, noradrenalin and serotonin have been described by Welsh (69) as neurohumors. They have short range activities on other neurons and are not released into the circulation to act on specific target organs. On the contrary, the chemical mediators or humoral agents of the hypothalamus, according to Harris, are secreted into the portal circulation to exert their activities in the anterior pituitary gland.

6. The Adenohypophysial Hormone Releasing Factors

(a) Corticotropin releasing factor (CRF)

One of the early studies devoted to the demonstration of a hypothalamic principle which regulates ACTH secretion was that of Slusher and Roberts (70). They were able to isolate a lipid or lipoprotein which stimulated the release of ACTH in the intact animal, but their work was criticized for nonspecificity of the test system. With the development of an *in vitro* method of ACTH assay, Saffran and Schally (71) found that the hypothalamic tissue in the presence of epinephrine stimulated the release of ACTH from the anterior pituitary gland. Guillemin and collaborators (72), employing an organ culture technique, demonstrated that the pituitary *in vitro* was unable by itself to release ACTH for any significant length of time, but ACTH secretion was considerably increased in amount and duration by the addition of hypothalamic extracts or explants to the culture medium.

Vasopressin, because of its ability to release ACTH *in vitro* and *in vivo*, was suggested as the natural agent regulating ACTH secretion (73,74). However, Saffran *et al.* (75) isolated an active principle from the posterior pituitary extract, which after separation from vasopressin by paper chromatography still contained the ACTH releasing activity. They named this compound corticotropin releasing factor (CRF). The hypothalamic CRF activity could be dissociated from the antidiuretic activity and was shown not to be due to vasopressin (76). Royce and Sayers (77) demonstrated that the CRF activity of an acid extract of the hypothalamus was lost after treatment with the proteolytic

enzyme, pepsin. They concluded that this pepsin-labile compound was distinct from ACTH and vasopressin. Guillemin et al. (78) isolated a substance with CRF activity from the hypothalamus and also from the posterior pituitary gland, and demonstrated it to be a small peptide different from oxytocin, vasopressin and ACTH.

The peptidic nature of the neurohypophysial CRF was demonstrated by Schally et al. (79). Two distinct peptides with CRF activity have been isolated from hog posterior pituitary extracts (80). Both of them are very basic. The one associated with the α-MSH-area in the countercurrent distribution pattern has been designated α-CRF, while the one associated with the vasopressin peak is known as β-CRF. The structure of β-CRF has been reported by Schally et al. (81). It has unique resemblance with α-MSH at the amino-terminal and with vasopressin in other parts of the molecule. Still another CRF, designated α_2-CRF, has been isolated from the hog posterior pituitary gland (82). It has identical amino acid composition and sequence to those of α-MSH, with a probable difference at the N-terminal. α_2-CRF may be an artifact resulting from α-MSH during isolation procedures (82). The posterior pituitary is now known to contain four different peptides which specifically release ACTH; they are vasopressin, α-CRF, β-CRF and α_2-CRF, all of them are isolated and characterized in full or in part (83).

Although a considerable number of studies has demonstrated the presence of CRF-activities in the hypothalamic extracts, very little is known about the chemical nature of hypothalamic CRF.

Fractionation of hypothalamic extracts by precipitation with ether and chromatography on ion exchange cellulose revealed the presence of oxytocin, vasopressin, α- and β-MSH, β-CRF, ACTH and ACTH-like peptides (84,85). It has also been shown that a CRF preparation from sheep hypothalamus can be resolved by countercurrent distribution into two active zones corresponding to those of α- and β-CRF of the posterior pituitary (86). Whether the hypothalamic corticotropin releasing factors are different from those of the posterior pituitary remains to be seen.

(b) Luteinizing hormone releasing factor (LHRF, LRF)

Much evidence has now been accumulated to confirm the presence of luteinizing hormone releasing factor(s) in the hypothalamus. McCann et al. (87) reported that the rat median eminence extract contained a substance with LH-releasing activity and demonstrated this material to be different from histamine, serotonin, substance P, epinephrine, vasopressin and oxytocin. Nikitovitch-Winer (88) showed that a direct intrapituitary infusion of the median eminence extract induced ovulation in rats. The presence of LHRF in sheep hypothalamic extracts has been reported by Johnson (89). Guillemin et al. (90) have reported the first partial purification of the LHRF-activity from sheep hypothalamic extracts. The biological activity is associated with a fraction of low molecular weight, heat-stable polypeptides and the activity is lost by digestion with trypsin or pepsin or by acid hydrolysis (91).

(c) Thyrotropic hormone releasing factor (TRF)

The secretion of thyrotropin (TSH) has been demonstrated to be under the control of the hypothalamus (92,93). Schreiber et al. (94) reported the isolation of TRF from bovine hypothalamus. The activity has been found associated with a principle which activates adenohypophysial acid phosphatase (95). Acid hydrolysis of the most active preparation showed the presence of at least five different amino acids (95).

(d) Growth hormone releasing factor (STH-RF, SRF)

The presence of a growth hormone releasing factor (SRF) in dog hypothalamic extracts, different from vasopressin, has been demonstrated by Franz et al. (96). Dauben and Meites (97) have shown that rat hypothalamic extracts contain a substance which significantly increases the release of growth hormone from rat adenohypophysis in tissue culture experiments. The factor responsible for causing release of STH is not destroyed by boiling. The chemical nature of SRF is not known.

Although none of the hypothalamic factors has been purified yet to the extent to be characterized fully, it appears that these release factors are peptides of relatively small size.

7. The Nature of the Present Work

The hypothalamus is now known to contain several low molecular

weight peptides with biological activities, which apparently constitute a small fraction of the total peptide content of hypothalamic extracts (84,85). The nature of the major portion of hypothalamic peptides is unknown. Previous work on the hypothalamus has been devoted to the demonstration and isolation of only the biologically active peptides. A systematic study of other peptidic materials has been overlooked so far.

The object of the present study is to isolate and characterize the major peptides in hypothalamic extracts, irrespective of their biological activities. Such a study would require the utilization of modern techniques of isolation and fractionation, applicable in the field of peptides and proteins. The following few pages contain a general consideration of peptide and protein chemistry, with respect to the methods of isolation and characterization.

8. General Characteristics of Peptides and Proteins

(a) L-amino acids

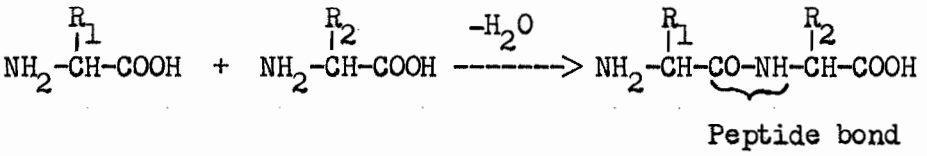
The fundamental units of peptides and proteins are α-amino acids, the general formula of which can be written as $R{-}\underset{\displaystyle NH_2}{\underset{|}{C}}H{-}COOH$, where R stands for a variety of structures. With the introduction of an amino group, the α-carbon becomes asymmetric, giving rise to stereoisomerism and optical properties (98). Except for glycine, all naturally occurring amino acids found in proteins are optically active and are of the L-configuration. Although there is no evidence for the existence of D-amino acids in proteins (99), some poly-

peptide antibiotics of bacterial origin contain both D- and L-amino acids (100).

Vickery and Schmidt (101) proposed a list of 22 different amino acids found in protein hydrolysates. This figure has undergone several modifications with the inclusion of newly discovered amino acids and with the omission of those of uncommon existence. A list of commonly occurring natural amino acids will be given below.

(b) The peptide bond

In 1902, Emil Fischer and Franz Hofmeister independently suggested the peptide bond hypothesis of protein structure. According to this, the α-amino group (or α-imino group in the case of proline) of one amino acid is linked to the α-carboxyl group of another amino acid, with the elimination of water, to form an amide linkage between them, known as the peptide bond:



Amino acids can join at either end of a peptide to form a long polypeptide chain. Each amino acid in a peptide loses either an H or an OH or both and thus becomes a "residue".

(c) Formulation of peptides

It is customary to write the formula of a peptide starting from the residue bearing a free α-amino group. Those residues whose

carboxyl groups form the peptide bonds are considered as radicals and bear a suffix -yl, for example, Alanylglycylvaline (99,102). Brand and Edsall (103) proposed the designation of the amino acid residues by the first three letters of their conventional names. By adding to these residue symbols a numerical coefficient equal to the number of residues per molecule, it is possible to write empirical formulae of proteins, e.g., $Ala_{30}Gly_{29}Pro_6$ .... etc. The residue having a free α-amino group is known as the N-terminal residue and the one bearing a free α-carboxyl group is called the C-terminal residue (104). The sequence, or in other words, the relative positions of the amino acid residues in a peptide, is expressed by joining the symbols with a period or hyphen, starting from the N-terminal and ending with the C-terminal residue. When the sequence is not known, the residues are separated by the comma (104). Often, for the sake of clarity, the N-terminal and C-terminal residues are written by adding NH_2- and -COOH to the respective residues. When the terminals are substituted, they are expressed likewise, e.g., Acetyl-Ser-Tyr-.... $GlyCONH_2$.

The following is a list of α-amino acids generally found in proteins with their residue names and symbols.

Amino acid	Residue name	Residue symbol
1. Alanine	Alanyl	Ala
2. Arginine	Arginyl	Arg
3. Aspartic acid	Aspartyl	Asp
4. Asparagine	Asparaginyl	$Asp(NH_2)$, Asn
5. Cysteine (Half-cystine)	Cysteinyl	CySH

Amino acid	Residue name	Residue symbol
6. Glutamic acid	Glutamyl	Glu
7. Glutamine	Glutaminyl	Glu(NH_2), Gln
8. Glycine	Glycyl	Gly
9. Histidine	Histidyl	His
10. Isoleucine	Isoleucyl	Ileu
11. Leucine	Leucyl	Leu
12. Lysine	Lysyl	Lys
13. Methionine	Methionyl	Met
14. Phenylalanine	Phenylalanyl	Phe
15. Proline	Prolyl	Pro
16. Serine	Seryl	Ser
17. Threonine	Threonyl	Thr
18. Tryptophan	Tryptophanyl	Try
19. Tyrosine	Tyrosyl	Tyr
20. Hydroxylysine	Hydroxylysyl	HO-Lys, Hylys
21. Hydroxyproline	Hydroxyprolyl	HO-Pro, Hypro.

(d) The peptide chain

Sanger (104) pointed out the possibility of three main types of polypeptide chains: open, cyclic and branched. In an open chain, the amino acid residues are linked in a linear array leaving a free α-amino group at one end and a free α-carboxyl group at the other end of the molecule. A cyclic chain may be derived from the formation of a peptide bond between the free terminals. Except for gramicidin (105) and tyrocidine

(106) no peptides or proteins of natural source are known to have cyclic structure in the truest sense of the term. The absence of a free α-amino group in proteins used to be taken as evidence of a cyclic structure, but this is not always true. Ovalbumin, considered a cyclic protein, has been shown to contain an acetylated N-terminal (107). Similar acetylated N-terminal residues have been found in tobacco mosaic (108), cucumber (109) and turnip yellow mosaic (110) virus proteins; bovine fibrinogen (111), α-melanocyte stimulating hormone (112) and cytochrome c (113). On the other hand, an amidated C-terminal residue occurs in oxytocin and vasopressin (30). Both the terminal residues of α-MSH (112) and β-CRF (81) are substituted. Branching of peptide chains through peptide bonds is not known, though there is a possibility of the formation of such bonds through the ϵ-NH_2 group of lysine and ω-carboxyl groups of aspartic and glutamic acids.

Peptide chains may be branched, looped or cyclized through disulfide bonds. The cyclic character of oxytocin and vasopressin is due to the disulfide bond. A disulfide bond can be formed between two distinct peptide chains or within the same chain. Insulin is an example of both inter- and intra-chain disulfide bonds (256).

(e) Peptides and proteins

Proteins are composed of one or more peptide chains. The properties exhibited by a protein are essentially the manifestation of the properties of its peptide chain or chains. From a chemical point

of view, therefore, there is not much difference between peptides and proteins, with the exception that the complexity of their properties is proportional to their molecular size.

The borderline between peptides and proteins has practically disappeared in recent years (114). In fact, proteins and peptides are being treated simultaneously nowadays and the terms are often used synonymously. Nevertheless, proteins are viewed as macromolecules and peptides as their relatively simpler counterparts. A molecule having more than one hundred amino acid residues or a molecular weight greater than ten thousand is generally considered a protein.

(f) Non-covalent bonds and denaturation of proteins

Two classes of non-covalent bonds are found in proteins: hydrogen bonds and hydrophobic bonds (115). Hydrogen bonds are formed between peptide groups, polar side chain groups or between peptides and side chain groups. Hydrophobic bonds, on the other hand, are formed between non-polar regions of the protein molecule by mutual repulsion of the aqueous solvent. Besides, there are ionic bonds which are formed between groups of opposite charges.

These non-covalent bonds determine the three dimensional structure of a protein molecule. Interactions between different parts of the molecule maintain the molecular integrity. The association of similar or dissimilar peptide chains is also due to non-covalent bonds. It has been shown that polymerization of fibrin monomers occurs through hydrogen bondings (116). There is much evidence of protein-protein

interactions and interactions between proteins and other small or large molecules.

Non-covalent bonds are weaker than covalent bonds and so their disruption may be caused by various simple means, such as changes in temperature, pH, or concentration of substances like urea, guanidine, etc. Even without any cleavage of covalent bonds, rupture of non-covalent bonds will destroy the molecular conformation of proteins. This phenomenon has been termed denaturation (117). Denaturation may be reversible or irreversible. The probability of reassuming the original configuration depends on the degree of denaturation. Cleavage of all non-covalent bonds and disulfide cross-linkages will lead to complete denaturation of proteins. Peptides, being relatively small in size, may have little or no specific three dimensional configuration and consequently are more stable towards denaturing agents.

9. Isolation and Purification of Peptides and Proteins

A. Isolation

A number of methods has been described in literature for the isolation of cellular proteins. Common to them is the phenomenon of disruption of the cell membrane. The choice of a particular method comes from the purpose of isolation and the source of the material itself. Bacterial cell membranes can be easily disrupted by mechanical grinding with sand or glass beads (118). Grinding of the frozen tissue has been found useful for the extraction of hormonal proteins from the pituitary gland (119). Dehydration by repeated freezing and thawing

is another method of disintegrating the cellular structure (122). Supersonic vibrations can be used to break cell membranes, but there is a danger of damage to sensitive peptide bonds (120). Homogenization of the tissue appears to be the most universally applicable method for the extraction of cellular components. Besides these physical methods of rupturing cell membranes, use can be made of chemical or enzymatic methods. For example, catalase has been isolated by digesting away the bacterial cell membrane with the enzyme, lysozyme (121). Organic solvents, like alcohol, acetone, and glycerol are known to destroy cell membranes and may be effectively used. Acetone is particularly useful for the dehydration of tissues. The acetone-dried tissue powder can be preserved for a long time without appreciable change in its protein composition. A great advantage of this procedure is that most of the lipids are extracted with acetone and denaturation of proteins is minimal (122).

Extraction of peptides and proteins from cellular materials largely depends on the solvent used. While most of the peptides and proteins are soluble in aqueous solvents, their solubility varies according to the ionic strength and pH of the solvent. The solubility of a protein depends on the proportion of polar hydrophilic and non-polar hydrophobic groups in the molecule and their resultant dipole moment (123). With a proper selection of the extracting solvent, the desired protein can be isolated mostly free of other interfering materials of the tissue. Butanol and chloroform have been found useful in removing lipids and denaturing other proteins in the extraction of

actin (124).

B. Fractionation

(a) Chemical methods

Solubility properties have long been used for the isolation and purification of proteins. Proteins are least soluble at their isoelectric pH and can be precipitated by the addition of neutral salts. Ammonium sulfate has been widely used for this purpose because of its high solubility in water. By repeated precipitation and solution, a certain degree of purification can be easily achieved. Crystallizable proteins may often be crystallized from their salt solutions. Thus, human hemoglobin (125), serum albumin (126), pancreatic enzymes (127) and a number of other proteins have been crystallized from ammonium sulfate solutions. Pituitary proteins have been fractionated by ammonium sulfate precipitation, in order to isolate various hormonal activities (128). A similar fractional precipitation method has been used for the isolation of papain from papya latex (129).

Water soluble organic solvents decrease the dielectric constant of aqueous solutions and thus facilitate the precipitation of proteins. With a careful control of pH, ionic strength and temperature, it has been possible to fractionate serum proteins (130) and egg white proteins (131)by employing ethanol. The use of organic solvents for fractional precipitation is well known in the isolation and purification of ACTH (132,133). Some proteins are relatively resistant to the denaturing agent, and this property can be used for

their isolation. For example, carbonic anhydrase has been isolated from erythrocytes by preferential denaturation and removal of hemoglobin with a chloroform-ethanol mixture (134).

Precipitation of proteins with trichloroacetic acid is a conventional method for the preparation of protein-free solutions. The use of trichloroacetic acid for the isolation of peptides or proteins in the natural state seems to be dangerous. Trichloroacetic acid, even in mild conditions, may hydrolyze sensitive peptide bonds. This has been definitely proved in the case of tobacco mosaic virus protein (135).

(b) Chromatography

According to the mechanism of separation, chromatography can be classified into four main groups: adsorption, partition, ion-exchange and exclusion chromatography. Before the advent of the ion-exchange technique, the only methods available to a protein chemist were those of adsorption and partition chromatography. The use of adsorption chromatography in protein purification is limited because of the unavailability of suitable adsorbents. Nevertheless, this method has been successfully used in the purification of many proteins, such as alkaline phosphatase (136) and DPNH-dehydrogenase (137). Partition chromatography also suffers from limitations. The choice of a solvent pair with a suitable partition coefficient and the risk of denaturation of proteins at the interface restrict the use of partition chromatography. The purification of insulin (138) and ribonuclease (139), however, has

been made possible by this method, with the use of a buffered glycol ether solvent system.

The development of ion-exchange chromatographic techniques by Moore and Stein (140) has brought a new era in the history of protein chemistry. Cross-linked polystyrene resins, bearing cation or anion exchange groups, are being used extensively for the separation of amino acids, peptides and proteins (141). The polysulfonic resins, because of their strong exchange capacity, are particularly suitable for the separation of complex mixtures of amino acids and peptides. The amount of cross-linkage in the resin determines the maximum size of the molecules which can be successfully chromatographed. With the use of a resin that contains low cross-linkage, e.g., Dowex 50-X-2, even larger peptides can be separated (141). Often, an elevated temperature may be necessary for a better resolution, as in the case of amino acid separation. Strong sulfonic acid groups of resins may hydrolyze susceptible peptide bonds at an elevated temperature (142). The use of high temperature, therefore, is not encouraging for the separation of peptides and proteins. However, the mild cation exchangers, such as IRC-50, have been used for the purification of histones (143), trypsinogen (144), prolactin (145) and other proteins. Anion exchangers, such as Dowex 2, have been used for the isolation of serum albumin (146), serum trypsin inhibitor (147), red cell phosphatase (148) and many other proteins.

Another major advance in the purification of proteins is the development of ion-exchange celluloses by Peterson and Sober (149).

The limitations imposed by polystyrene resins in protein fractionation have been greatly overcome by ion-exchange celluloses. With the introduction of ionizing groups, such as carboxymethyl (CM-), phosphate (P-), or diethylaminoethyl (DEAE-) into the cellulose backbone, cation and anion exchange celluloses with high binding capacities have been prepared (149). Because of their hydrophilic nature and enormous surface, ion-exchange celluloses are particularly suitable for the purification of proteins. Moreover, the conditions necessary for the ion-exchange cellulose chromatography are very mild, and the chances of protein denaturation, therefore, are greatly reduced. There are innumerable examples of the application of ion-exchange celluloses. To mention only a few: CM-cellulose has been used for the purification of trypsin (150), myoglobin (151) and hemoglobin (152); and DEAE cellulose, for pancreatic enzymes (153), serum proteins (154) and growth hormone (155). Thrombin has been purified on phosphocellulose (156). Phosphocellulose, being a relatively stronger cation exchanger, can be used for the separation of peptides (157).

Porath and Flodin (158) developed a new method of protein fractionation, known as gel filtration. This technique falls under the subdivision of exclusion chromatography. Cross-linked dextran (Sephadex) gel offers separation on the basis of molecular size. Larger molecules which cannot enter into the gel matrix are excluded from the column first, while smaller molecules are progressively retarded. This property of Sephadex is also known as molecular sieving. By the

use of gels containing different degree of cross-linking, separation of proteins from peptides is possible. Highly cross-linked dextran, Sephadex G-25, can be used for the separation of small peptides (159). The combined effects of molecular sieving and ion-exchange properties have been obtained by the introduction of ion-exchange groups in Sephadex (160).

(c) Electrophoresis

Electrophoresis is used as an analytical rather than a fractionation procedure, although electrophoretic convection and zone electrophoresis on various supportive media have been in use for the separation of proteins and peptides into groups of similar charge. Starch gel electrophoresis (161) is a valuable analytical method and sometimes can be used for the preparative purposes (162). Though the separation is basically dependent on charge, starch gel also imposes a molecular sieving effect. Electrophoresis in a vertical column of starch or cellulose permits separation and subsequent elution of the separated bands (163). Because of high reproducibility and repetitive use of the same column, this method can be employed for both analytical and isolation purposes. Electrodialysis separates components on the basis of both charge and size. Its use in the protein field has been tried for many years, but only with limited success (164).

(d) Countercurrent distribution (CCD)

The countercurrent distribution technique of Craig (165) is

a powerful tool for peptide separation. The efficiency of this method has been demonstrated by the separation of desamino insulin from insulin (166). The CCD technique has been found very useful for the purification and characterization of ACTH (133), oxytocin and vasopressin (28,29), lysozyme and ribonuclease (167), the melanocyte stimulating hormones (168), and growth hormone (169). The α- and β-chains of human hemoglobin (170) and the peptide fragments of the β-chain (171) have also been separated by CCD. The countercurrent technique has some limitations when it is applied to large molecular weight proteins, even though it has been used successfully in a number of instances. The limitations arise from the difficulty of finding a suitable solvent pair which will not denature the protein in question.

C. Homogeneity

It is obvious that a pure preparation is necessary before a chemical compound can be fully characterized. The criteria of purity, however, depend on the methods available to demonstrate heterogeneity. It has always been said that no single physicochemical criterion is enough to prove homogeneity in macromolecules like proteins. The development of improved methods for examining homogeneity, such as electrophoresis, sedimentation, ion-exchange chromatography, countercurrent distribution, etc. has led to the recognition of "microheterogeneity" in otherwise pure proteins. A new term, "sequential purity", has been introduced to describe homogeneity with respect to the amino acid sequence or other covalently

bonded structures of the molecule (172). The degree of homogeneity to be achieved is directed by the purpose and methods of study. Presence of the slightest impurity in crystallographic studies will be misleading, while the same preparation may be useful for chemical or biological studies.

10. Estimation of Proteins

(a) The Kjeldahl method

Proteins, on the average, contain 16 per cent nitrogen. By knowing the total nitrogen content of a preparation, it is possible to calculate the amount of protein present. Contamination of proteins with other nitrogen containing substances will give erroneous results. Since biological preparations or tissue extracts are almost always contaminated with non-protein nitrogenous materials, a correct estimation of protein concentration is not possible before purification of such preparations.

The total nitrogen content is generally determined by the Kjeldahl method. This consists of the conversion of organic nitrogen into ammonium sulfate by digestion of proteins with sulfuric acid in the presence of a catalyst, and the subsequent estimation of ammonia liberated by the addition of alkali to the digest. Modifications of the original method have been described in order to achieve reliable results (173).

(b) Biuret method

In a strongly alkaline solution, proteins react with copper sulfate to produce a purplish-violet color having an absorption maximum at 330 mμ. The color is due to the formation of a complex between copper ions and peptide bonds. The biuret test is not very sensitive. A negative reaction does not always mean the solution is devoid of proteins; on the other hand, a positive test is given by histidine (122). However, a suitable method has been described (174) by which a certain estimate of protein and peptide concentrations in biological preparations can be obtained.

(c) The Folin-Lowry method

The color produced by the reaction of proteins with Folin's phenol reagent is also used to measure protein concentrations. The intensity of the color depends chiefly on the tyrosine and tryptophan content of the protein. The method has been improved by Lowry et al. (175), combining it with the biuret method and is generally known as the Folin-Lowry method. This is probably the most widely used and reliable method known.

(d) Ninhydrin method

Ninhydrin reacts with amino acids to give a purple color, with the concomitant release of CO_2 (176). The color intensity is proportional to the concentration of amino acids. Ninhydrin also reacts with peptides and proteins, but the color value is variable due to progressive destruction

and liberation of free amino groups from the peptide chain during the course of the reaction. For this reason, the method cannot be used for quantitative determinations of peptides or proteins. Nevertheless, the increment of ninhydrin color in the hydrolysate may be used as a measure of peptide or protein concentration in the original preparation. Incomplete hydrolysis of the protein or destruction of amino acids during hydrolysis has to be considered. The ninhydrin method has been used to measure peptides even in the presence of free amino acids by differential destruction of the free amino acids with ninhydrin before hydrolysis of the peptides (177).

11. The Study of Protein Structure

Linderstrøm-Lang (178) pointed out that the structure of proteins can be considered in terms of three distinct categories: primary, secondary and tertiary structure. The term "primary structure" refers to the fixed amino acid sequence of the polypeptide chain or chains building up the backbone of the protein molecule. In other words, this is the covalently bonded structure of the molecule. The "secondary structure" arises from the helical coiling of the polypeptide chain, stabilized by -CONH-hydrogen bonds. Amongst several possible helices, the right-handed α-helix has been considered to be the most probable in protein structure (179). A complete turn of the helix contains 3.7 amino acid residues, contributing 5.4 Å in linear translation along the central axis (179). The helical structure is

strengthened by the "tertiary structure" which constitutes further folding of the polypeptide chain, properly anchored by disulfide bridges and non-covalent bonds. The secondary and tertiary structures give rise to the specific three dimensional configuration of a protein molecule, which depends on the environmental conditions.

A. The study of the secondary and tertiary structures of proteins

(a) X-ray crystallographic studies

Kendrew and his co-workers (180) have made extensive studies of the crystalline structure of sperm whale myoglobin. They have shown that the single polypeptide chain of myoglobin is coiled into a very compact form and there is no hole or channel. They have been able to calculate that 60 to 72 per cent of the polypeptide chain is in the right-handed α-helical configuration. Almost all the polar groups have been shown to be on the surface of the molecule. A similar study on horse oxyhemoglobin, though at a lower resolution, led Perutz et al. (181) to conclude that the four polypeptide chains are made up of two apparently identical pairs, and the convolutions and three dimensional aspects of these polypeptide chains are closely similar to each other and to the myoglobin chain. The heme groups of both hemoglobin and myoglobin have been reported to emerge from pockets in the surface of the protein. The success of this method with hemoglobin and myoglobin has led to the application of this technique in the study of crystalline structures of other proteins, such as chymotrypsin (182) and ribonuclease (183). Both have been reported to contain a low α-helix content. The

question as to how much of the crystalline structure of a protein persists in solution still remains unanswered.

(b) Optical methods

Moffitt and Yang (184) developed an equation for the calculation of α-helix content in proteins from optical rotatory dispersion data. In the case of myoglobin, the α-helix content measured by optical rotatory dispersion techniques (185) agrees nicely with the value derived from x-ray studies. A negative Moffitt constant (b_o) value indicates the α-helical conformation. The possibility of the presence of both right-handed and left-handed helices have been suggested for those proteins in which the Moffitt constant is zero (186). But Jirgensons has (187) pointed out that the non-helical globular proteins ($b_o = 0$) do not seem to possess both right- and left-handed α-helices; instead, they may possess some order other than the α-helix in the folding of the polypeptide chains. Tanford et al. (188) have shown that globular proteins have an intrinsic tendency to form α-helices only in solvents rich in organic substances.

The spectra of proteins in the region 240 to 320 mμ differ in detail from those of the constituent aromatic amino acids (189). Changes in secondary and tertiary structures of proteins produce small changes in the spectra, which can be detected by the technique of difference spectroscopy. The spectral shift observed on denaturation of insulin (190) and ribonuclease (191) has been ascribed to the disruption of tyrosine-carboxylate hydrogen bonds. Similar difference

spectroscopic studies on other proteins suggest that the spectral shift is due to alterations in the dispersive interaction of hydrophobic bonds (192).

(c) Titration

The hydrogen ion titration of proteins reveals many characteristic features of the secondary and tertiary structures. It is often found that some of the reactive groups, such as phenolic and imidazole, are so hidden or buried in the molecule that they can be exposed only after denaturation. For example, approximately 6 of 12 histidyl residues in native metmyoglobin are available for reaction with H^+ or p-nitrophenyl acetate; the masked imidazole groups are released on acid denaturation (193). Crammer and Neuberger (194) found that only 2 of the 10 tyrosyl groups of ovalbumin are titrated at pH 12, but after alkaline denaturation all the groups behave normally. Similar abnormal tyrosyl groups have been found in ribonuclease in which 3 of the 6 tyrosyl groups are titratable in the native protein (195). It has been found that when denaturation exposes masked tyrosyl groups, an equal number of titratable carboxyl groups are also liberated. This suggests that there are tyrosyl-carboxylate interactions in the native proteins, which are broken on denaturation (196,197).

The secondary and tertiary structures depend, in fact, on the primary structure which is fixed. The reason for the formation of a helix in certain parts of the polypeptide chain or of folding at specific points is not clearly established. However, Blout et al.

(198) have suggested that some amino acid residues are helix formers while others are not. The general belief that only proline turns the corner of polypeptide chains may need revision in view of the fact that there are seven corners in the myoglobin molecule (180) but only four proline residues (199).

B. Methods for the determination of primary structure of proteins

(a) Amino acid composition

The first requirement in the study of protein structure is to know the correct composition of its constituent amino acids. The amino acid composition is usually determined in an acid hydrolysate. The development of ion-exchange chromatography by Moore and Stein (140) and its automation by Spackman et al. (200) have made amino acid analysis simple but accurate. All the amino acids except tryptophan can be separated and estimated. Since tryptophan is completely destroyed by acid hydrolysis, it is generally determined by spectrophotometric methods (201). Destruction of serine and threonine during acid hydrolysis may be corrected by extrapolation to zero time of hydrolysis (202). An enzymatic method for the complete hydrolysis of proteins has been described (203). This obviates the difficulties of hydrolytic loss of amino acids.

(b) Disulfide and sulfhydryl groups

Free sulfhydryl groups in proteins can be estimated by spectrophotometric titration with p-chloromercuribenzoate (204) or by

amperometric titration with silver or mercury ions (205). Alkylation of thiol groups with iodoacetate, bromoacetate or their amides at pH 8.5 converts cysteine residues into S-carboxymethyl derivatives which can be estimated in the hydrolysate (206). Moore (207) described an improved method for the determination of total cystine and cysteine content as cysteic acid, after performic acid oxidation of the protein. The disulfide content of a protein is usually determined indirectly by subtracting the number of free thiol groups from the total number of half-cystine in the molecule.

To facilitate enzyme actions for the cleavage of polypeptide chains it is necessary to disrupt disulfide bridges. Sanger (208) used performic acid oxidation to convert cystine residues of insulin to cysteic acid. The same method has been used with ribonuclease (209). One drawback of the oxidative cleavage is that methionine and tryptophan are completely oxidized, and an incomplete removal of hydrogen peroxide, used in the reaction, may lead to the conversion of tyrosine to chlorotyrosine during subsequent hydrolysis of the protein with HCl (210). These difficulties do not arise with the reductive cleavage. Of several reducing agents, β-mercaptoethanol appears to be the most satisfactory, being itself an effective denaturing agent (211). Alkylation of the liberated thiol groups prevents their reoxidation to disulfide bonds. It has been found that iodoacetate used for alkylation slowly reacts with methionine, histidine and tyrosine; but this can be minimized by rapid removal of the reagent, employing gel filtration (212).

(c) Cleavage of polypeptide chains

(i) Enzymic hydrolysis

Our knowledge of the primary structure of proteins comes from the degradative studies. It is, therefore, necessary to break the polypeptide chain into easily manageable fragments. Proteolytic enzymes are very useful for this purpose. They can produce a relatively uniform population of small peptide fragments. Proteolysis proceeds in mild conditions so that the integrity of labile residues, such as tryptophan, glutamine, and asparagine, is preserved. Moreover, by the use of enzymes of different specificities, like trypsin and chymotrypsin, the resulting peptide fragments, after further studies, may be reassembled into their original order (213).

Trypsin is by far the most specific enzyme available for selective cleavage of the polypeptide chain. It splits only those peptide bonds whose carboxyl groups are contributed by either lysine or arginine (214). Peptide bonds formed by adjacent lysine or arginine residues are often found to be resistant to trypsin (213). Neighboring negatively charged residues, such as glutamic acid, aspartic acid, cysteic acid, or S-carboxymethylcysteine also offer resistance to trypsin action (213). Lys-Pro and Arg-Pro bonds are not hydrolyzed by trypsin (215). The action of trypsin may be selectively restricted to the cleavage of only arginine peptide bonds by blocking the ϵ-NH_2 groups of lysine (216,217). Alkylation of thiol groups with β-haloethylamine produces S-β-aminoethylcysteine, the side chain of which resembles that of lysine, and thus creates new sites susceptible to trypsin (218). Most commercial trypsin preparations are known to

exhibit some chymotrypsin-like activity. This may be due to the presence of actual chymotrypsin in the preparation or to an inherent chymotryptic action of trypsin (219).

Chymotrypsin has a broader specificity than trypsin. It rapidly hydrolyzes peptide bonds on the carboxyl side of aromatic residues, such as tyrosine, phenylalanine, and tryptophan (213,214a). Like trypsin, the action of chymotrypsin is retarded by the presence of neighboring acidic residues. Thus, a Phe-Asp bond in lysozyme was not hydrolyzed by chymotrypsin (222). On the other hand, chymotrypsin hydrolyzes leucyl, methionyl, asparaginyl and histidyl bonds (220,221,213). Both in human heart cytochrome c (223) and in the α-chain of human hemoglobin (221) a Lys-Gly bond has been hydrolyzed by chymotrypsin. A similar trypsin-like cleavage has been observed in a chymotryptic digest of α-corticotropin (224). Chymotrypsin has also been found to hydrolyze repetitive lysyl bonds (223). Whether this is due to tryptic contamination in chymotrypsin preparation is not known. An unexpected cleavage of Thr-Gly bond has also been noted (223).

Other proteolytic enzymes used for structural studies are papain, pepsin and subtilisin. Papain is non-specific in its proteolytic action and is generally used for further fragmentation of tryptic or chymotryptic peptides (220,225). At pH 4, papain hydrolyzes the peptide bond involving carboxyl groups of glutamic acid, whereas at a higher pH, when the γ-COOH group is ionized, such bonds are resistant to papain (226). The specificity of pepsin is unpredictable. It is able to hydrolyze all the linkages that occur in the C-terminal

sequence of α-corticotropin (227). Of the common sequence, Glu-His-Phe, pepsin hydrolyzes Glu-His bond in corticotropin and His-Phe bond in MSH (228). However, it has been confirmed that pepsin mainly acts at the NH_2 or the COOH side of aromatic residues and leucine (171). The bacterial protease, subtilisin, is apparently non-specific. It has been found to hydrolyze 10 out of 28 peptide bonds in glucagon (229) and 4 out of 17 bonds in β-MSH (228). Subtilisin has been found useful in the sequence study of lysozyme (230).

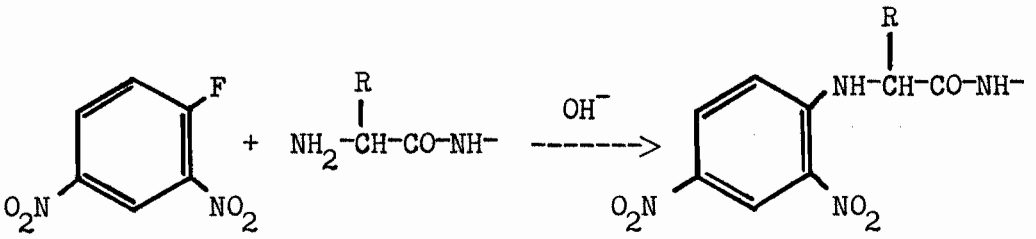
(ii) Chemical methods

Sanger et al. (231) used partial acid hydrolysis to cleave polypeptide chains of insulin. Since a large number of peptide fragments are generally produced, this method is not suitable for the cleavage of long peptide chains. Several chemical methods for specific cleavage of polypeptide chains have been developed, but their use is limited. However, N-bromosuccinimide has been used to cleave specifically the C-tryptophanyl bonds in lysozyme, TMV-protein, bovine serum albumin (232) and glucagon (233). Cyanogen bromide has been successfully used for the cleavage of methionyl bonds in the study of ribonuclease (234) and sperm whale myoglobin (235). The high degree of specificity of the preferential release of aspartic acid by dilute HCl (236) has been used in the study of tryptic peptides of the γ-chain of fetal hemoglobin (237).

(d) End group analysis of proteins and peptides

(i) Dinitrophenylation

In 1945, Sanger (238) developed the first chemical method for the identification of the N-terminal residues of peptides and proteins by reacting them with 1,2,4-fluorodinitrobenzene (FDNB). The dinitrophenylated (DNP) protein or peptide upon acid hydrolysis yields DNP-derivative of the N-terminal amino acid, which may be separated, identified and estimated by one of several chromatographic methods (239). Besides reacting with the free α-NH_2 groups, FDNB also reacts with the ϵ-NH_2 group of lysine, imidazole group of histidine and HO group of tyrosine. The reaction occurs in alkaline pH as follows:



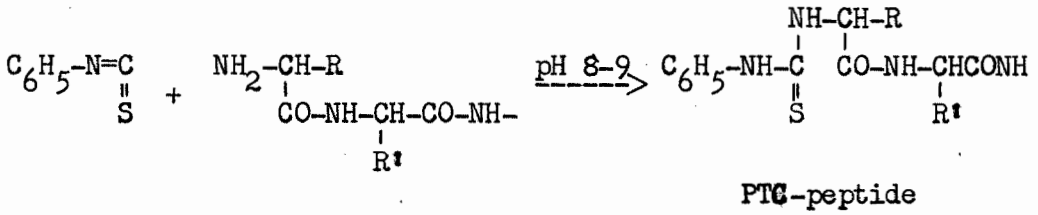
In neutral or slightly acidic medium, FDNB specifically reacts with the SH-group of cysteine; the use of this reaction for the specific cleavage of peptide chains at cysteine residues by β-elimination has been reported (240).

In his studies on the structure of insulin, Sanger (241) invariably used the dinitrophenylation reaction to determine the N-terminal sequences of peptides. Later studies (239,242) have shown that this method has some limitations. Premature release of DNP-amino acids from DNP-proteins did not permit unequivocal identification of

N-terminal residues (239). Decomposition of DNP-derivatives of glycine, tryptophan, threonine and serine during acid hydrolysis of DNP-proteins results in poor yield of those derivatives. In the case of cytochrome c which has an acetylated N-terminal glycine (113), the FDNB method led to the erroneous conclusion that the N-terminal residue was histidine (243) or arginine (244).

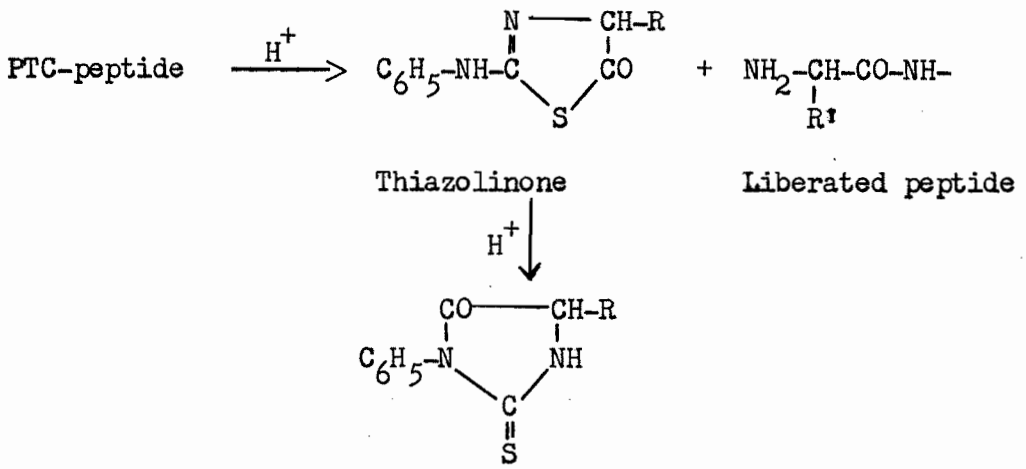
(ii) The Edman reaction

Edman (245) developed a method for stepwise removal of N-terminal residues from peptides by reacting them with phenylisothiocyanate. The reaction proceeds in two steps. In the coupling reaction, the α-NH_2 group of a peptide is allowed to react with phenylisothiocyanate at alkaline pH to yield phenylthiocarbamyl (PTC) peptide:



PTC-peptide

In acid solution, the PTC-peptide cyclizes to form a thiazolinone, which then rearranges to the phenylthiohydantoin (PTH) derivative of the amino acid, and thus liberates the remainder of the peptide, with an α-NH_2 group available for a second reaction:



Phenylthiohydantoin

Edman performed the cyclization reaction in strictly anhydrous conditions (in nitromethane saturated with HCl gas), which favor a rapid rearrangement of thiazolinones to PTH derivatives and also prevent hydrolysis of peptide bonds. In later studies, however, the cyclization has been performed in various conditions; for example, in 3N HCl (239), 5:1 glacial acetic acid and concentrated HCl mixture (222) and in glacial acetic acid-anhydrous HCl (246). It has been shown that when the cyclization in the stepwise Edman degradation is performed in glacial acetic acid-anhydrous HCl,(a) a newly liberated N-terminal glutamine residue cyclizes to a pyrrolidone carboxylic acid residue, thus blocking subsequent degradations; (b) an internal asparagine or aspartic acid may rearrange from an α- to a β-peptide; (c) peptides containing serine or threonine are acetylated in hot HCl-acetic acid, and may undergo an O----->N acyl migration to the extent that the subsequent degradation is hindered (247). However, these difficulties have been overcome by an improved method of cyclization, described by

Konigsberg and Hill (225). Insolubility of PTC-proteins in acid solution is a major problem of this technique, when applied to proteins. This may be avoided by the paper strip method in which both coupling and cyclization reactions are performed while the material is absorbed on filter paper (239).

The PTH amino acids can be extracted with organic solvents and identified by paper chromatography (248,249). The identification of PTH amino acids is often overshadowed by the presence of various products of side reactions, such as PTC-amino acids, diphenylthiourea, etc. To circumvent this difficulty, the subtractive Edman procedure has been introduced (246). In this method, the missing amino acid is detected by performing quantitative amino acid analyses on aliquots of the peptide sample before and after each Edman degradation. The Edman degradation is the most useful tool available at present for sequence studies.

(iii) Leucine aminopeptidase

Availability of highly purified leucine aminopeptidase permits stepwise removal of amino acids from the NH_2-terminal of peptides and proteins (250). The enzyme acts so rapidly that it is sometimes difficult to follow the sequence. The action of leucine aminopeptidase is halted when it comes to a proline residue. The enzyme has been successfully used in sequence studies of cytochrome c (220) and lysozyme (230).

(iv) The cyanate procedure

Recently, Stark and Smyth (251) developed a method for the identification of the N-terminal residues in proteins by coupling them with cyanate at pH 8. The carbamyl derivative on subsequent treatment with acid forms a hydantoin of the N-terminal residue. After isolation, the hydantoin may be hydrolyzed and the liberated amino acid can be identified.

(v) Carboxypeptidase

Carboxypeptidase A has a broad specificity, although it favors the removal of aromatic residues. It does not act when the C-terminal residue is lysine, arginine or proline (252). Carboxypeptidase B, on the other hand, specifically liberates only C-terminal lysine, arginine or ornithine (252,253). A free α-COOH group is the necessary requirement for these enzymes to be active. Carboxypeptidase A and B are universally employed for the determination of C-terminal sequences of peptides and proteins.

(vi) Hydrazinolysis

The reaction of peptides and proteins with anhydrous hydrazine (254) gives rise to amino acid hydrazides with all amino acids except the one bearing a free α-COOH group. The unreacted C-terminal amino acid may be separated from the mixed hydrazides and estimated quantitatively. The original method has been modified by Bradbury (255) to achieve consistent results and has been applied to determine the C-

terminal residues in insulin, lysozyme and wool proteins.

(e) Deduction of complete sequence of polypeptides

Chymotrypsin and trypsin have different specificities and they will break the polypeptide chain at different points producing two series of peptides from the same chain. Some parts of peptides of one series will have a common sequence with those parts of peptides of the other series which have originated from the same part of the chain. These overlapping sequences in two series of peptides can be used to reassemble the peptide fragments in an order in which they were originally present in the parent polypeptide chain (213). Once the fragments are arranged in the proper order by joining one another through the regions of overlapping sequence, the complete sequence of the polypeptide is known. But there still remains the problem of allocating the disulfide bridge between cysteine residues, which had been broken before fragmentation of the parent polypeptide chain. Ryle et al. (256) established the three disulfide bonds in insulin by isolating and identifying each cystine-containing peptide, after partial acid hydrolysis and enzyme hydrolysis of the intact molecule. The pairing of the half-cystine residues in ribonuclease has been elaborated by identifying the cystine-containing peptides, produced by partial hydrolysis of the native enzyme with pepsin, followed by trypsin digestion (257).

(f) Peptides and proteins of known primary structure

The following list summarizes some of the peptides and proteins whose primary structures have been established.

Proteins	Total No. of amino acids	Terminal Sequences	Reference
Horse heart cytochrome c	104	Acetyl-Gly-Asp ....Asp(NH_2)-Glu-COOH	(113)
Ribonuclease	124	NH_2-Lys-Glu.... ..Ser-Val-COOH	(247)
Insulin	51	NH_2-Gly-.....-Asp(NH_2)-COOH NH_2-Phe-.....-Ala-COOH	(256)
Glucagon	29	NH_2-His-Ser...-Asp-Thr-COOH	(258)
TMV protein	158	Acetyl-Ser-Tyr.....Thr-COOH	(259)
Corticotropin	39	NH_2-Ser-Tyr-...Glu-Phe-COOH	(260,261, 262)
Oxytocin Vasopressin	8	NH_2-Cys-Tyr.....Gly-$CONH_2$	(30)
α-MSH	14	Acetyl-Ser-Tyr.....Pro-Val-$CONH_2$	(263)
β-MSH	18	NH_2-Asp-Glu.....Lys-Asp-COOH	(264)
Human hemoglobin			
α-chain	141	NH_2-Val-Leu.....Tyr-Arg-COOH	(265)
β-chain	146	NH_2-Val-His.....Tyr-His-COOH	(266)
Bradykinin	9	NH_2-Arg-Pro.....Phe-Arg-COOH	(267)
Angiotensin I	10	NH_2- Asp.....His-Leu-COOH	(268)
Lysozyme	129	NH_2-Lys-Val.....Ang-Leu-COOH	(230)

EXPERIMENTAL

1. Extraction of Peptides from Fresh Hypothalamus

One kilogram of fresh hog hypothalamus suspended in one liter of 0.1N HCl, was obtained from Nordic Biochemicals Ltd., Montreal. The tissue was homogenized with the aid of an additional volume (500 ml) of 0.1N HCl in a Waring Blendor. A thick blend was formed. The emulsion so produced could not be broken by centrifugation immediately, but after storing in a polyethylene bottle for several days in the refrigerator, the mixture separated, yielding a clear supernatant. The straw-colored supernate was decanted, neutralized with ammonium hydroxide and lyophilized in batches. A total of 6 g of yellowish powder was obtained. The lyophilized material was then subjected to various methods of fractionation.

2. Extraction of Peptides from Hypothalamus Acetone Powder

Acetone-dried hog hypothalamus tissue powders were obtained from Nordic Biochemicals Ltd. Peptide materials were extracted by the method of Bates *et al.* (269) as modified by Saffran *et al.* (270). In this method, the tissue powder was percolated with a discontinuous gradient of water and acetic acid in ethanol. Three hundred and ninety g of hypothalamus powder were mixed with an equal weight of Hyflo Super-Cel and the mixture was sized by passing through a flour sieve. A slurry was made by adding 4 liters of commercial 95% ethanol to the mixture and was poured into a glass percolator plugged with glass-wool

covered by a layer of Hyflo Super-Cel. The excess ethanol was allowed to drip from the bottom of the percolator and collected. The difference between the total volume of ethanol used for making the slurry and the volume recovered gave a retention volume of 2 liters. Double the retention volume of each solvent mixture was used for percolation, starting with 95% ethanol. Subsequent elution was carried out with ethanol diluted stepwise by 5% with water until the ethanol concentration reached 70%. Acetic acid was then added to the percolating solvent mixtures to final concentrations of 0.01, 0.05, 0.20, 0.50, and 1.00 molar in 70% ethanol. The percolation was concluded with 1M acetic acid in 50% ethanol. Percolates were collected in 1 liter fractions and there were altogether 52 fractions. The fractions were concentrated in a rotary evaporator under water-pump vacuum at a temperature below 40° to final volumes of 250, 100 or 50 ml. The concentrated percolates were stored in polyethylene bottles at 4°.

3. Analysis of Percolate Fractions and the Acid Extract

Aliquots of the individual fractions were analyzed for peptide content by the Folin-Lowry (175) method in a Technicon automatic analyzer. Crystalline bovine serum albumin was used as standard. The fractions were also analyzed by ninhydrin reaction directly and after hydrolysis with HCl or NaOH (271,272) against a leucine standard.

The acid (0.1N HCl) extract and the percolate fractions were examined by two-dimensional electrophoresis and chromatography (273, 274), hereinafter referred to as "Fingerprint", on a full sheet (18 1/2 x

21 1/4 inch) of Whatman No. 3MM filter paper. Electrophoretic separation was carried out in the first dimension and chromatography, in the second. The material was applied on the buffer soaked paper at a point 3 and 6 inches from the longer and shorter edges of the paper respectively and subjected to electrophoresis along the longer side. The buffer used was pyridine-glacial acetic acid-water (1:10:289, by volume), pH 3.7 (274). Electrophoresis was performed in a high voltage electrophoretor (Gilson Medical Electronics, Model D) at 3000 volts for 60 minutes. During the run, the current changed from 160 to 180 milliamperes. At the end of the electrophoretic run, the paper was dried and subjected to descending chromatography along the second dimension with the upper phase of n-butanol-glacial acetic acid-water (4:1:5, by volume) mixture for 16 hours. The dried chromatograms were sprayed evenly with 0.1% ninhydrin solution in 95% ethanol-glacial acetic acid-collidine (50:15:2, by volume) mixture and heated for 3 to 5 minutes at 95° to visualize the spots (275).

4. Gel Filtration

Sephadex G-25, G-50 and G-75, obtained from Pharmacia Fine Chemicals, New York, were used. Gel columns were prepared essentially according to Porath (276). The Sephadex was suspended in 0.2M acetic acid and was allowed to swell overnight. The fines were removed by decantation or by siphoning off the liquid from the top of the settled gel. The gel slurry was poured into Pyrex chromatographic tubes of various dimensions ranging from 0.9 x 150 cm to 2.5 x 105 cm. At least

3 to 4 column volumes of solvent (0.2M acetic acid) were passed through before the column was used. Samples were applied to the gel column in a minimum possible volume. The columns were developed at room temperature with a suitable flow rate. The column eluates were collected in fractions of 3 to 4 ml by a fraction collector (Gilson Medical Electronics). Enough fractions were collected in each chromatography to ensure that all the materials applied on the column were eluted.

When 50% acetic acid was used as a developing solvent, the gel was prepared in the same solvent before pouring into the column.

5. Ion-exchange Chromatography

Ion-exchange celluloses were obtained from Calbiochem, Los Angeles. The cation exchanger, CM-cellulose (exchange capacity 0.74 mEq/g), was washed successively with 0.5N NaOH, water, 0.5N HCl, water and finally several times with the starting buffer. DEAE cellulose (exchange capacity 0.94 mEq/g) was washed with 0.5N NaOH, water, followed by the starting buffer. Columns of suitable size were packed under an air pressure of one atmosphere and thoroughly washed with the starting buffer until the pH of the effluent corresponded to that of the buffer employed. Materials to be fractionated were dissolved in the buffer, and after adjustment of the pH, the solution was applied to the column. The columns were developed at room temperature with a smooth gradient of increasing molarity, achieved by allowing buffers of higher concentration to flow into a mixing chamber containing buffers of lower concentration. Adequate mixing of

buffers was achieved by a magnetic stirrer. A gradient in pH was obtained by employing buffers of different pH. When the gradient was not applied, the elution was performed with a stepwise change of buffers.

The cation exchanger, Dowex 50W-X2 (200-400 mesh), was used for separation and isolation of peptides derived from a tryptic digestion of a purified polypeptide. The resin was converted to the hydrogen form by washing twice successively with 2.0N NaOH, water, 2.0N HCl and water (277). The H^+ form resin was suspended in the starting buffer and stirred magnetically with intermittent buffer change until equilibrium was reached, as indicated by the pH of the supernatant. The fines were removed by decantation and the resin was poured into a jacketed column (0.9 x 135 cm) in five portions. Water at 37° was circulated through the jacket during the preparation and development of the column. Pyridine acetate buffers (278) were employed. The starting buffer was 0.2M with respect to pyridine at pH 3.1. A gradient of increasing molarity and pH was obtained through a 500 ml mixing chamber. Gradients were applied from 0.2M, pH 3.1 to 0.5M, pH 4.1 and from 0.5M, pH 4.1 to 2.0M, pH 5.1. The column was then washed with 2.0M pyridine at pH 6.0. Fractions of 3.7 ml were collected. A constant flow rate of 20 ml per hour was maintained throughout by the use of a Technicon proportionating pump. The resin volume had been found to shrink by about 10 cm of the column length at the end of the run.

Dowex 50W-X2 chromatography of a fraction, obtained from gel

filtration of the acid (0.1N HCl) extract, was performed essentially under the same conditions as described above. The column dimension, in this case, was 1.8 x 150 cm. The gradient was obtained by employing a constant volume (1 liter) mixing chamber. Three and a half ml fractions were collected at a flow rate of 20 ml per hour.

6. Analysis of the Column Eluates

The column eluates were analyzed by absorption at 280 or 278 mμ in a Beckman DU spectrophotometer, and by ninhydrin reaction before and after alkaline hydrolysis (272). The peak materials of column chromatography were examined by paper electrophoresis or by the fingerprint (peptide map) technique as described before or by a modified procedure. In the latter method, electrophoresis was performed in pyridine-acetic acid-water (200:8:1800, by volume) buffer, pH 6.4 in an apparatus designed by Kimmel et al. (278) at 1000 volts for 120 minutes. The solvent used for descending chromatography was n-butanol-acetic acid-water (200:30:75, by volume) and was run for 17 hours. This method was routinely employed for comparison of the tryptic peptides as they were eluted from the Dowex 50 column.

7. Amino Acid Analysis

Peptides were hydrolyzed with three times glass-distilled 6N HCl in evacuated sealed tubes for 24 hours at 110°. The hydrolysates were evaporated to dryness in a rotary evaporator at 50°, the residue was dissolved in distilled water and evaporated twice. The final residue was quantitatively transferred to the analyzer column with small portions

of 0.1N HCl. The amino acid analysis was performed in a Technicon automatic amino acid analyzer (279).

Tryptophan was estimated spectrophotometrically by the method of Goodwin and Morton (201).

8. Dinitrophenylation

The method employed for dinitrophenylation was that described by Parcells and Li (280). Peptide samples were dissolved in 400 µl of 5% $NaHCO_3$ solution and 800 µl of 2% fluorodinitrobenzene in ethanol was added. The reaction mixture was shaken mechanically for 2 hours, acidified with HCl and the insoluble dinitrophenylated peptide was washed successively with water, ethanol and ether. The derivative, after removal of most of the dinitrophenol by exposing to high vacuum, was hydrolyzed with 6N HCl in evacuated sealed tubes at 100° for 16 hours. The hydrolysate was diluted 6 times with water and extracted with peroxide-free ether until no more yellow color appeared in the extract. The combined ether extracts and the aqueous phase were taken to dryness. The ether extract was submitted to two-dimensional paper chromatography using "toluene" and 1.5M phosphate buffer of pH 6 as developing solvents (239). The aqueous phase was chromatographed on Whatman No. 4 filter paper with ter-amyl alcohol-phthalate buffer system of Blackburn and Lowther (281). Whenever possible, the DNP-amino acids were eluted from the paper chromatograms with 4% $NaHCO_3$ and estimated spectrophotometrically (239).

9. Carboxypeptidase Digestion

Diisopropylfluorophosphate-treated carboxypeptidase A was obtained from Worthington Biochemical Corporation as a suspension containing 50 mg of protein per ml. The digestion was carried out either in 1 per cent $NaHCO_3$ at 25° (282) or in 0.04M Tris buffer, pH 8.2, at 40° (220). The enzyme-substrate molar ratio varied from 1:25 to 1:10. Analysis of the products of hydrolysis was done either by dinitrophenylation (282) or by the fingerprint technique (220).

The combined digestion with carboxypeptidase A and carboxypeptidase B (Worthington) was performed in 0.02M phosphate buffer, pH 7.6 as described by Guidotti et al. (277). The enzyme-substrate ratio was 1:25. Aliquots at different times of hydrolysis were analyzed by the automatic amino acid analyzer.

10. The Edman Degradation

The coupling reaction was routinely performed by a modified procedure (220) of the Edman reaction. Suitable quantities of peptides (0.5 to 1.0 μmole) were dissolved in 500 μl of water and 50 μl of 25% aqueous trimethylamine was added followed by the addition of 500 μl of 2% phenylisothiocyanate in redistilled pyridine. The single phase reaction mixture was kept at 40° for 4 hours in 3 ml glass-stoppered tubes (Kimax). The excess reagent was extracted five times with thiophene-free benzene. The aqueous phase, containing the PTC-peptide, was dried over NaOH, H_2SO_4 and paraffin shavings in a vacuum desiccator.

The dried material was further exposed to high vacuum at 60°

for 5 minutes. Cyclization was carried out with 1 ml of anhydrous trifluoroacetic acid at 25° for one hour (225). After the removal of trifluoroacetic acid in a rotary evaporator, the cyclized peptides were dissolved in 0.5 ml of 0.01N HCl and phenylthiohydantoin (PTH) amino acids were extracted once with water-saturated ethylacetate and five times with peroxide-free water-saturated ether (220). The peptide solution was then taken to dryness as before, dissolved in a known volume of water and then a precalculated aliquot was hydrolyzed and analyzed by the automatic amino acid analyzer. The remainder of the material was dried and subjected to the next step of degradation. PTH amino acids were identified by paper chromatography in solvent systems A and F (248,249). This identification was confirmed by the subtractive method of analysis (246). Often, after 2-3 steps of the Edman degradation, the residual peptides were purified on a short column of Dowex 50-X2, as recommended by Konigsberg and Hill (225).

11. Digestion with Leucine Aminopeptidase

The digestion mixture contained 5 mg of protein in 450 µl of water, 50 µl of 0.025M $MgCl_2$, 50 µl of 0.4M Tris-HCl buffer, pH 8.2 and 0.26 mg of leucine aminopeptidase (Worthington) in 50 µl of water (220). The mixture was incubated at 37°, and 100 µl aliquots were removed at 10, 20, 35, 60, 90 and 120 minutes of digestion directly into tubes containing 20 µl of glacial acetic acid. After drying in vacuum over KOH, the aliquots were analyzed (220) by the peptide map technique. A blank experiment, i.e., digestion mixture omitting the protein, was run side by side to check autodigestion of the enzyme.

12. Digestion with Trypsin

A sample of 100 mg of protein was dissolved in 3.125 ml of 0.024M Tris-HCl buffer, pH 8.2 and a 5 µl aliquot was taken out. To the protein solution was then added 1.92 mg of crystalline trypsin (Sigma Chemical Co.) dissolved in 1.0 ml of Tris buffer, and the total volume was brought to 6.240 ml with the buffer. The digestion mixture was kept at 25° (278). Aliquots representing 10 µl were removed at intervals of one hour during the first 16 hours and then at intervals of 4 hours up to 24 hours of digestion. At the end of this time, the digestion was terminated by bringing the solution to pH 3 (pH paper) with 0.1N HCl and the digest was lyophilized.

The aliquots were taken directly into tubes containing 100 µl of 0.01N HCl to stop the enzyme action immediately. The course of hydrolysis was followed by the increment in ninhydrin color value of the aliquots. Portions of the digest were analyzed by the peptide map technique and the rest was saved for Dowex 50-X2 chromatography.

13. Digestion with Chymotrypsin

Chymotryptic digestion was carried out in 0.1M ammonium carbonate-bicarbonate buffer, pH 8.0 for 24 hours at 25° (225). α-Chymotrypsin (Sigma Chemical Co.) was added to the digestion mixture from a freshly prepared 1% solution in 0.001N HCl. The enzyme-substrate molar ratio was either 1:20 or 1:100. Larger polypeptides were generally hydrolyzed with a 2% enzyme concentration (275). No appreciable change in pH occurred during the digestion. At the end of the digestion period, the solution was brought to pH 4 (pH paper) with the addition of a few

drops of glacial acetic acid and freeze dried. Portions of the digest were subjected to ionophoresis-chromatography.

14. Nomenclature

Peptides isolated from the acid extract of fresh hypothalamus will be designated by letters, such as peptide A, B or C, except for the preparation "peptide 9"; and those isolated from the percolates of acetone-dried tissue will be designated by the number of the percolate fraction(s), such as polypeptide 50-52.

RESULTS AND COMMENTS

1. Peptides in the Acid Extract of Hog Hypothalamus

(a) Fingerprint

Fig. 1 shows the fingerprint pattern of 0.1N HCl extract of fresh hog hypothalamus. Comparison with a similar fingerprint of the standard amino acid mixture suggests the presence of many free amino acids in the extract. Glutamic acid, glutamine, glycine, alanine, leucine (or isoleucine) and serine were found in higher quantities than other free amino acids. γ-Aminobutyric acid was also present in appreciable amounts. The only peptide zone recognized on the fingerprint was the one that streaked from the origin towards the cathode in electrophoresis and did not move in chromatography. This was verified by hydrolysis of the extract. Fingerprint of the acid hydrolysate did not show any ninhydrin positive material in this area. The disappearance of the peptide zone was compensated by increased intensities of the existing amino acids and by the appearance of new amino acid spots.

If a peptide and a free amino acid have identical mobilities on fingerprint, it is difficult to differentiate them. The so-called peptide stain (283) is not specific for peptides alone; most of the free amino acids give a positive reaction, making it virtually impossible to distinguish between peptides and free amino acids. Moreover, peptides which do not give a positive ninhydrin reaction will escape detection. For these reasons, it was necessary to separate each component

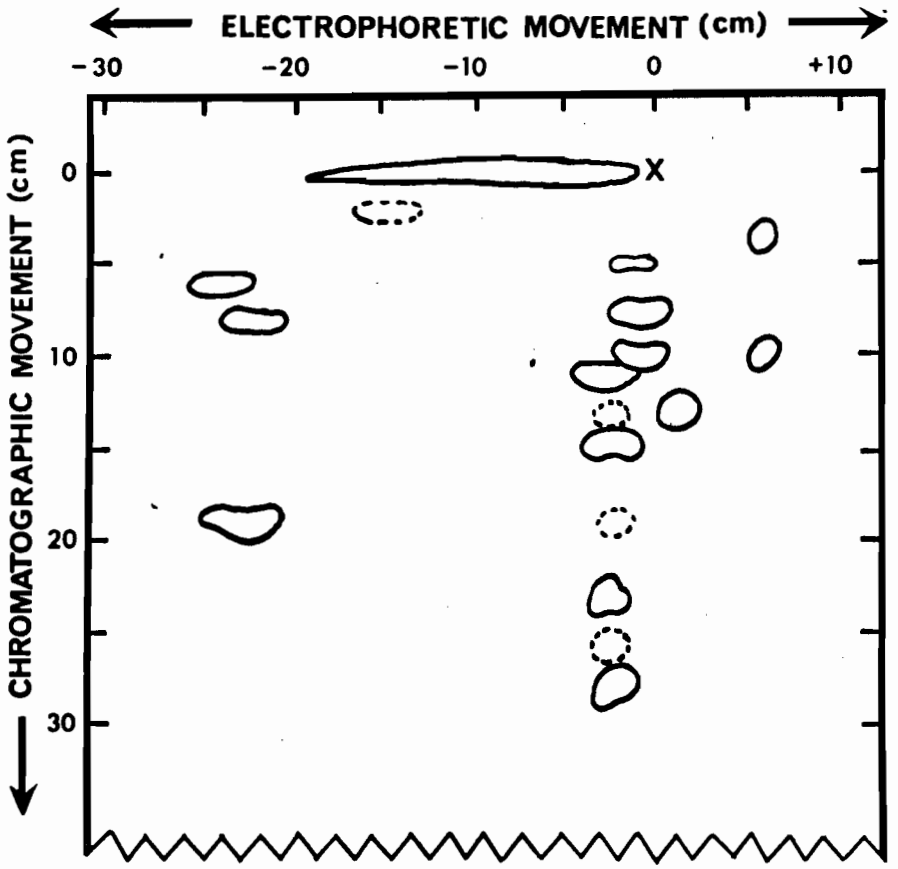


FIG. 1. Electrophoresis-chromatography of 0.1N HCl extract of fresh hog hypothalamus. Electrophoresis was carried out in pyridine-acetate buffer, pH 3.7 at 3000 volts for 1 hr. and descending chromatography was performed in the n-butanol-acetic acid-water (4:1:5, v/v) system for 18 hrs. The sample was applied at a point marked X. Spots were visualized by ninhydrin spraying.

before it could be definitely identified to be a peptide.

(b) Chromatography on Sephadex G-25

The choice of Sephadex G-25 for the initial fractionation came from the fact that it excludes large molecular weight (> 5000) peptides, giving an opportunity to investigate relatively small peptides. The developing solvent used for gel filtration was 0.2M acetic acid. This solvent has been found to be satisfactory for the separation of small peptides in many instances (225,247,277). Free amino acids are not generally separated from small peptides under these conditions.

Fig. 2 shows the gel filtration pattern of the lyophilized acid extract. The crude powder (500 mg) was extracted twice with 2 ml portions of 0.2M acetic acid. About 50 per cent of the material was insoluble. Only the soluble portion was chromatographed. Some colloidal material was always present, which could not be separated by centrifugation, but came out of the gel column as the first peak. As can be seen in the figure, there were some UV absorbing substances in the extract, which did not show ninhydrin color. These UV absorbing materials were retarded much more than the free amino acids and were tentatively identified to be nucleotides by their absorption maxima at 260 mμ. The fourth peak contained mostly ammonium chloride, arising from neutralization of the extract with ammonia, and free amino acids. Free amino acids could be separated from NH_4Cl by gel filtration with 50% acetic acid, as shown in Fig. 3.

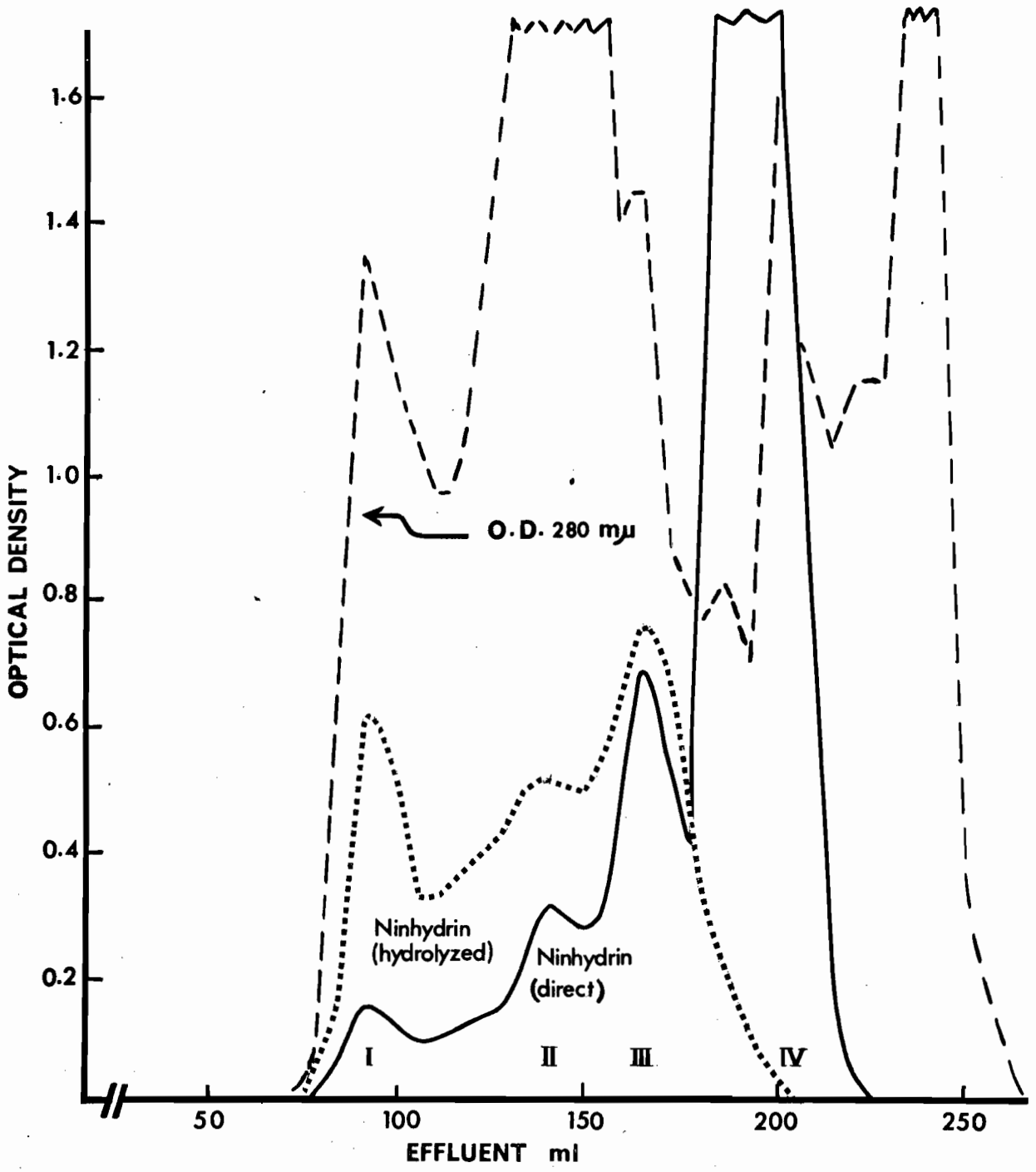


FIG. 2. Gel filtration of 0.1N HCl extract of fresh hog hypothalamus on a Sephadex G-25 column (1.8 x 110 cm). The column was developed with 0.2M acetic acid at a flow rate of 8 ml per hour, and 3.5 ml fractions were collected. Aliquots from individual fractions were analyzed by ninhydrin reaction directly (——) and after hydrolysis (·····) with 2.5N NaOH.

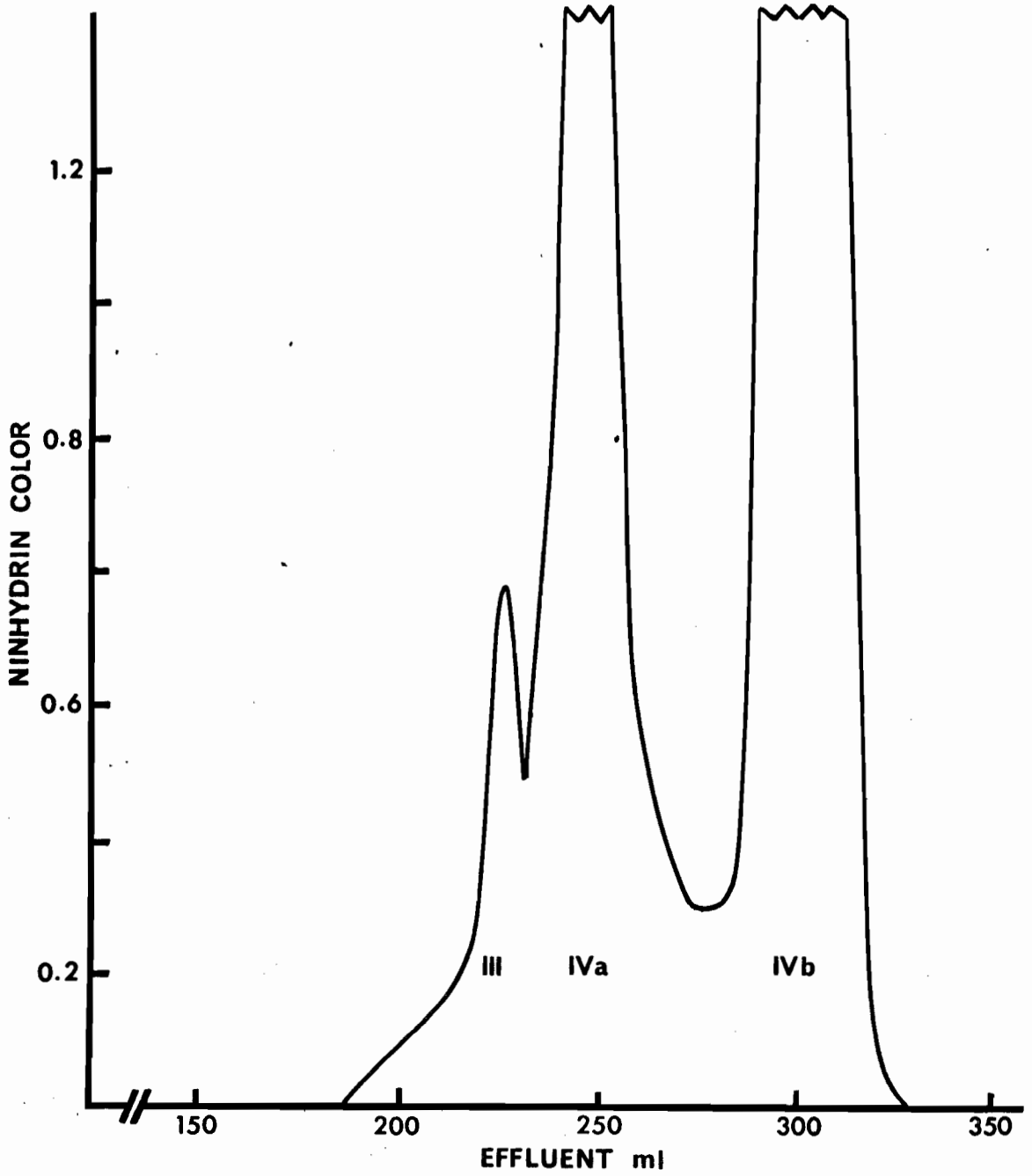


FIG. 3. Separation of the materials of peak IV (Fig. 2) with 50% acetic acid on the Sephadex G-25 column (1.8 x 110 cm). Fractions were analyzed directly by ninhydrin reaction.

The third peak of Fig. 2 showed an increase in ninhydrin color after basic hydrolysis, suggesting the presence of peptides; but this peak also contained free amino acids, evident from the fingerprint analysis. Separation of free amino acids from peptides, such as vasopressin, by chromatography on Sephadex G-25 with pyridine-acetic acid-water (15:55:30, v/v) mixture has been reported (284). Model experiments showed that this system could not separate tri-peptides from free amino acids. Without further attempts to separate free amino acids from peptides of the third peak, the whole mixture was chromatographed on a Dowex 50-X2 column.

(c) Chromatography of Sephadex G-25 peak III on Dowex 50-X2

The separation achieved by chromatography of the third peak (Fig. 2) on Dowex 50-X2 is shown in Fig. 4. Ninhydrin analysis of the fractions before and after alkaline hydrolysis indicated the presence of peptides only in two zones (peaks 2 and 9) which were ninhydrin negative before hydrolysis. No increment in ninhydrin color was observed in other peaks. Fingerprint analysis of each peak showed that free amino acids were separated in groups. The electrophoretic and chromatographic mobilities of peak 10 corresponded to those of γ-amino butyric acid.

Materials of peak 2 (Fig. 4) after acid hydrolysis, yielded large quantities of aspartic acid and comparatively little glutamic acid by amino acid analysis. These values were not in stoichiometric relation to represent a dipeptide or tripeptide of aspartic and glutamic

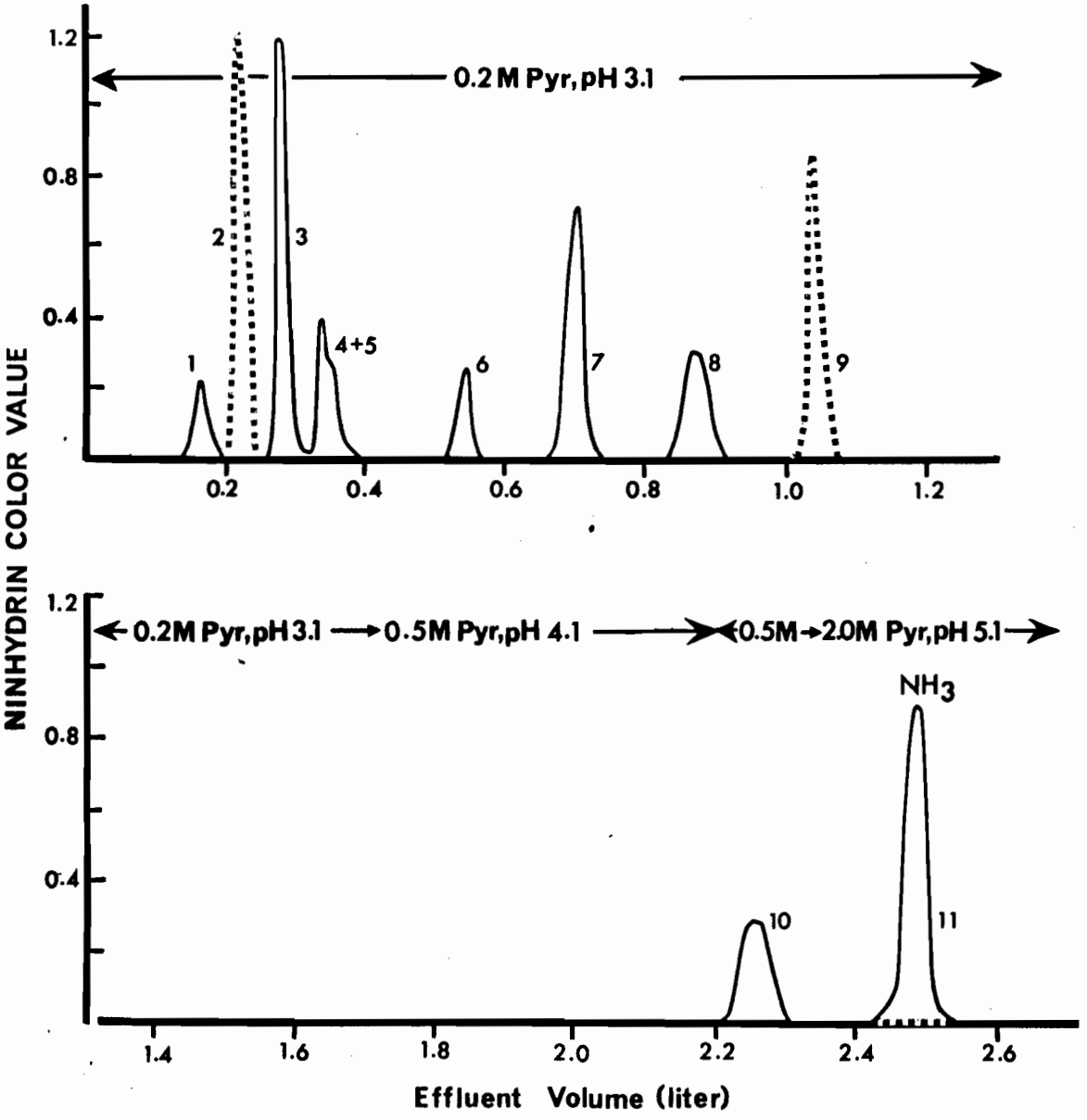


FIG. 4. Chromatography of peak III (Fig. 2) on a Dowex 50X-2 column (1.8 x 150 cm) with pyridine-acetate buffers. The gradient was obtained through a constant volume (1 liter) mixing chamber. A flow rate of 20 ml per hour was maintained. 3.5 ml fractions were collected and aliquots from each fraction were analyzed with ninhydrin directly (———) and after alkaline hydrolysis (·····). Except for peaks 2 and 9, no increment in ninhydrin color was observed in other peaks after basic hydrolysis.

acids. Moreover, it was found in some chromatographic runs that the materials of peak 2 were separated representing two ninhydrin negative components, but they were never separated completely. The fast moving component was probably N-acetyl aspartic acid and the slowly moving one might be glutamic acid or glutamine, the α-NH_2 group of which appeared to be blocked either by acylation or by cyclization with the formation of a pyrrolidone carboxylic acid. These substances were eluted from the column very early preceded only by a highly acidic component. Tallan et al. (285) reported the presence of significant quantities of N-acetyl aspartic acid in mammalian brain. They have also found pyrrolidone carboxylic acid and other highly acidic compounds like glycerophosphoethanolamine and phosphoethanolamine in brain extracts. Whether pyrrolidone carboxylic acid was an artifact produced during extraction is not known. But the presence of ninhydrin negative acidic compounds appears to be a common occurrence in the extracts of neural tissues. Since we were primarily interested in peptides, no further investigations on these materials were carried out.

The second ninhydrin negative zone, namely, peak 9, was eluted in the neutral or slightly basic area of the elution sequence. The isolated material was further purified on Sephadex G-25, using 0.2M acetic acid as eluant (Fig. 5). A single symmetrical peak was formed. The material of this peak was designated "Peptide 9". The yield of "peptide 9" was 162 mg per kilogram of the fresh tissue.

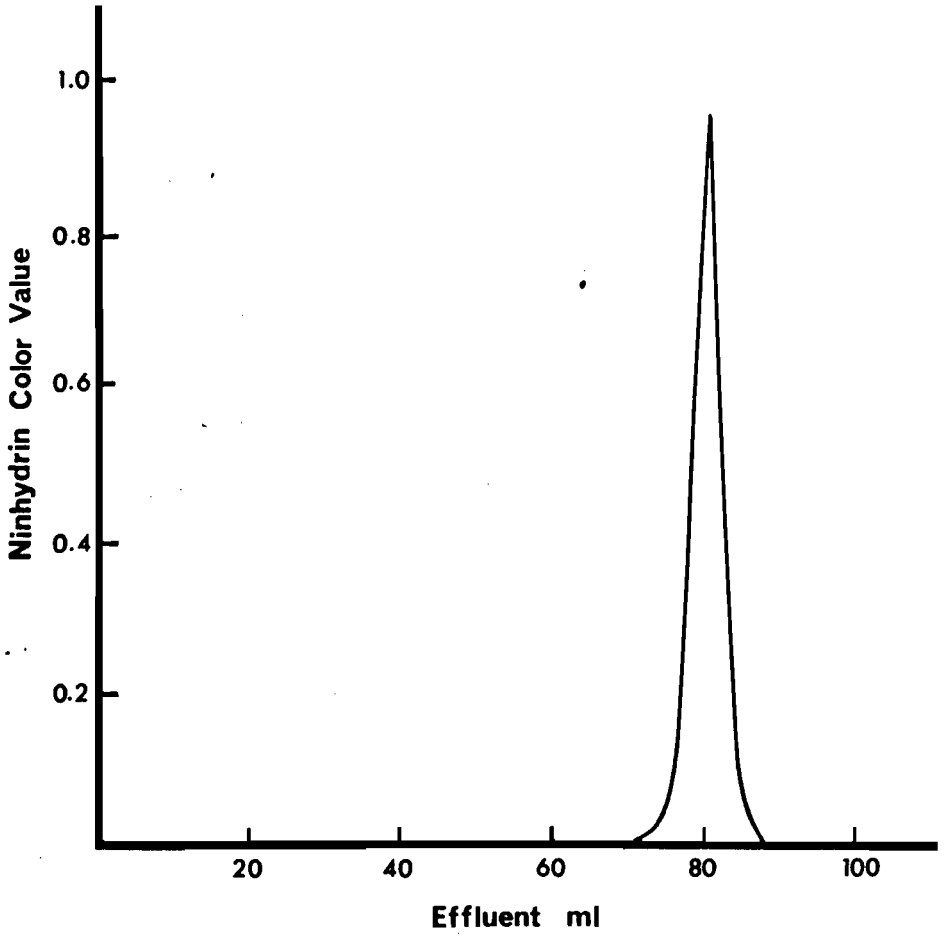


FIG. 5. Chromatography of peak 9 (Fig. 4) on a Sephadex G-25 (1.0 x 145 cm) column. The elution was performed with 0.2M acetic acid. 2 ml fractions were collected. Aliquots from individual fractions were analyzed by ninhydrin reaction after hydrolysis with NaOH.

(d) Amino acid composition of "peptide 9"

Weighed samples (3-4 mg) were hydrolyzed in 6N HCl at 110° for 24 and 48 hours. Results of the amino acid analyses are given in Table I. One unknown ninhydrin positive material was eluted at a position half-way between serine and glutamic acid. The color value of this peak was of the same order as that of isoleucine. The molar ratios of valine and isoleucine are too low for integral values and are not included in the total (Table I).

The Kjeldahl nitrogen (286) of "peptide 9" was found to be 5.7 to 5.8 per cent. Considering that peptides, in general, contain 16% total nitrogen, 36% of the "peptide 9" preparation may be assumed to be composed of peptide. The determination of carbohydrate content of this material by the orcinol-H_2SO_4 method using 1:1 galactose-mannose standard (287) gave a value of only 1.6 per cent. This definitely does not account for the rest of the molecule. A glyco-peptide would have a higher percentage of carbohydrate.

Although no conclusion can be made regarding the non-peptide portion of "peptide 9", some idea may be derived from its solubility behavior. "Peptide 9" is freely soluble in water. The dried material (67.4 mg) was extracted twice with 12 ml portions of chloroform-methanol (2:1) mixture. The insoluble material was separated by centrifugation. To the combined supernatant was added one-fifth of its volume of water and the mixture was vigorously agitated. After centri-fugation, the upper phase was collected, and the lower phase was extracted twice with an equal volume of the "ideal-upper-phase",

TABLE I

Analyses of the acid hydrolysates of "peptide 9"

Amino acid	Amino acid residue (grams per 100 g of peptide)			Calculated molar ratio	Nearest integral value
	24-hour hydrolysate	48-hour hydrolysate	Average or extrapolated value		
Aspartic acid	12.20	12.66	12.43	3.20	3
Threonine	3.64	3.64	3.64	1.05	1
Serine	4.18	3.05	5.66	1.89	2
Glutamic acid	13.43	13.95	13.69	3.07	3
Glycine	3.48	3.19	3.33	1.71	2
Alanine	2.84	2.70	2.77	1.14	1
Valine	1.98	1.49	1.73	0.46	
Isoleucine	1.13	1.47	1.25	0.34	
Leucine	2.38	2.26	2.32	0.60	1
Lysine*	7.02	9.38	8.20	1.88	2
Total			52.04		15

* Lysine-peak was eluted earlier than usual.

chloroform-methanol-water (3:48:47, v/v) mixture (288). The extracts were combined and evaporated. This gave 60.6 mg, representing about 90% of the starting material. Nothing could be recovered in the lower phase. The insoluble material remained after 2:1 chloroform-methanol extraction, the residue, weighed 5.2 mg, i.e., 7.7% of the starting material.

Table II shows the amino acid compositions of the residue and the chloroform-methanol extracted material. A comparison of the amino acid compositions of the original preparation of "peptide 9" and those of the residue and the extract indicates that the preparation has been resolved into two components. This resolution was not achieved by ion-exchange chromatography. Chloroform-methanol mixtures are widely used for the fractionation of lipids (288). That the major portion of the "peptide 9" preparation is soluble in 2:1 chloroform-methanol mixture suggests the characteristics of a lipid. On the other hand, "peptide 9" as a whole is soluble in water. The chloroform-methanol soluble portion can be extracted completely from the solution with water-rich solvents, like the "ideal-upper-phase". This suggests that the major component of the "peptide 9" preparation is a lipopeptide. Thin layer chromatography (289) of "peptide 9" on silica gel G, with chloroform-methanol-acetic acid-water (65:25:8:4, v/v) as developing solvent, showed a very faint ninhydrin-positive spot near the origin and just ahead of it, not well separated, a zone which gave a positive test for phosphorus with perchloric acid and ammonium molybdate. It is supposed that this lipopeptide is a complex of a phospholipid and

TABLE II

Amino acid analyses of chloroform-methanol (2:1) insoluble and soluble portions of the preparation "peptide 9"

Amino acid	Insoluble (residue)		Soluble (extract)	
	Calculated molar ratio	Nearest integer	Calculated molar ratio	Nearest integer
Aspartic acid	1.02	1	2.06	2
Threonine	0.16		0.86	1
Serine	0.74	1	0.92	1
Glutamic acid	1.92	2	1.41	1
Glycine	1.01	1	1.01	1
Alanine	0.52	?	1.31	1
Valine	0.29		0.87	1
Isoleucine	0.19		0.50	?
Leucine	0.34		0.94	1
Lysine*	1.28	1	0.28	
Total		6		9

* Lysine-peak appeared earlier.

a peptide.

An attempt was made to split the peptide chain by digestion with pepsin. Seven mg of "peptide 9" was incubated with 1.2 mg of pepsin (3 x crystallized, Sigma Chemical Co.) in 1.2 ml of 0.2M acetic acid at 40° for 18 hours (220). Aliquots were tested after every 2-hr. interval with ninhydrin, but no detectable color was produced even after 18 hours of digestion. Fingerprint did not show any ninhydrin spots, except for that produced by the enzyme itself. The undigested substance was recovered by gel filtration on Sephadex G-25.

(e) Chromatography of peak II of the Sephadex column on CM-cellulose

Peptides eluted in peak II of Sephadex G-25 chromatography (Fig. 2) were fractionated on Sephadex G-50. The elution pattern is shown in Fig. 6. The second peak contained most of the material applied on the column. This was chromatographed on a CM-cellulose column (1 x 30 cm) with ammonium acetate buffers (Fig. 7). The column was developed in a stepwise fashion. The material (150 mg) was absorbed onto the cellulose from a solution (5 ml) made up with 0.005M ammonium acetate buffer at pH 6. The same buffer was used to develop the column until no more material was eluted with it. The concentration and pH of the buffer were then increased to 0.05M and 7.0 respectively. The column was finally eluted with 0.1M ammonium acetate at pH 7.0. A flow rate of 10 ml per hour was maintained by applying hydrostatic pressure.

The first peak that came out of the CM-cellulose column was resolved into two ninhydrin-positive zones on paper electrophoresis

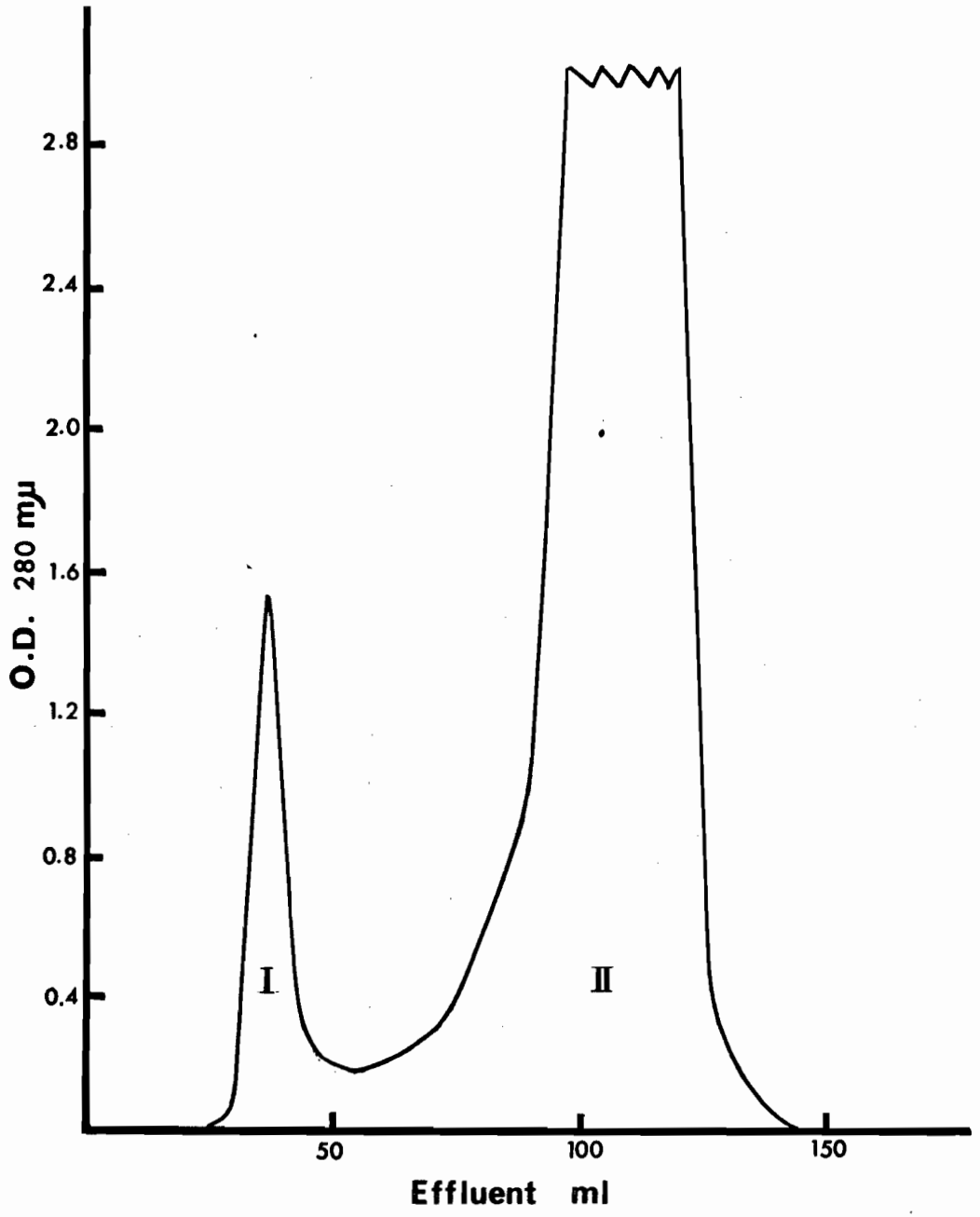


FIG. 6. Chromatography of peak II (Fig. 2) on Sephadex G-50. The column (2.0 x 40 cm) was developed with 0.2M acetic acid and 3 ml fractions were collected.

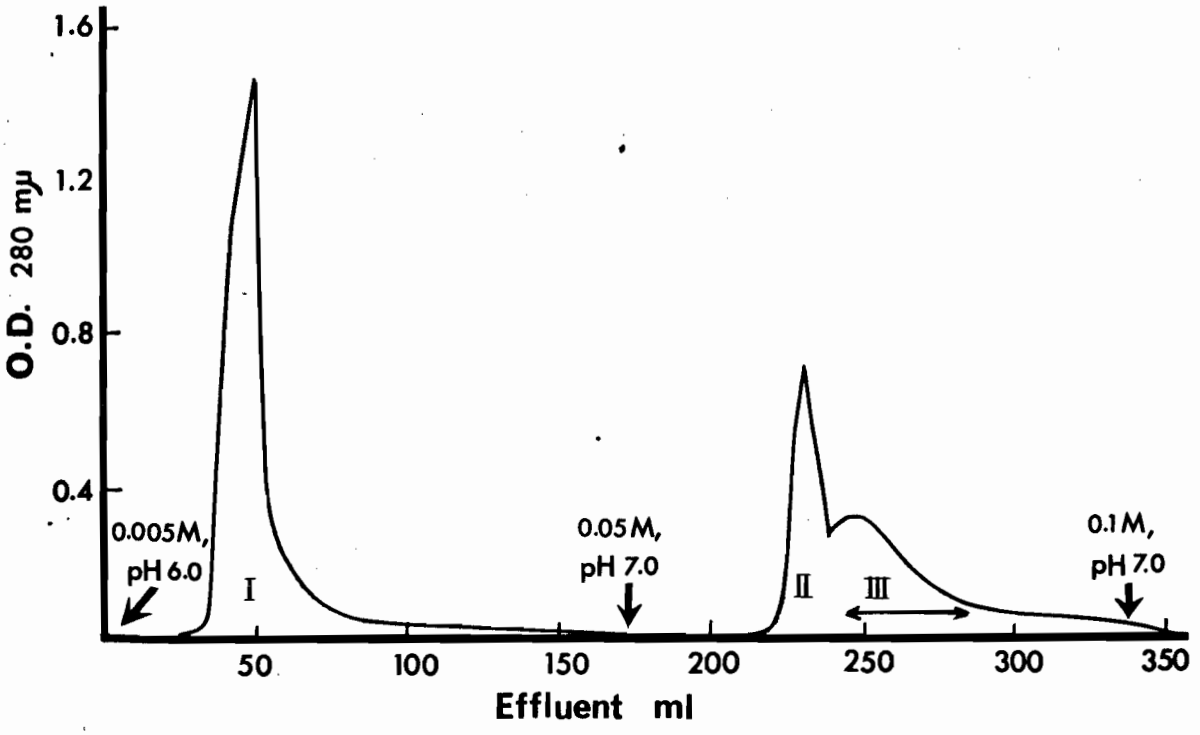


FIG. 7. Chromatography of peak II (Fig. 6) on a carboxymethyl cellulose column (1 x 30 cm) with ammonium acetate buffers. The starting buffer was 0.005M at pH 6.0. The column was developed with stepwise changes of buffers as indicated by arrows. 3.5 ml fractions were collected at a flow rate of 10 ml per hour.

(Fig. 10, E). Separation of these two compounds will be described later. The second peak constituted a minor fraction of the total peptides. The electrophoretogram showed this peak to be a mixture of slightly basic and neutral components (Fig. 10, F). The third peak showed a trailing in CM-cellulose chromatography (Fig. 7) and only those fractions were pooled which had an absorption higher than 0.10. Electrophoresis of the pooled fractions gave a homogeneous band at pH 3.5 and 6.4 (Fig. 10, G).

Other conditions of chromatography were tried, such as gradient elution starting with 0.01M ammonium acetate buffer, pH 4.5 and increasing the buffer concentration to 0.5M at pH 7.0; but no better separation could be achieved than in the stepwise elution. In gradient elution, the third peak which contained the major portion of the peptides appeared broader and more diffuse, and the first and second peaks came closer.

The peptide of the third peak (Fig. 7) was freed of ammonium acetate by gel filtration on Sephadex G-25. The yield of this peptide was 36 mg from about one gram of the lyophilized acid-extract, which represented 166 g of the fresh tissue. One kilogram of the fresh tissue, therefore, contains 220 mg of the peptide. This peptide will be referred to as peptide C.

(f) Chromatography of peak I (CM-cellulose) on DEAE cellulose

Peptides of the first peak (Fig. 7) were chromatographed on a DEAE cellulose column with a sodium phosphate buffer gradient. The

separation obtained is shown in Fig. 8. Paper electrophoresis of the peaks revealed that neither was homogeneous. The first peak contained both the neutral and the acidic component of the original material. The second peak had mostly the acidic peptide, but the amount recovered was negligible compared to that put on the column. Attempts at the separation of these two peptides on DEAE cellulose with altered conditions were not successful. Their separation was then achieved by preparative paper electrophoresis in pyridine-acetate buffer at pH 6.4. The neutral peptide has been designated peptide A and the acidic one, peptide B.

Fig. 9 shows a flow diagram of the steps involved in the preparation of different peptides from 0.1N HCl-extract of the hypothalamus. Yields have been shown for one kilogram of the fresh tissue. Fig. 10 represents a comparative study of the compositions of various fractions obtained at different stages of purification. The main peak of DEAE cellulose chromatography did not differ in its electrophoretic pattern from peak I of the CM-cellulose column and, therefore, was not shown in the figure.

(g) Amino acid analysis of peptide C

Lyophilized, air-equilibrated samples (0.5 mg) were hydrolyzed in 6N HCl for 24 hours and the hydrolysates were analyzed. Duplicate analyses were performed. An average value was used for calculations (Table III). The hydrolytic losses of amino acids were not corrected for. The recovery was 88.47%. Examination of a tryptic hydrolysate of the

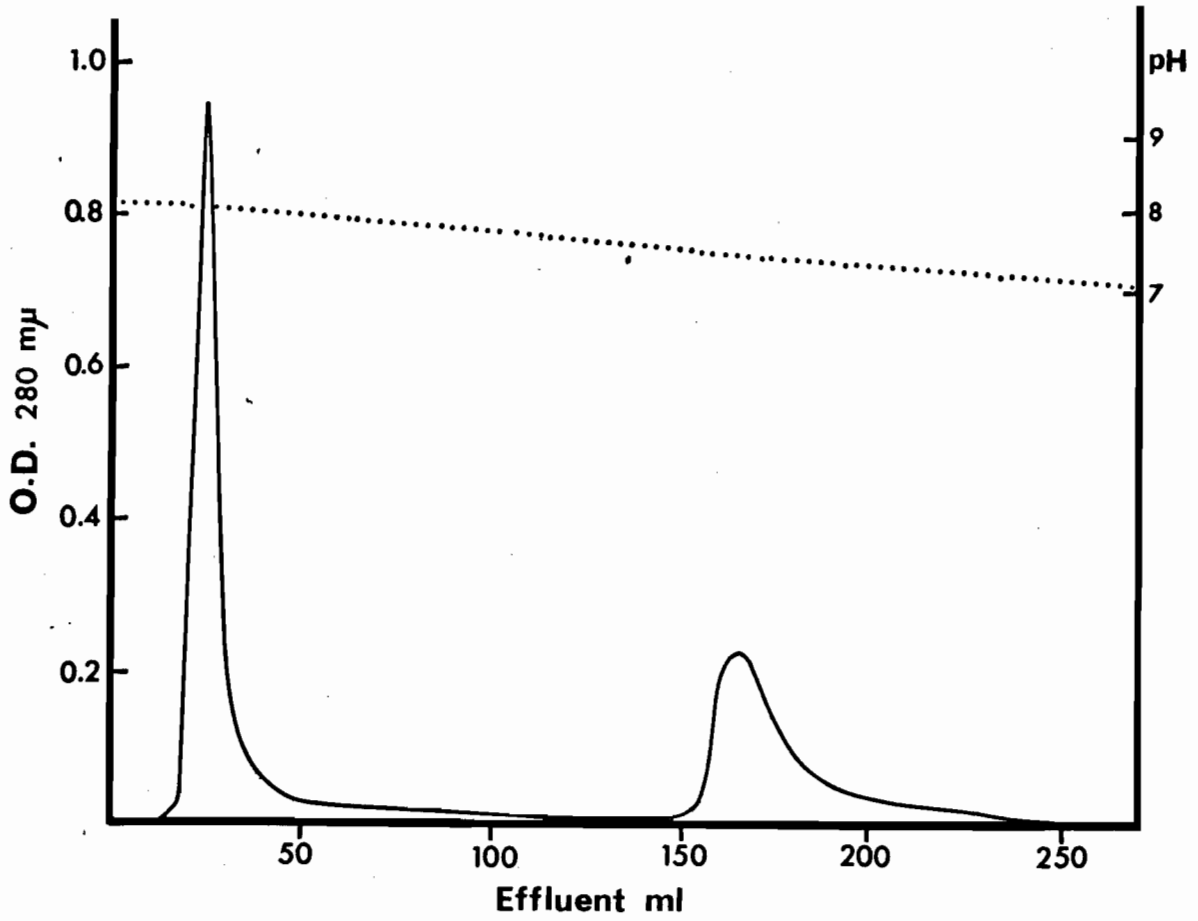


FIG. 8. Chromatography of peak I of the CM-cellulose column (Fig. 7) on DEAE cellulose. The column (1 x 35 cm) was developed with a gradient of sodium phosphate buffer (0.005M, pH 8.1 ---> 0.05M, pH 7.0), obtained through a 200 ml mixing chamber. The gradient was applied at the start. 3 ml fractions were collected at a flow rate of 24 ml per hour.

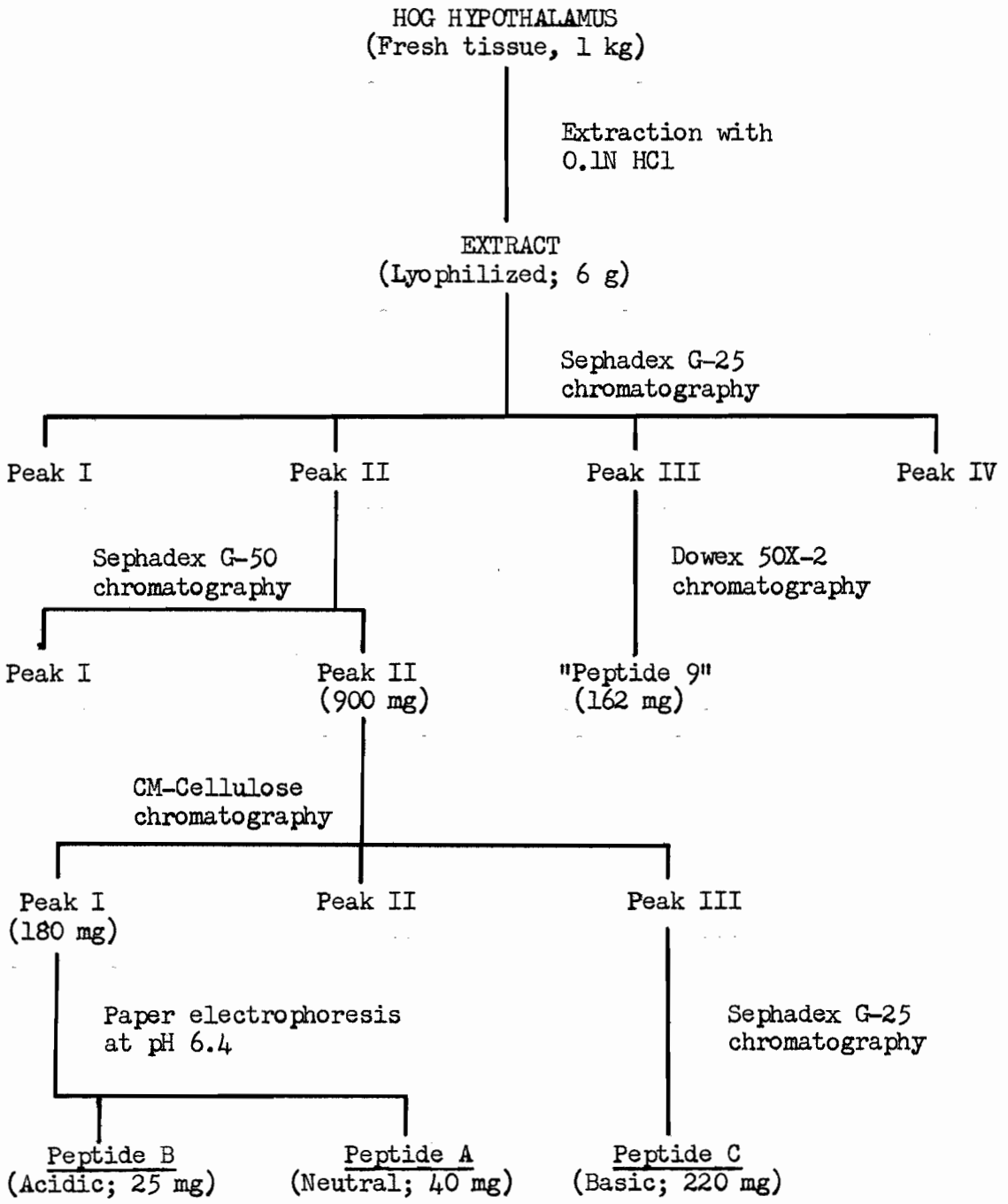


FIG. 9. Flow sheet for the isolation of peptides from fresh hypothalamus.

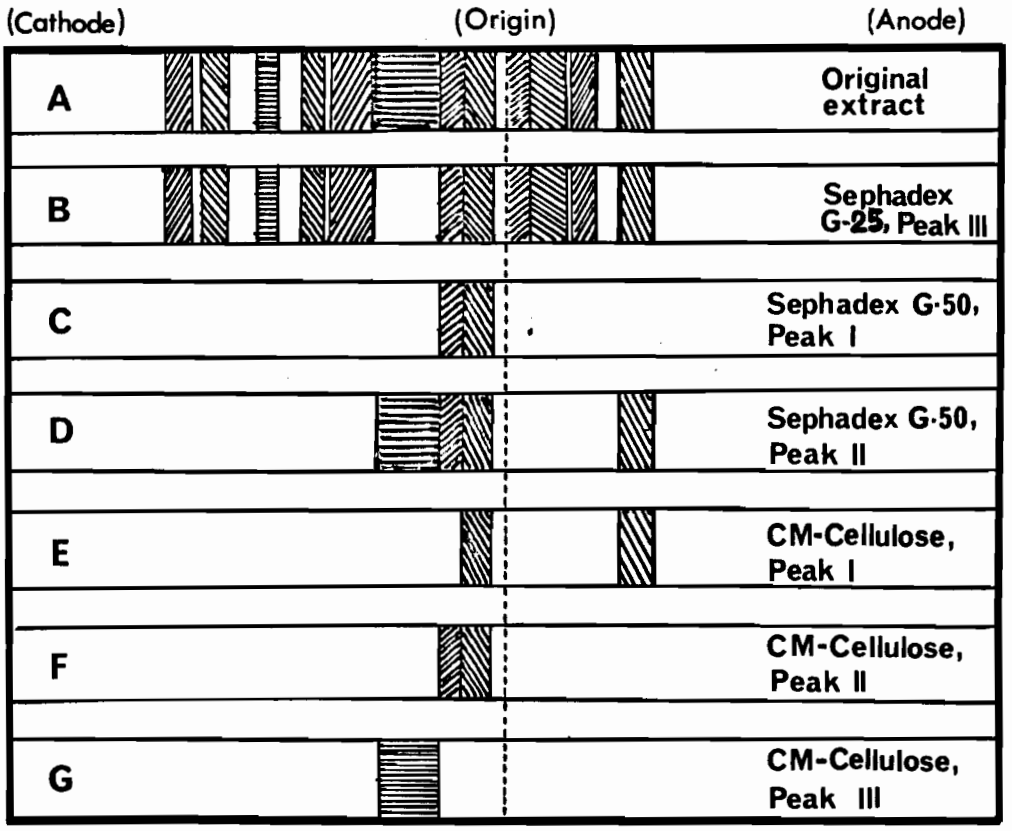


FIG. 10. Schematic representation of electrophoretic patterns at various stages of fractionation of the acid extract of fresh hypothalamic tissue. Electrophoresis was performed on Whatman No. 3MM paper in pyridine-acetate buffer, pH 6.4, at 750 volts for 150 min. Bands were located by ninhydrin spray.

TABLE III

Analysis of the acid hydrolysate of peptide C

Amino acid	Amino acid residue (grams per 100 g of peptide)[a]	Calculated molar ratio	Nearest integral value (No. of residue per mole)
Aspartic acid	6.78	5.87	6
Threonine	4.13	4.77	5
Serine	4.18	5.24	5
Glutamic acid	7.94	6.23	6
Proline	6.30	5.87	6
Glycine	4.73	8.09	8
Alanine	5.71	7.92	8
Valine	5.36	5.11	5
Methionine	2.10	1.35	1
Isoleucine	3.66	3.09	3
Leucine	7.05	6.05	6
Tyrosine	2.19	1.46	1
Phenylalanine	3.28	2.21	2
Lysine	10.78	8.11	8
Histidine	4.98	3.45	3
Arginine	9.30	5.65	6
Tryptophan	1.93		1[b]
Total	90.40		80[c]

a Average of two determinations.

b Assumed value.

c Calculated molecular weight, 8,696.

polypeptide revealed the presence of one tryptophan-containing peptide. When the tryptophan value is added, the recovery is 90.40%. Ammonia was not calculated, because its value was not reliable. The low recovery might be due to the moisture content of the sample. The number of residues of individual amino acids was calculated from the molar ratio and expressed as nearest integers. The molar ratio was obtained by finding a suitable average value by trial. The number of amino acid residues represents a minimum value. Peptide C contains a total of 80 amino acid residues and its calculated molecular weight is 8,696. All the amino acids except cystine are present in this peptide. The absence of cystine suggests that the molecule is composed of a single peptide chain.

(h) Isoelectric pH of peptide C

Electrophoresis on Whatman No. 3MM paper was used for the determination of the isoelectric pH of peptide C. Movement due to electroendosmosis was corrected by the use of a neutral compound, DNP-ethanolamine, as marker. The electrophoretic movement of the peptide at different values of pH is shown in Fig. 11. The peptide has a net zero charge at pH of about 8.2. The basicity of peptide C is also indicated by its amino acid composition. There is a net excess of 2 basic residues, if all the α-carboxyl groups of aspartic and glutamic acids are assumed to be free.

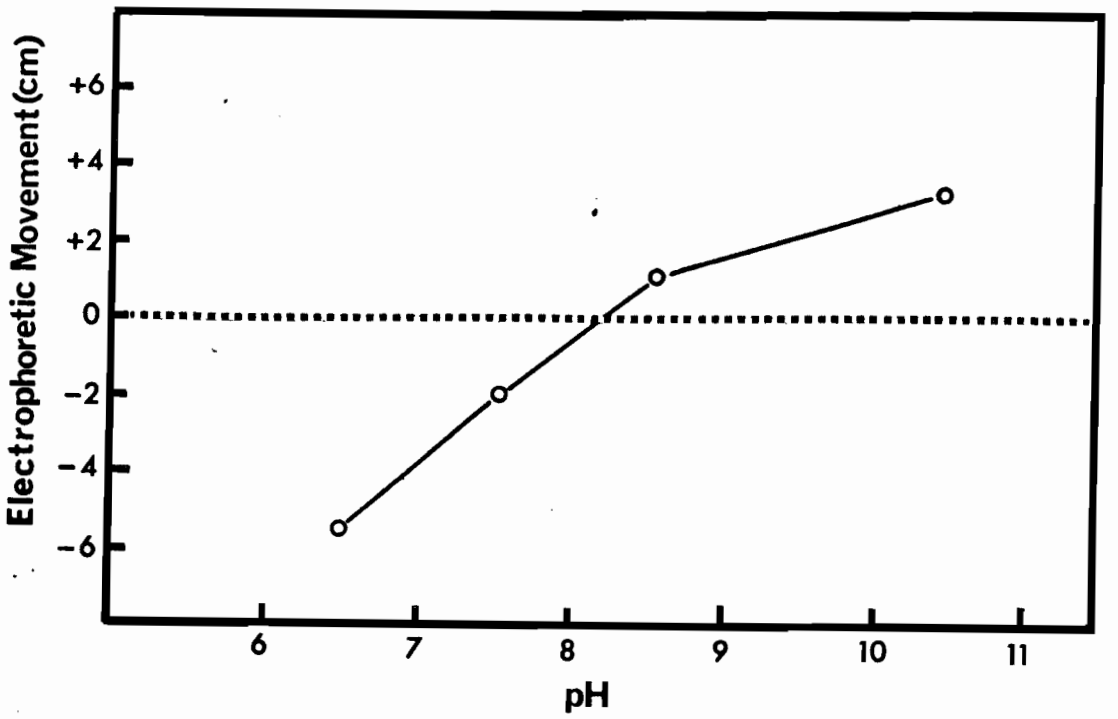


FIG. 11. Electrophoretic mobilities of peptide C at different pH. Pyridine-acetate (pH 6.5), collidine-acetate (pH 7.55) and sodium bi-carbonate (pH 8.6 - 10.5) buffers of ionic strength 0.10 were used. Electrophoresis was performed at 800 volts for 2.5 hrs. Electro-endosmotic movements were corrected by using DNP-ethanolamine as marker.

(i) Digestion of peptide C with trypsin

The tryptic digestion was carried out in 0.1M ammonium bicarbonate buffer, pH 8.0 at 37° for 16 hours (290). The enzyme-substrate ratio was 1:100 by weight. Fig. 12 shows the fingerprint of the tryptic digest. Peptide C contains 8 lysine and 6 arginine residues. At least 15 peptides can be expected if all the lysyl and arginyl bonds are hydrolyzed. Peptide C yielded 12 peptides, one of which gave a positive reaction for tryptophan (291). Inability of trypsin to hydrolyze three peptide bonds in peptide C may be due to some special sequences in those regions of the molecule.

(j) End groups of peptide C

The digestion of peptide C with carboxypeptidase A was carried out in Tris buffer as described in the Experimental. Aliquots were analyzed by the fingerprint method. Detectable quantities of leucine were liberated within 2 minutes of digestion. At 9 min., free amino acids found were leucine > alanine > valine, and traces of phenylalanine, tyrosine, glycine and glutamine. At 25 min., leucine levelled off, but alanine, valine and glutamine increased. Visual estimations were checked by scanning the cut-strips of fingerprints in a Densicord (Photovolt Corp.) scanner. Attempts were made to integrate the peak areas for comparison with the standard but without success, since even with the utmost care the area of the spots could not be kept the same in different fingerprints. However, it is apparent from this study that the polypeptide contains a C-terminal

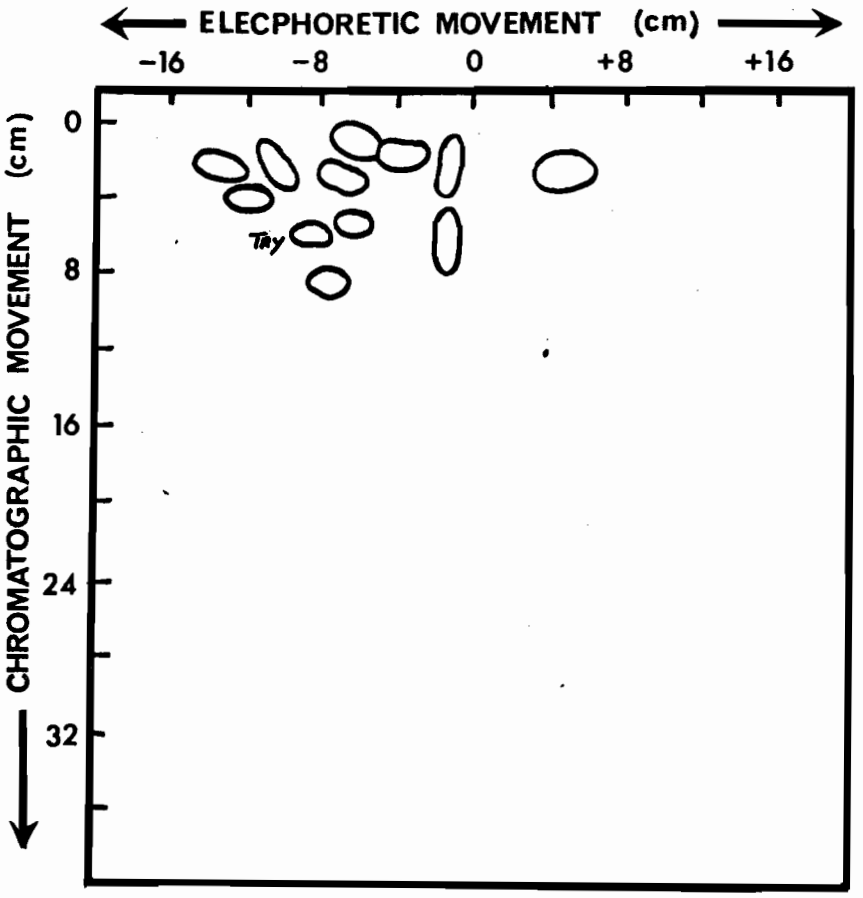


FIG. 12. Fingerprint of the tryptic digest of peptide C. Electrophoretic separation was carried out in pyridine-acetate buffer, pH 6.4 at 1000 volts for 2 hrs. The upper phase of n-butanol-acetic acid-water (4:1:5, v/v) mixture was used for a 16 hr. descending chromatography. Try = Tryptophan.

leucine and the C-terminal sequence is probably Val-Ala-Leu-COOH.

Preliminary studies with 2,4-dinitrofluorobenzene suggested that the peptide contained a free N-terminal aspartic acid residue. Quantitative studies were not possible due to unavailability of the material. The peptide was found to be labile in the laboratory conditions. Even when stored in the dry state in the cold, it slowly degraded forming a neutral and more basic component detectable by electrophoresis at pH 6.4. For this reason, the peptide had to be purified by electrophoresis before use and this encountered a heavy loss of material. The amino acid composition of the neutral component did not differ significantly from that of the original peptide. The basic component showed traces of most of the amino acids in amounts too small to calculate plus a large quantity of ammonia.

(k) Amino acid composition of peptide A and peptide B

Table IV shows the amino acid compositions of the neutral peptide A and the acidic peptide B. The molar ratio and the number of residues were calculated as described previously. Peptide A contains a minimum of 81 residues with a calculated molecular weight of 8,582. This peptide is as large as peptide C described before. There is a similarity between these two peptides in regard to the distribution of hydrophobic residues. The major difference appears in the content of basic residues. The isoelectric pH of peptide A in collidine acetate buffer, ionic strength 0.10, was found to be near 7.2. This indicates that of the total 14 acidic residues, 4 or 5 may be amidated.

TABLE IV

The amino acid analyses of peptide A and peptide B

Amino acid	Peptide A		Peptide B	
	Molar ratio	Nearest integer	Molar ratio	Nearest integer
Aspartic acid	7.44	7	1.86	2
Threonine	4.62	5	0.94	1
Serine	5.18	5	3.93	4
Glutamic acid	6.81	7	2.41	2
Proline	6.50	7		
Glycine	7.06	7	2.27	2
Alanine	8.09	8	1.86	2
Valine	9.15	9	0.89	1
Methionine	1.25	1		
Isoleucine	3.68	4	0.40	
Leucine	7.06	7	1.02	1
Tyrosine	0.89	1		
Phenylalanine	1.90	2		
Lysine	4.62	5	0.47	
Histidine	2.21	2	0.24	
Arginine	3.50	4		
Total		81		15
Calculated Molecular Weight		8,582		1,424

Peptide B was acidic. The amino acid analysis of this peptide showed it to contain a minimum of 15 residues with a calculated molecular weight of 1,424. It appears that peptide B does not contain a lysine or isoleucine residue; the molar ratios of these amino acids are far from integral values. Due to unavailability of substance, no further studies could be made with this peptide.

2. Peptides in the Hypothalamic Percolates

Fig. 13 shows the percolation pattern of hog hypothalamic powder. The percolate fractions were analyzed by the Lowry and ninhydrin methods. Results were expressed as mg Lowry peptide and mg leucine equivalent per fraction. The earlier fractions, fraction nos. 1 to 29, contained much lipid, which became butter-like on concentration. It is interesting that even the acetone-dried powder contains lots of fatty materials. The amount of lipid progressively decreased in the course of percolation. As for peptides, earlier fractions showed variable Lowry and ninhydrin colors. The quantity of peptides represented by the Lowry reaction was always higher than the actual amounts present in these fractions. This was due to the formation of turbidity upon reaction with the alkaline reagent, which could not be completely removed by centrifugation. Fractions 4 to 16 were analyzed by ninhydrin after HCl-hydrolysis. The increase in ninhydrin color after acid hydrolysis was very little in these fractions. Whether this slight increment in color is due to the presence of peptides or other materials which on acid hydrolysis liberate ammonia or ninhydrin-positive compounds other than amino acids, is hard to ascertain. Fractions 17

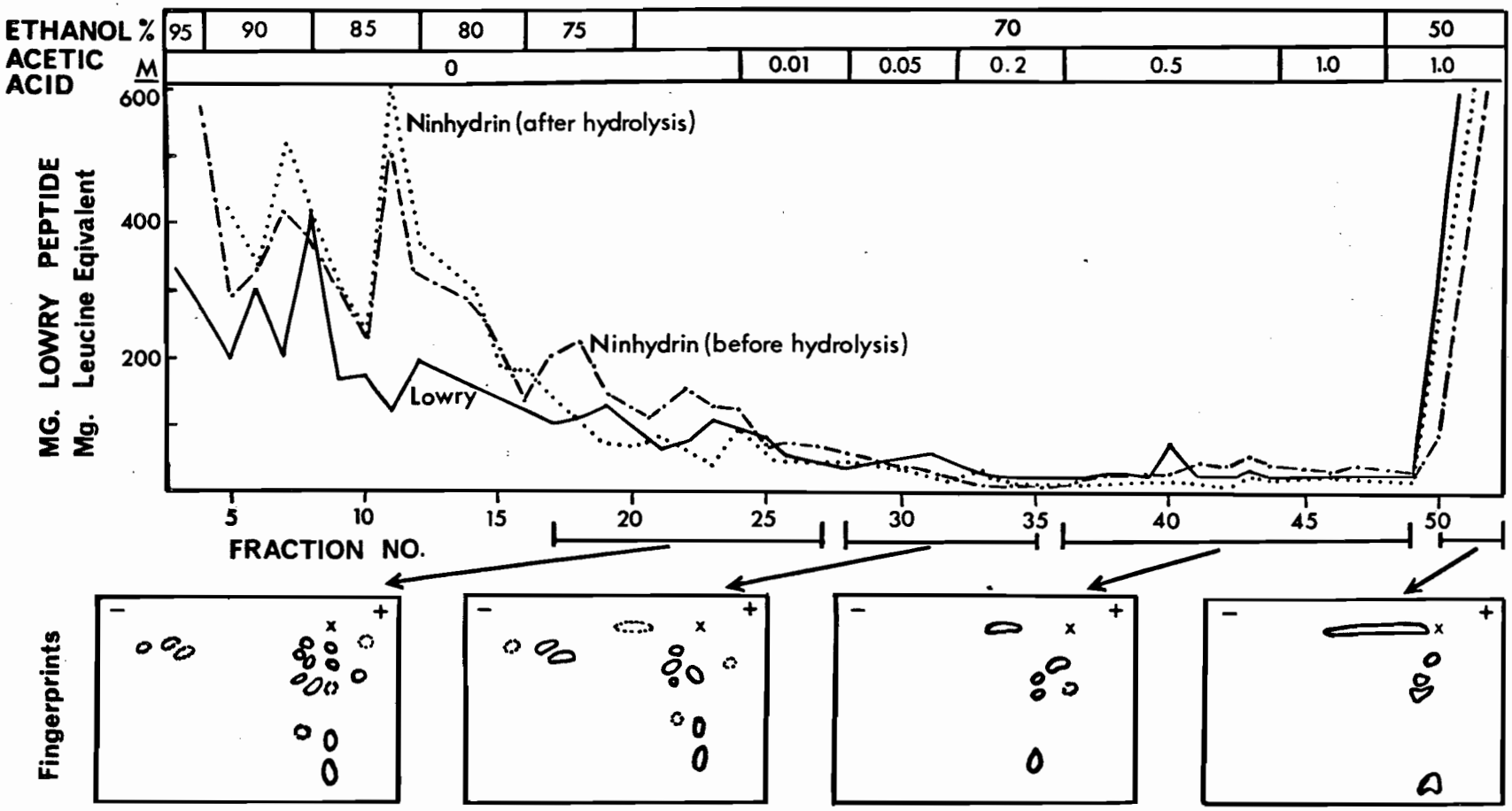


FIG. 13. Percolation of 390 g of hog hypothalamus acetone powder with the solvents indicated in the upper part of the figure. Peptide content and ninhydrin-reacting materials are shown as mg per fraction (1000 ml). Fingerprints of fractions having similar patterns are given. Samples were applied at a point marked X. Electrophoresis was performed in pyridine-acetate buffer, pH 3.7 at 3000 volts for 1 hr. and chromatography, in butanol-acetic acid-water (4:1:5, v/v) for 16 hrs.

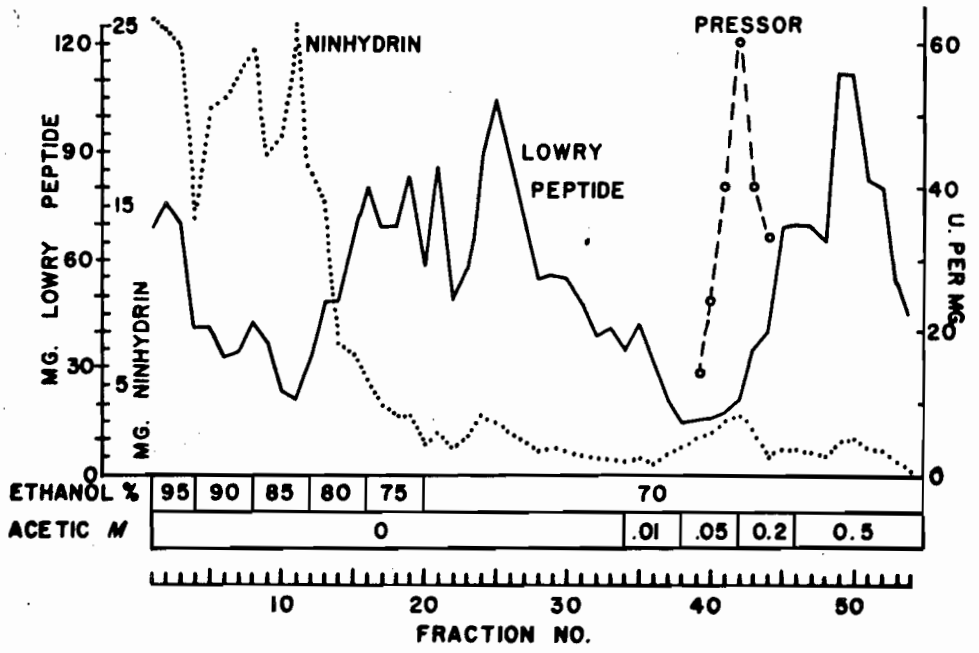


FIG. 13a. Ninhydrin reacting materials, peptide content and pressor activity in fractions obtained by percolation of 20 g of beef posterior pituitary powder with solvents indicated. The fraction size was 50 ml. (Reproduced from Saffran et al. (270)).

NOTE: Large quantities of peptide substances appeared in fractions eluted with 70-80% ethanol (cf. Fig. 13, the percolation pattern of 390 g of hog hypothalamus powder. Though the scales are different, it can be seen that the peptide content of the hypothalamus is less than 1/10 that of the posterior pituitary).

onward were hydrolyzed with NaOH. The ninhydrin color values of the hydrolyzed samples were lower than those of the unhydrolyzed samples, except for the last few fractions. It seems probable that acid hydrolysis trapped ammonia, giving increased ninhydrin color values in earlier fractions. The source of ammonia may be the destruction of amides, notably glutamine. Fingerprints showed that these fractions contained mostly amino acids. Peptides were detected on fingerprints of fractions 28 onward, but their concentrations were insignificant compared to those of free amino acids. Fractions 50 to 52, eluted with 50% ethanol in 1.0M acetic acid, contained significant amounts of peptides. This is evident by the sharp rise in ninhydrin and Lowry curves (Fig. 13). Fingerprints of these fractions showed relatively low concentrations of free amino acids.

The percolation pattern and the fingerprint analyses clearly showed that the major peptides of the hypothalamic tissue powders were eluted only in the last few fractions. These fractions were free from lipid materials as opposed to the earlier fractions. In order to isolate and purify hypothalamic peptides, our first choice was to start with these fractions.

3. Isolation of Peptides from Percolate Fractions 50-52

(a) Chromatography on Sephadex G-25

Concentrated fractions 50 to 52 were lyophilized in batches. The total yield of the dried material was 2.34 g. A 500 mg sample of the lyophilized powder was extracted twice with 1.5 ml portions of 0.2M

acetic acid, spinning down the insoluble substances. About 60% of the material went into solution. This was chromatographed on Sephadex G-25 and the eluate was analyzed by UV absorption and ninhydrin reactions (Fig. 14). Some UV absorbing materials were eluted after free amino acids. They did not show ninhydrin color before or after alkaline hydrolysis. The peptidic material came out of the column unretarded in the first three peaks, marked zone A. Although the resolution of the peaks was very poor, it seemed probable that their separation could be achieved by filtration through gels of lower cross-linkage. Sephadex G-25 chromatography, however, separated free amino acids and other non-peptide materials from the peptide preparation.

(b) Chromatography on Sephadex G-50

Peptides of zone A (Fig. 14) were chromatographed on Sephadex G-50. The resolution obtained is shown in Fig. 15. Electrophoretic mobilities of the peaks are shown in Fig. 17(B-D). Zone A contained about 45% of the starting material. The relative distribution of this material in peaks I, II and III was 7%, 25% and 68% respectively. Since most of the peptides appeared in the third peak, it was decided to purify this fraction further. Electrophoresis showed at least three different peptides in this peak (Fig. 17,D). A highly basic component, appearing in this peak, had mobility like free lysine and presumably was not a peptide. This basic substance might have been carried over from Sephadex G-25 chromatography and was not separated on Sephadex G-50.

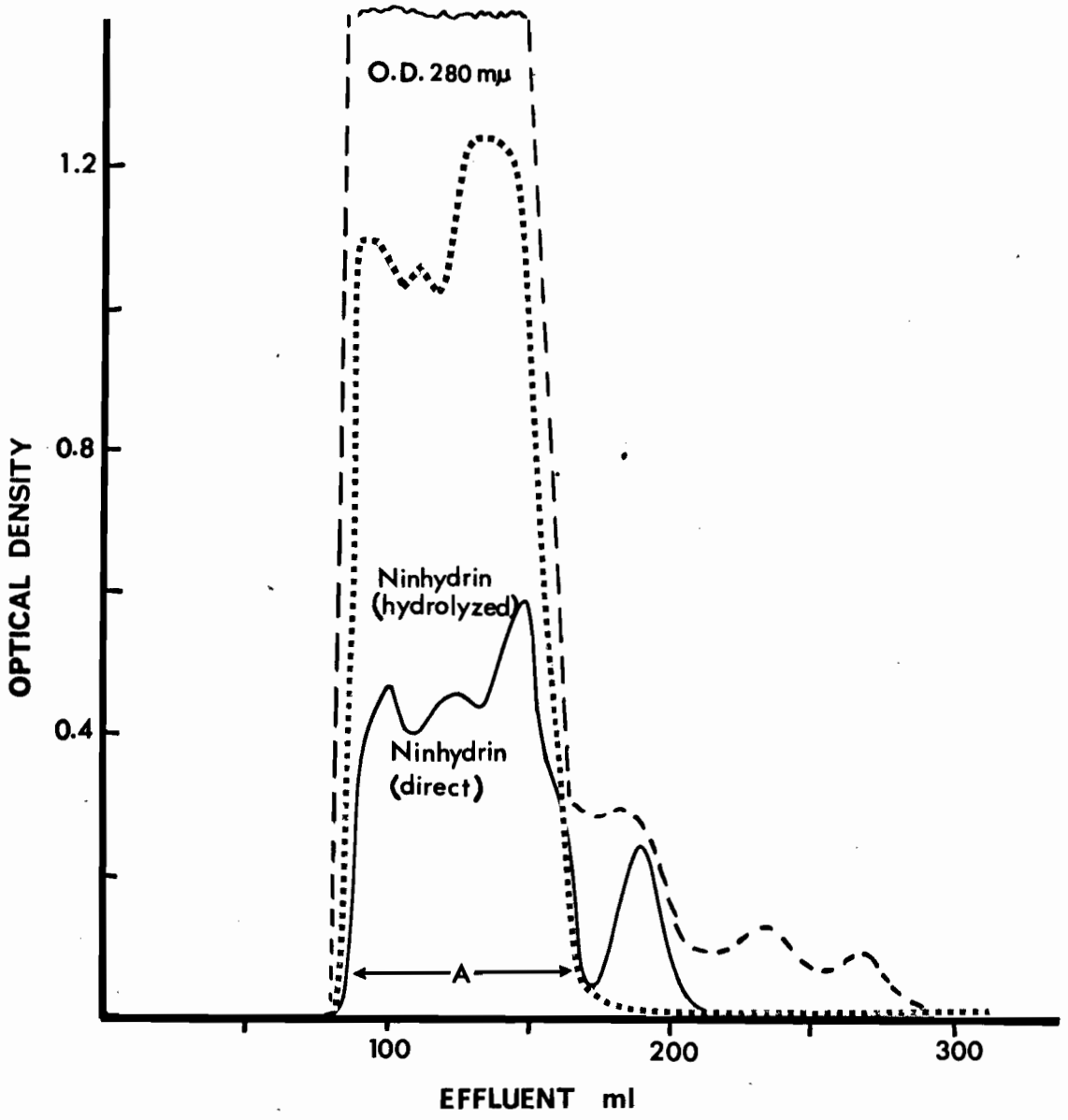


FIG. 14. Gel filtration of percolate fractions 50-52 on a Sephadex G-25 column (1.5 x 105 cm). The elution was performed with 0.2M acetic acid and the effluent was collected in 3.5 ml fractions. Aliquots from individual fractions were analyzed by ninhydrin reaction directly (——) and after hydrolysis with NaOH (·····). The optical density of the effluent is shown (- - -).

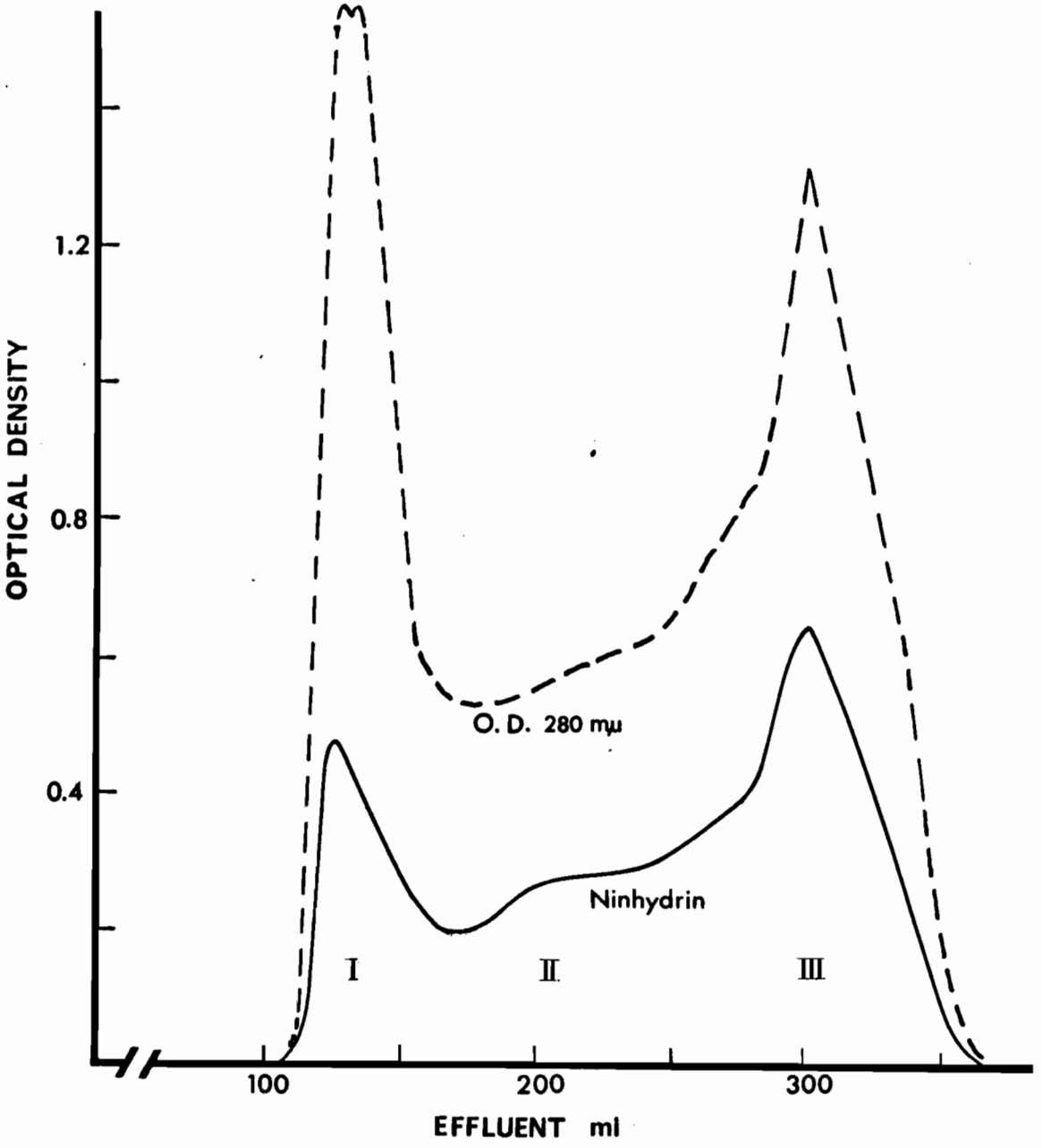


FIG. 15. Chromatography of zone A (Fig. 14) on a Sephadex G-50 column (2.5 x 105 cm) with 0.2M acetic acid. 3.5 ml fractions were collected. Fractions were analyzed by absorption at 280 mμ and with ninhydrin (——).

Sephadex gel filtrations were reproducible so long as the columns were not overloaded with the materials. The same column was used for different batches and practically no variation was observed in the elution pattern.

(c) Chromatography of peak III on CM-cellulose

Peptides eluted in the third peak of Sephadex G-50 chromatography were chromatographed on CM-cellulose columns with ammonium acetate buffers. In a typical experiment, 300 mg of peptides were absorbed on a column (1.5 x 37 cm) at pH 5.0 and the column was developed with a gradient of pH and molarity (Fig. 16). Two peaks, appearing at the very outset, were unabsorbed or slightly absorbed. They comprised the neutral components of the peptide mixture (Fig. 17, E-F). The major component was eluted at a pH higher than 7.0 and at a molarity of about 0.4. On electrophoresis, this material (peak II, Fig. 16) showed two bands; the highly basic component, mentioned previously, was also eluted along with the peptide (Fig. 17,G). Separation of this basic material from the peptide was achieved by gel filtration on Sephadex G-25 (Fig. 17,H). Gel filtration also removed ammonium acetate. The yield of this peptide was 147 mg; that is, about 49% of the total material put on the CM-cellulose column. Peak I contained about 6% and peak X, less than 1% of the total peptides. The recoveries from CM-cellulose columns were variable in different batches, ranging from 35 to 55%.

The peptide isolated from peak II (Fig. 16) represents the

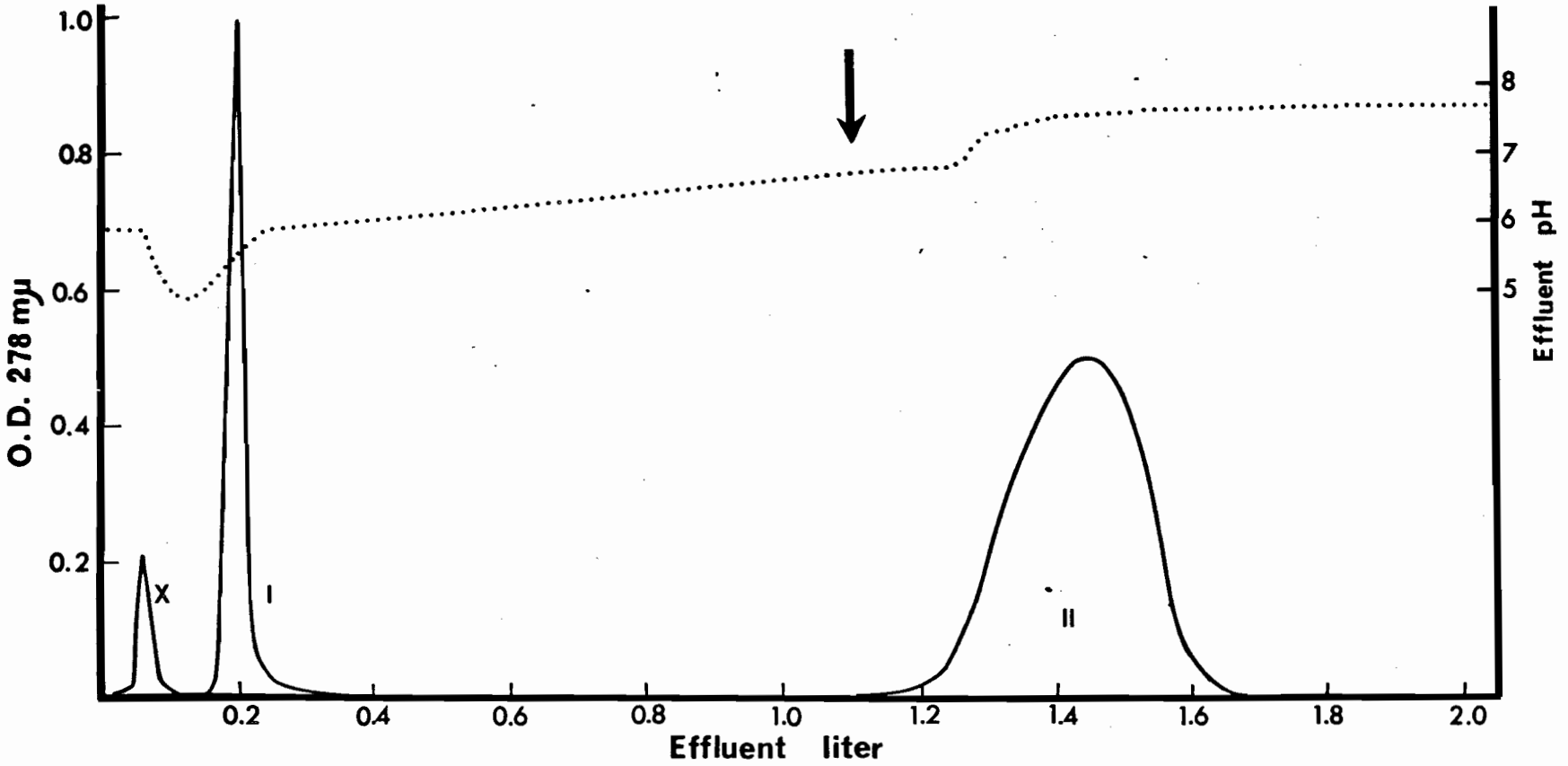


FIG. 16. CM-cellulose column (1.5 x 37 cm) chromatography of peak III (Fig. 15). The column was developed at a flow rate of 15-20 ml per hr. Ammonium acetate buffer gradient (0.05M, pH 5.9 ---> 0.1M, pH 6.9) was applied, after 50 ml of elution, through a 500 ml mixing chamber. The second gradient (0.1M, pH 6.9 ---> 1.0M, pH 8.0) was applied at a point indicated by the arrow. The pH of the effluent is shown by the upper curve.

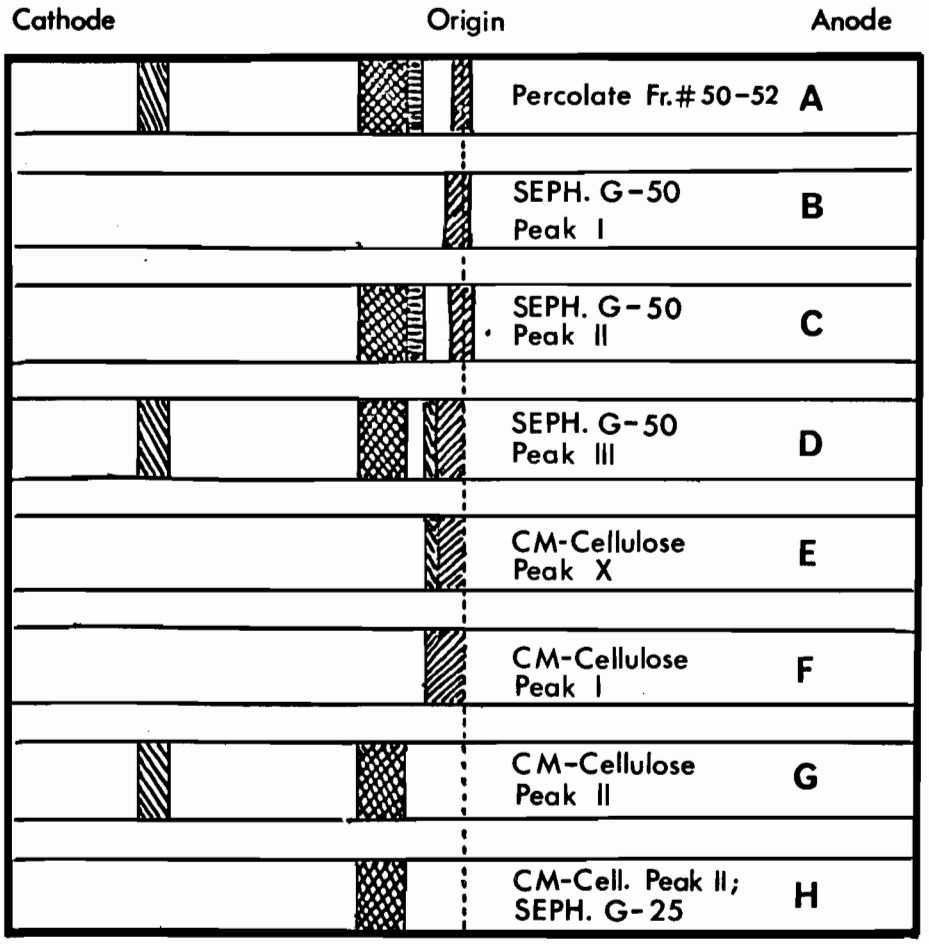


FIG. 17. Schematic diagram of electrophoretic patterns of fractions obtained at various stages of purification of a polypeptide from percolate Fr. No. 50-52. Electrophoresis was carried out in pyridine-acetate buffer, pH 6.4 at 750 volts for 150 min. The bands were located by ninhydrin spray.

major peptide of percolate fractions 50 to 52, and accordingly, has been designated Polypeptide 50-52. Fig. 18 shows a flow diagram of the steps involved in the isolation of this polypeptide. Electrophoretic patterns of fractions containing the peptide at different stages of purification are shown in Fig. 17.

4. Characterization of Polypeptide 50-52

(a) Electrophoretic mobility

Fig. 19 shows the electrophoretic mobility of the polypeptide at different values of pH. The 2,4-dinitrophenyl derivative of ethanolamine was used as a marker to correct movements due to electroendosmosis. The polypeptide has an isoelectric point near pH 9.4 in sodium bicarbonate buffer of ionic strength 0.10. It moved as a single band over the pH range 6 to 12. At a lower pH, the polypeptide had a tendency to diffuse and trail on paper.

(b) Ultracentrifugal study

The sedimentation pattern (Fig. 20) was obtained in a Spinco, model E, analytical ultracentrifuge. The protein concentration was 1% in 0.15M NaCl. A single peak appeared with an observed sedimentation coefficient of 1.240×10^{-13}.

(c) Ultraviolet absorption spectrum

The polypeptide, dissolved in 0.15M NaCl, was scanned in the region of 240 to 310 mμ in a Beckman DU spectrophotometer. It had an

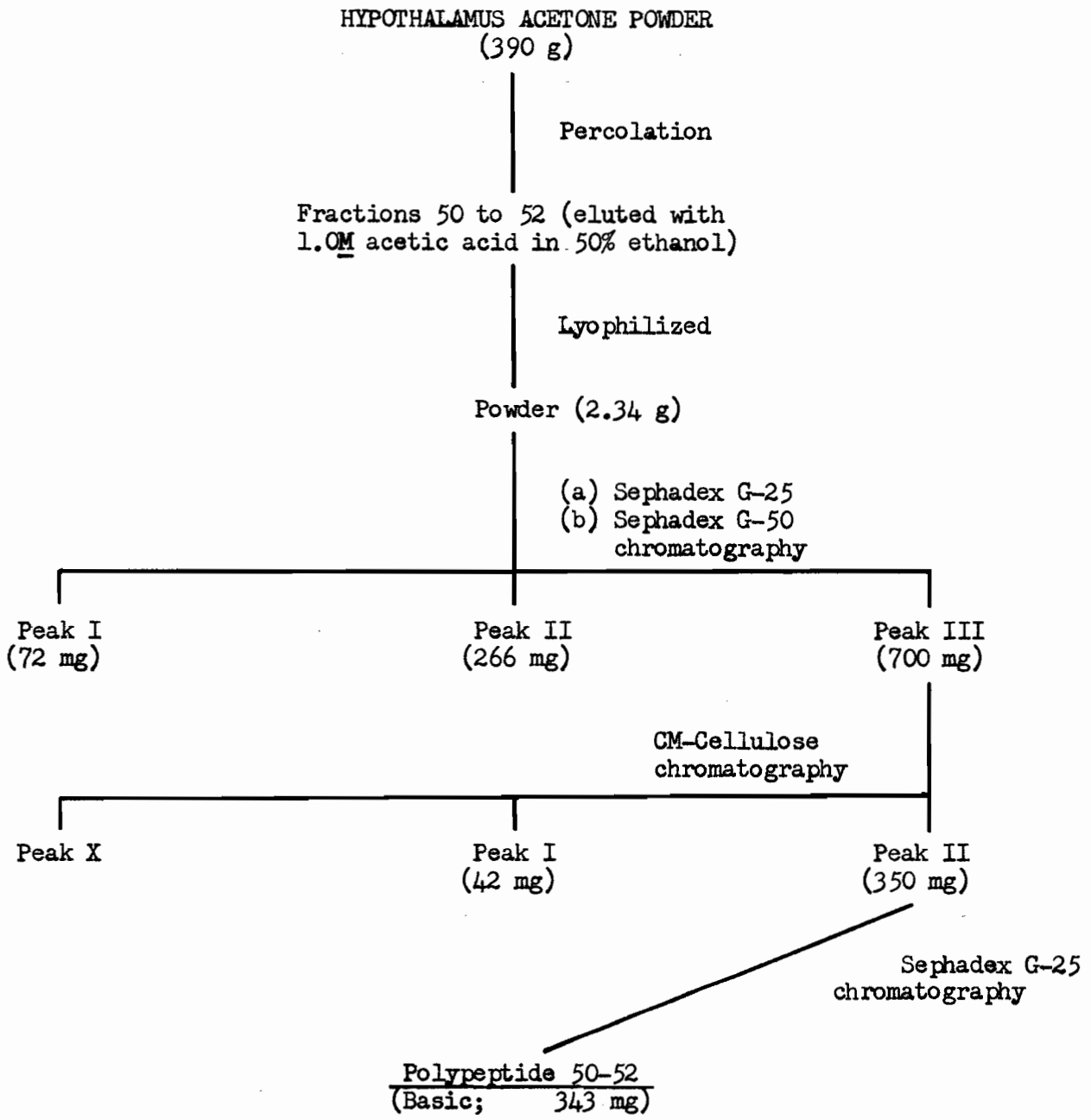
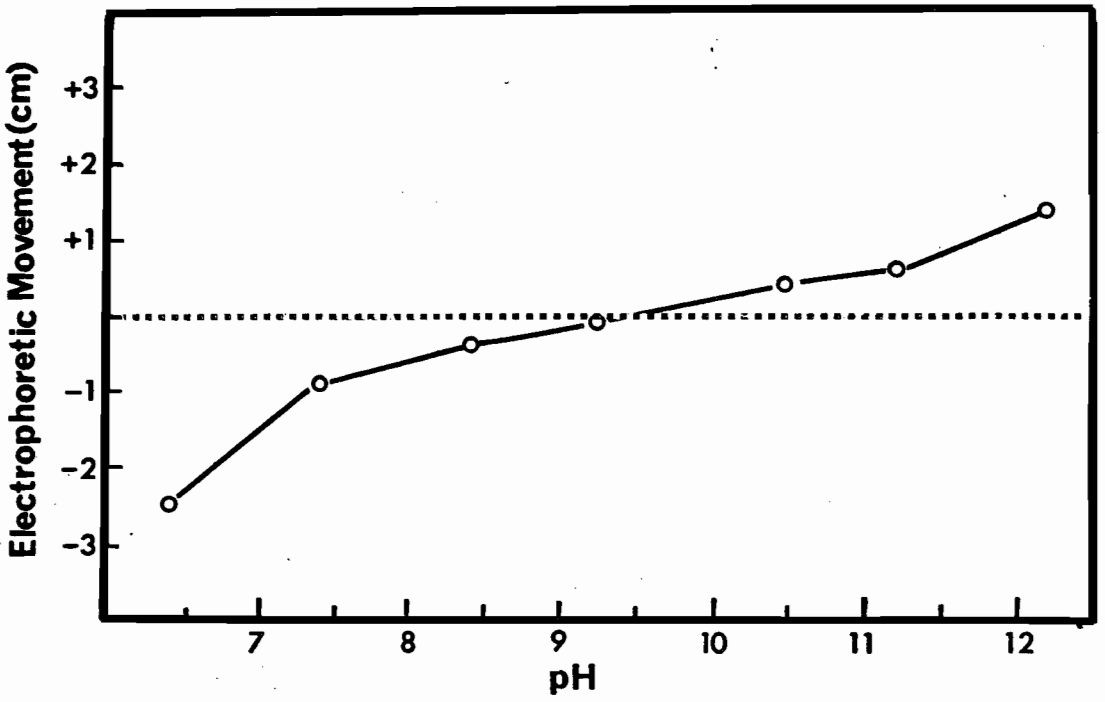


FIG. 18. Flow diagram of the steps involved in the purification of a polypeptide from percolate fractions No. 50 to 52.



BUFFERS

pH	μ	Composition
6.4	0.10	NaH_2PO_4/
7.4	0.10	Na_2HPO_4
8.43	0.10	$NaHCO_3$/
9.25	0.10	Na_2CO_3
10.50	0.10	"
11.2	0.05	Na_2CO_3
12.2	0.025	NaOH/HOAc

FIG. 19. Electrophoretic mobilities of polypeptide 50-52 at different values of pH. Electrophoreses were performed on Whatman No. 3MM paper at 1000 volts for 2 hrs. The buffers employed are shown. The ionic strength (μ) of buffers of pH higher than 10 had to be lowered to keep the current within the limit. Electroendosmotic movement was corrected by the use of 2,4-dinitrophenylethanolamine as marker.

FIG. 20. Ultracentrifugal pattern of polypeptide 50-52. Sedimentation proceeds from left to right. Pictures were taken at 12, 27, 42, 57 and 72 minutes after the rotor speed reached 59,780 r.p.m. The Protein concentration was 1% in 0.15M NaCl. A synthetic boundary cell was used. Temperature 25°; observed S = 1.240.

absorption maximum at 275 mμ and a minimum at 255 mμ. The protein had $E_{cm}^{1\%} = 6.98$ at λ_{max}, and at 280 mμ, $E_{cm}^{1\%}$ was 6.68. Assuming a molecular weight of 14,300 for the protein, the molar extinction coefficient, ε, is equal to 9.981×10^3.

(d) Amino acid composition

A sample of 2.5 mg of the lyophilized polypeptide was weighed in a glass stoppered test tube and dried at 105° for two hours. The dried sample was allowed to cool in the stoppered-tube to room temperature and a final weight was taken. The material was then dissolved in a known volume of three times glass-distilled 6N HCl and aliquots representing 0.48 mg were hydrolyzed for 24, 48 and 72 hours at 110°. The hydrolysates, after the removal of HCl, were quantitatively transferred to the analyzer column and analyzed by the usual procedure. Table V shows the amino acid analyses. An average or extrapolated value was used to calculate the molar ratios of the constituent amino acids and hence the number of residues per mole of the polypeptide. The recovery, calculated as amino acid residues, amounts to 83.81%, and when the tryptophan content is included, the recovery is 85.11%.

Tryptophan was estimated by the spectrophotometric method. The value for tyrosine, estimated by this method, was in good agreement with the value obtained from the amino acid analysis. For example, 4.8 mg of the polypeptide yielded 0.87 μmole of tyrosine by the spectrophotometric method and 0.91 μmole by the amino acid analysis method. This gives an indication of the reliability of the spectrophotometric method

TABLE V

Analyses of the acid hydrolysates of polypeptide 50-52

Amino acid	Amino acid residue (grams per 100 g of protein)				Amino acid residues per mole	
	24-hr. hydrolysate	48-hr. hydrolysate	72-hr. hydrolysate	Average or extrapolated value	Calculated	Assumed
Aspartic acid	6.33	6.21	6.33	6.25	9.06	9
Threonine	3.44	3.23	3.23	3.44[a]	5.55	6
Serine	5.49	5.14	4.88	5.83[a]	11.06	11
Glutamic acid	6.97	6.97	7.10	7.01	9.00	9
Proline	4.37	4.56	4.56	4.39	7.65	8
Glycine	6.50	6.50	6.62	6.54	18.96	19
Alanine	4.69	4.76	4.76	4.73	11.03	11
Valine	1.19	1.49	1.68	1.45	3.10	3
Methionine	1.05	0.92	0.79	0.92	1.20	1
Isoleucine	1.58	1.70	1.81	1.69	2.40	2
Leucine	5.54	5.43	5.66	5.54	8.13	8
Tyrosine	3.10	3.10	3.10	3.10	3.14	3
Phenylalanine	5.74	5.74	6.03	5.83	6.55	7
Lysine	8.33	8.46	8.59	8.46	10.89	11
Histidine	6.31	6.31	6.31	6.31	7.62	8
Arginine	12.34	12.34	12.03	12.23	13.17	13
Ammonia	1.29	1.34	1.45	1.23[ab]	12.69	13[b]
Tryptophan				1.30	0.89[c]	1
Total				85.11		130[d]

a Extrapolated value for zero hydrolysis time.

b Not included in the total.

c Spectrophotometric value.

d Calculated molecular weight, 14,300.

for the determination of tryptophan which was estimated in conjunction with tyrosine. The tyrosine-tryptophan ratio was found to be 3.3.

The total number of amino acid residues, calculated from the molar ratios, gives a minimum value of 130 residues per mole of the polypeptide, with a calculated molecular weight of 14,300. Since only one tryptophan-containing peptide was found among the tryptic peptides, it is probable that the protein contains only one tryptophan residue. The calculated molecular weight will then approach very near the actual molecular weight of the protein. A sedimentation coefficient of 1.24S (uncorrected) is not incompatible with the assumed molecular weight.

The low recovery of the protein, as calculated from the amino acid analyses, is probably a reflection of its moisture content. The presence of some non-peptide group or groups in the molecule may lower the percentage recovery. Such groups, however, were not identified; but the NH_2-terminal of this protein was found to be unreactive. The possibility exists that the N-terminal is covered by a non-peptide moiety.

(e) End groups of polypeptide 50-52

(i) Dinitrophenylation

Repeated attempts failed to identify any DNP derivative of the N-terminal residue of the polypeptide either in the ether phase or in the aqueous phase of a hydrolysate of the dinitrophenylated protein. On the other hand, the aqueous layer contained ε-DNP-lysine and Im-DNP-histidine. Yields of these two compounds, calculated without correction

for hydrolytic losses, were 87 and 59 per cent respectively. Since the hydrolytic recovery of DNP-amino acids is usually in the range of 50 to 90 per cent (239), it may be assumed that most, if not all, of the ε-NH_2 groups of lysine and imidazole groups of histidine reacted. It is difficult to see why a free α-NH_2 group would not react under these conditions. A possible explanation is that the protein does not have a free α-NH_2 group, or the α-NH_2 group may be so hidden that it is not available by the reagent. In view of the latter possibility, the experiment was repeated with the denatured protein. Denaturation was carried out by heating a suspension of the protein in 95% ethanol at 60° for 15 minutes. Again, no reactive α-NH_2 group was found.

(ii) Leucine aminopeptidase

Digestion with leucine aminopeptidase did not liberate any amino acid from the protein. The enzyme is known to act rapidly and the conditions of digestion were also optimum.

Inability of leucine aminopeptidase to hydrolyze the protein, together with the negative results of the dinitrophenylation experiments, suggests that the protein is devoid of a free α-NH_2 group.

The Edman degradation could not be successfully employed in this protein. After the first step of degradation, approximately 0.5 residue of tyrosine was lost, but after the second step, losses of several residues at a time were observed.

(iii) Carboxypeptidase

A sample of 7.5 mg of protein was digested with 0.5 mg of carboxypeptidase A in 1% $NaHCO_3$ at 25°. Aliquots, taken out at 1, 2, 4 and 8 hours of digestion, were analyzed by the dinitrophenylation procedure (282). No detectable quantities of DNP-amino acids were found. The chromatograms showed a large quantity of dinitrophenol and very faint but recognizable yellow spots corresponding to di-DNP-lysine, and the DNP derivatives of Try, Asp or Glu, Asp-NH_2, Ser, Thr, Gly and leucine. These spots did not increase in intensity during the course of digestion, which suggests that they were artifacts.

Since carboxypeptidase A would be ineffective in case the C-terminal residue is lysine or arginine, attempts were made to hydrolyze the protein with carboxypeptidase B. The polypeptide was digested, at first, with carboxypeptidase B for 60 minutes and then with carboxypeptidase A. The combined digestion was carried out in 0.04M Tris buffer, pH 8.2 with an enzyme substrate ratio of 1:25. Aliquots were taken out at different times of digestion and were freed of protein by precipitating with trichloroacetic acid (final concentration 5%). The supernatant was extracted six times with peroxide-free water-saturated ether to remove TCA and analyzed by the automatic amino acid analyzer. A control experiment was performed concurrently to check autodigestion of the enzymes. Analyses of the aliquots from the control experiment showed the presence of almost all the amino acids. In the calculation of the molar ratios of amino acids supposedly liberated from the polypeptide by carboxypeptidase digestion, the values were corrected for the

presence of amino acids, produced by the enzymes themselves. Results are given in Table VI. The molar ratios were calculated on the basis of arginine. Even after 270 minutes of digestion, the molar ratios of amino acids did not change, except for histidine and glycine. If arginine is assumed to be the C-terminal residue, then, next to it occurs probably histidine or glycine.

Due to the presence of spurious amino acids in the digestion mixture, even when carboxypeptidase B alone was used, it was difficult to identify which amino acid was actually released from the polypeptide. The presence of free amino acids in the control digestion might be the result of autodigestion of the enzyme or an actual contamination in the enzyme preparation. Results of carboxypeptidase digestion, though corrected for spurious amino acids, could not be explained satisfactorily. The polypeptide probably contains a C-terminal arginine residue; in that case, this may explain the inability of carboxypeptidase A to liberate amino acid from the polypeptide.

(f) Chymotryptic digestion

The digestion mixture contained 6 mg of polypeptide 50-52 and 0.12 mg of α-chymotrypsin in a total volume of 900 µl of 0.1M ammonium bicarbonate buffer, pH 8.0. The mixture was incubated at 25° for 24 hours. At the end of the digestion period, the product was acidified with glacial acetic acid and taken to dryness over H_2SO_4 and KOH pellets. The digest was dissolved in 120 µl of pyridine-acetate buffer, pH 6.4 and 20 µl aliquots were analyzed by the peptide map

TABLE VI

COOH-Terminal amino acids of polypeptide 50-52

Carboxypeptidase B was added at zero time;
Carboxypeptidase A, after 60 minutes.

Amino acid	Amino acid residue released			
	Carboxypeptidase B	Carboxypeptidase A + Carboxypeptidase B		
	60 min.	90 min.	150 min.	270 min.
Arginine	1.00	1.00	1.00	1.00
Leucine	0.90	0.80	0.93	0.90
Alanine	0.80	0.72	0.83	0.82
Phenylalanine	0.75	0.65	0.73	0.73
Lysine	0.61	0.57	0.56	0.65
Serine	0.51	0.40	0.53	0.56
Threonine	0.41	0.24	0.37	0.42
Histidine	0.38	0.49	0.52	0.58
Isoleucine	0.34	0.31	0.31	0.28
Tyrosine	0.26	0.14	0.22	0.25
Valine	0.15	0.11	0.19	0.19
Glycine	0.11	0.18	0.23	0.38
Aspartic acid	0.10	0.00	0.07	0.07
Proline	0.06	0.03	0.12	0.15
Methionine	0.06	0.00	0.06	0.08
Glutamic acid	0.02	0.00	0.05	0.04

technique.

The peptide map of the chymotryptic digest is shown in Fig. 21. It was found that a prolonged chromatography was necessary to separate the peptides. At least 28 peptides could be visualized by ninhydrin spraying. Among them, only one peptide was stained with the specific reagent for tryptophan (291). Sakaguchi reagent (292) revealed 4 arginine-containing peptides and the sulfanilic acid reagent (213) showed 6 histidine-containing peptides.

The polypeptide contains 20 major points susceptible to chymotrypsin. Since chymotrypsin also hydrolyzes some of the histidyl and asparaginyl peptide bonds, the number of possible sites of attack will be more than 20. The formation of 28 chymotryptic peptides, therefore, is not incompatible with the amino acid composition of the polypeptide. Although this does not prove a complete hydrolysis of all the susceptible bonds, a fair degree of hydrolysis had been achieved under the conditions employed.

(g) Tryptic digestion

The digestion of polypeptide 50-52 with trypsin was carried out as described in the Experimental. The course of tryptic hydrolysis was followed by ninhydrin color increase of the hydrolysate. Fig. 22 shows the rate of hydrolysis of the polypeptide. It appears that not more than 12 peptide bonds were hydrolyzed. The polypeptide contains 11 lysine and 13 arginine residues. A complete tryptic hydrolysis would break 24 peptide bonds, producing 25 peptides. The ninhydrin method

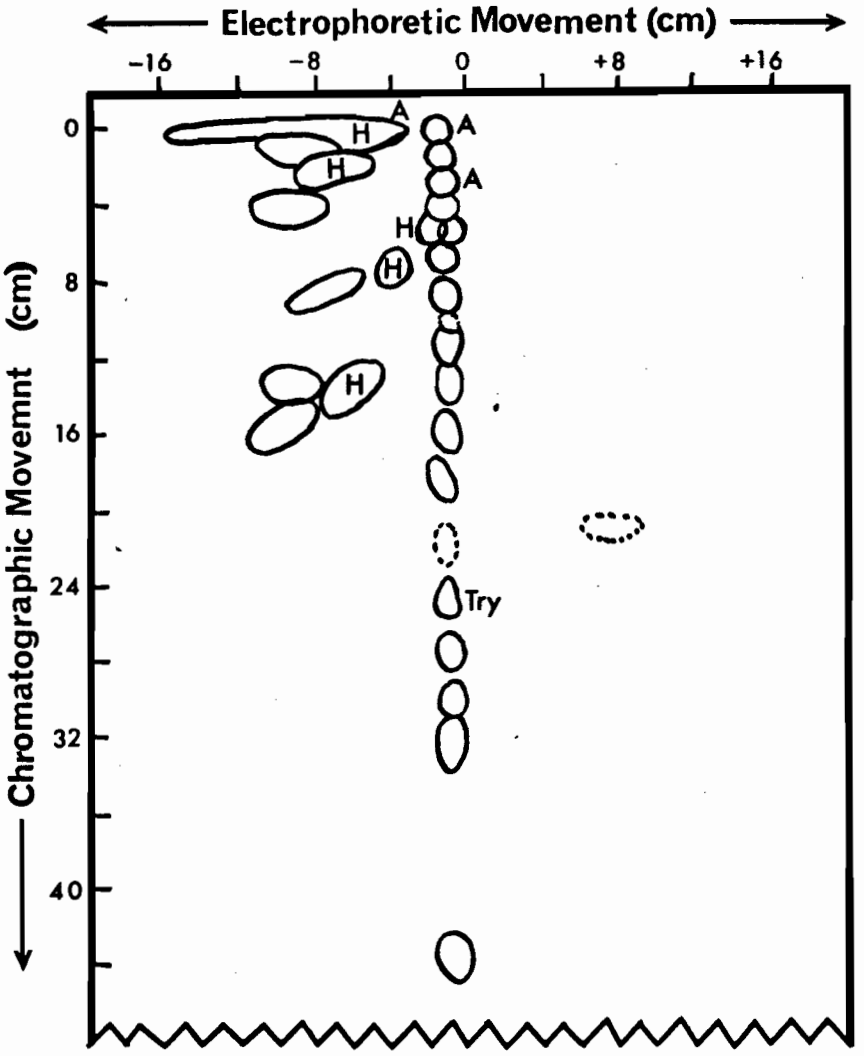


FIG. 21. Ionophoresis-chromatography of a chymotryptic digest of polypeptide 50-52. Electrophoresis was performed in pyridine-acetate buffer, pH 6.4 at 1000 volts for 2 hrs. and chromatography was carried out for 24 hrs. with n-butanol-acetic acid-water (200:30:75) mixture. Spots were visualized with ninhydrin spray. Peptides giving specific color reactions are shown: arginine, A; histidine, H; and tryptophan, Try.

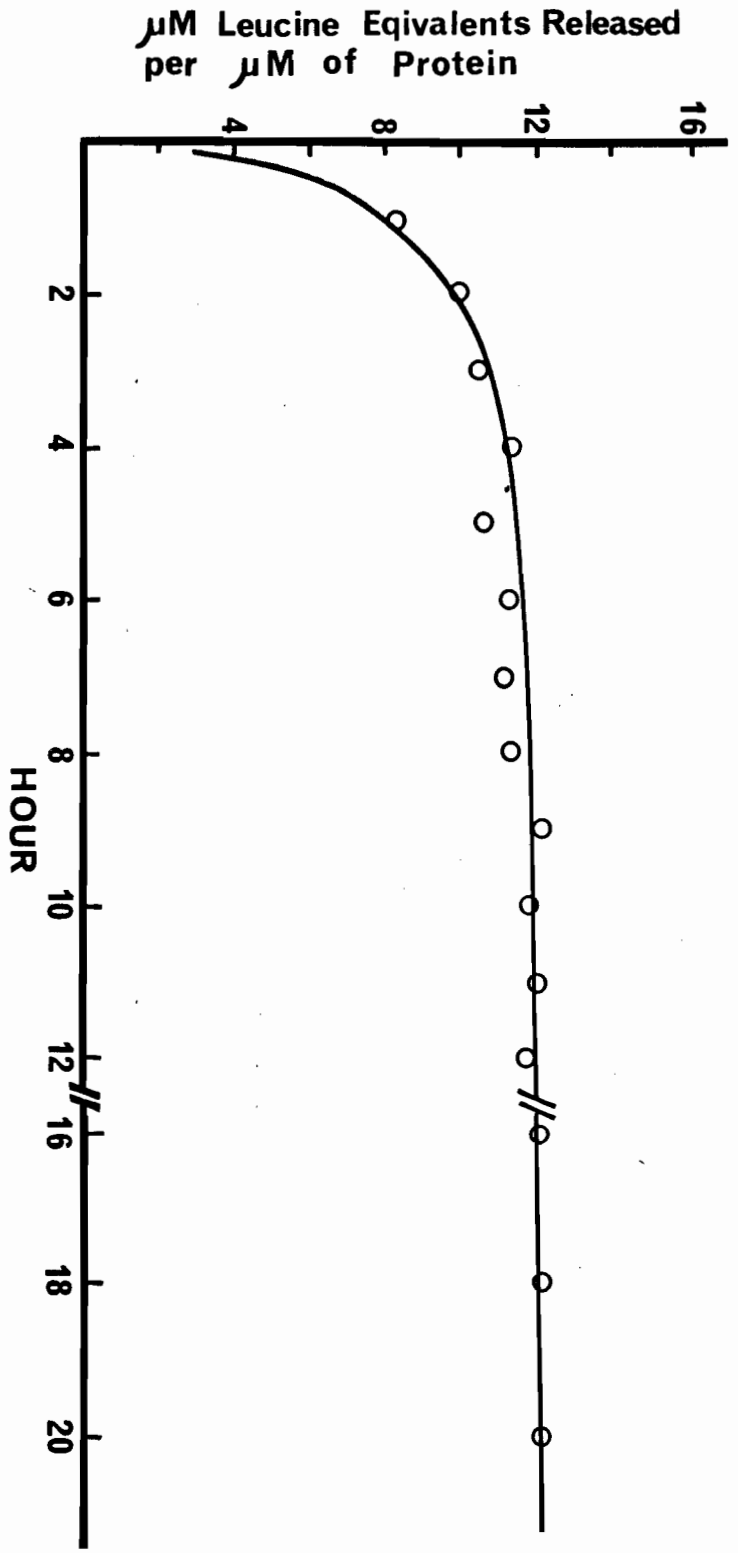


FIG. 22. The rate of hydrolysis of polypeptide 50-52 with trypsin. The liberation of NH_2 groups at different times of digestion was estimated by ninhydrin reaction.

indicated hydrolysis of about 50% of the susceptible bonds, but a peptide map of the hydrolysate (Fig. 23) definitely proved that the extent of cleavage far exceeded the number. In fact, the ninhydrin analysis cannot be a true measure of the degree of hydrolysis (278). The actual number of peptide bonds hydrolyzed may be calculated from the alkali uptake at a constant pH during the course of hydrolysis. However, the ninhydrin method showed that trypsin catalyzed hydrolysis was over after 9-10 hours of digestion.

Fig. 23 is a composite peptide map of the tryptic digest. The spots were located by ninhydrin spraying (275) and by specific color reactions for arginine (292) and tryptophan (291). The peptide map showed 27 major spots and 10 to 11 minor spots. Overlapping peptides appeared as a single large spot on the fingerprint. Such peptides were identified after column chromatographic separation. Trypsin produced only one tryptophan-containing peptide. At least 10 spots could be counted on the fingerprint, which gave a positive reaction with Sakaguchi reagent. But after column chromatographic separation and isolation of the peptides, it was evident that there were 15 arginine-containing peptides in the digest. They have been marked "A" in the peptide map (Fig. 23). Peptides were numbered according to their emergence from the Dowex 50 column. The formation of 27 major and 11 minor peptides indicated that some of the bonds were slowly attacked by trypsin.

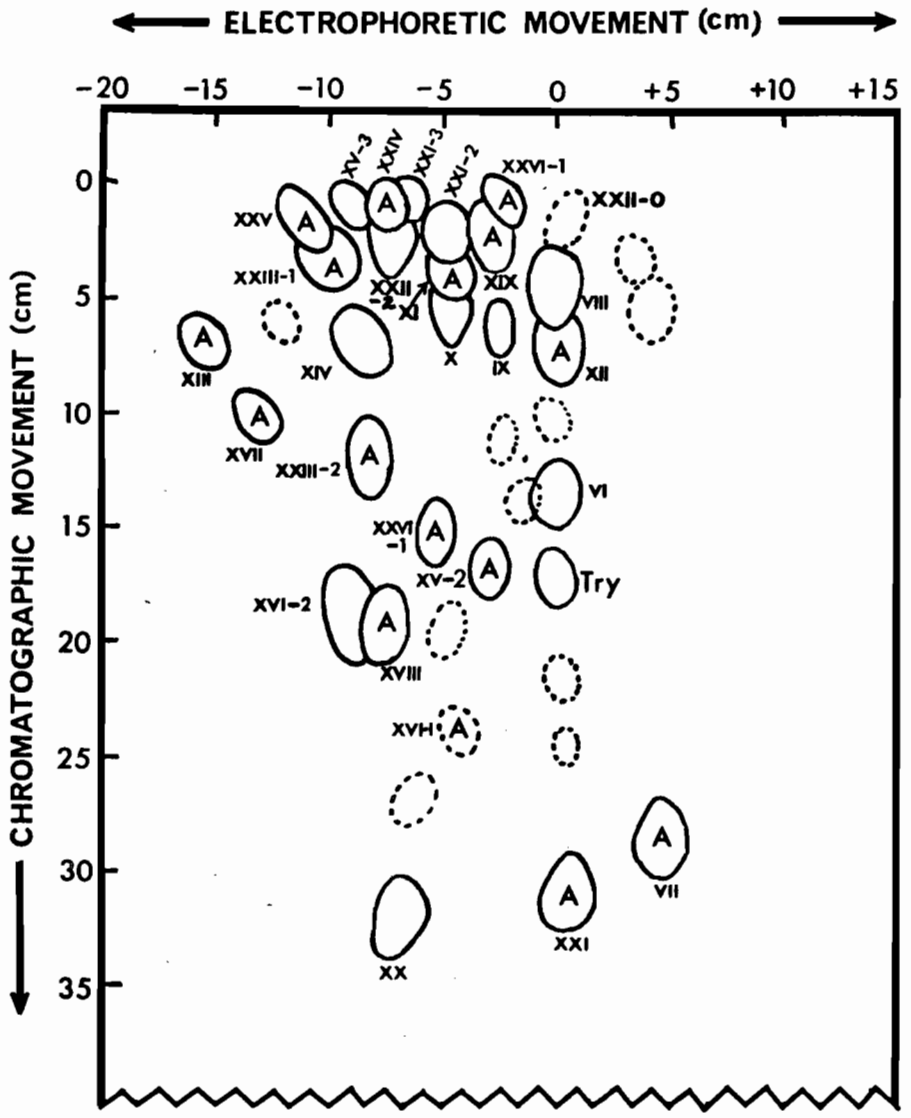


FIG. 23. Composite peptide map of the tryptic digest of polypeptide 50-52. Electrophoresis was performed in pyridine-acetate buffer, pH 6.4 at 1000 volts for 2 hrs. The chromatographic solvent was n-butanol-acetic acid-water (200:30:75, v/v) and was run for 17 hours. Isolated peptides have been numbered according to their description in the text. Arginine-containing peptides are marked A. The peptide containing tryptophan has been marked Try.

5. Isolation and Sequence of the Tryptic Peptides of Polypeptide 50-52

(a) Column chromatography of the tryptic digest

The separation of the tryptic peptides on a Dowex 50-X2 column is shown in Fig. 24. The recovery from the column, on a weight basis, was about 80 per cent. The peaks were numbered in Roman numerals in the order of their emergence from the column. The same numbering system was employed to indicate peptides in the peptide map (Fig. 23). Fractions under individual peaks were pooled, discarding the overlapping zones as much as possible, concentrated in a rotary evaporator and lyophilized. Peptides were obtained as brown powders. The color came from the concentration of large volumes of pyridine buffer. The actual recovery, therefore, will be less, if corrected for chromophores.

Peaks III-V were in poor yield and could not be analyzed. They probably represented the minor acidic peptides observed on the peptide map. Peaks I-II yielded a ninhydrin-negative solid product which on acid hydrolysis did not give any amino acid. These peaks were probably artifactual and the solid product might have come from the resin.

No more peptides were eluted from the column after the elution of peak XXVI. Examination of the peaks showed that almost all the peptides of the tryptic digest were eluted. The tryptophan-containing peptide, one of the major peptides of the digest, was not eluted; instead, there appeared a peptide in peak XXII, which gave a positive reaction for tryptophan. It was neutral like the original tryptophan-containing peptide, but its chromatographic R_f value was much less. The yield of this peptide was very low. This suggests that the peptide

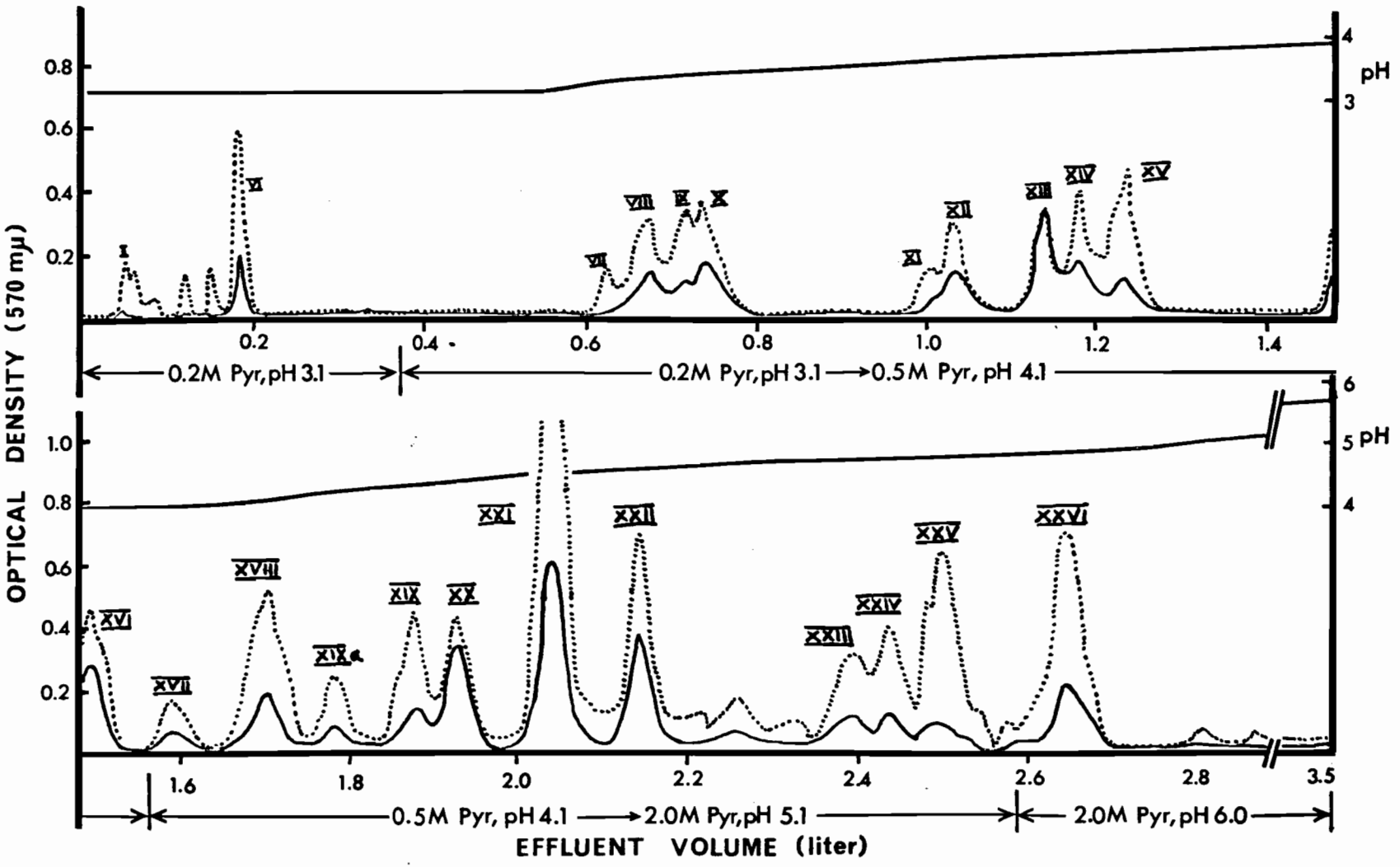


FIG. 24. Chromatography of the tryptic digest of polypeptide 50-52 on a Dowex 50X-2 column (0.9 x 135 cm). The elution was performed with pyridine-acetate buffers at 37°. Gradient was obtained through a 500 ml mixing chamber. 3.7 ml fractions were collected. Aliquots from individual fractions were analyzed with ninhydrin directly (——) and after alkaline hydrolysis (·····).

could be formed from the original tryptophanyl peptide by degradation, or it might have been in the original digest and escaped detection because of its poor yield.

(b) Purification of the tryptic peptides

In order to identify the peptides of the tryptic digest as they were separated by column chromatography, each peak was examined by the peptide map technique under standard conditions. In these conditions, electrophoresis was performed in pyridine-acetate buffer, pH 6.4 at 1000 volts for 2 hours, and descending chromatography was carried out at room temperature in n-butanol-acetic acid-water (200: 30:75, by volume) mixture for 17 hours.

All the peaks, except for peaks VI and VIII, contained more than one peptide. Purification of peptides occurring in individual peaks was achieved by preparative paper electrophoresis or chromatography or by successive use of both of these techniques. Whatman No. 3MM paper was washed with 30% acetic acid by descending chromatography for at least 24 hours and dried free of acid. The acid-washed paper was used for preparative purposes. Solvents used for electrophoresis or chromatography were those described above. Often a longer period of run was allowed for better separation of the closely moving peptides. Guide strips were cut from both ends and from the center, and stained with ninhydrin to locate peptide bands. Peptide-containing areas were cut out and eluted with 0.5% acetic acid for over 24 hours, collecting

30 to 50 ml of eluate from each 6-inch strip. Eluates were concentrated in a rotary evaporator under vacuum to about 1 ml, transferred into screw-cap vials and lyophilized. Peptides were stored dry in the cold until use. Complex peaks were first separated by unidimensional electrophoresis. The isolated peptides were then purified by chromatography. In no case were more than two steps necessary to attain the desired purity.

Procedures used for the purification of peptides of individual peaks are summarized below. The chromatographic (Ch) and electrophoretic (El) movements of peptides, under standard conditions mentioned above, are given. The electrophoretic movement is shown with a positive or negative sign to indicate movement towards the anode or cathode. Peptides isolated from a peak have been designated by the peak number, followed by Arabic numerals to distinguish different peptides of the same peak. To signify that these peptides are originated from a tryptic digest, the "T" has been placed before the peak number (Roman numerals). For example, a peptide, prepared from peak XXI and subfraction 3, will be called T-XXI-3. Peptides have been described in the order of their elution from the column.

Peak VI: Single neutral component; Peptide T-VI (El, 0 cm; Ch, 14 cm).

Peak VII: One major acidic peptide; Peptide T-VII (El, + 4.5 cm; Ch, 28.5 cm). This peptide was separated from a minor neutral component by electrophoresis.

Peak VIII: Single neutral component; Peptide T-VIII (El, 0 cm; Ch, 5 cm).

Peaks IX and X: These two peaks were similar and contained one acidic and one basic component. The acidic peptide was identical to Peptide T-VII. The basic peptide, Peptide T-IX (El, - 2.5 cm; Ch, 6 cm), was isolated by electrophoresis.

Peaks XI and XII: These two peaks were also similar in the peptide map pattern and contained a basic and a neutral peptide. Peak XI had more of the basic peptide, whereas peak XII was rich in the neutral peptide. Peptide T-XI (El, - 5 cm; Ch, 4 cm) and Peptide T-XII (El, 0 cm; Ch, 7 cm) were isolated by electrophoresis.

Peak XIII: One major basic component; Peptide T-XIII (El, - 14.5 cm; Ch, 6.5 cm), was isolated by electrophoresis.

Peak XIV: One major basic component; Peptide T-XIV (El, - 8.5 cm; Ch, 6.5 cm), was prepared by electrophoresis.

Peak XV: Three basic peptides; one of them was identical to Peptide T-XIV and constituted a minor fraction of the peak. Peptide T-XV-2 (El, - 3.5 cm; Ch, 17 cm) and Peptide T-XV-3 (El, - 9.5 cm; Ch, 1 cm) were isolated after electrophoretic separation.

Peak XVI: Three minor neutral components and two basic peptides. The basic peptides, Peptide T-XVI-1 (El, - 4.5 cm; Ch, 24 cm) and Peptide T-XVI-2 (El, - 9.5 cm; Ch, 19 cm), were prepared by electrophoresis.

Peak XVII: This peak contained minor amounts of all the components of peak XVI plus a basic peptide, Peptide T-XVII (El, - 13.5 cm; Ch, 10 cm). Peptide T-XVII was isolated by electrophoresis.

Peak XVIII: Two basic components; one of them was identical to Peptide T-XVII. The main component, Peptide T-XVIII (El, - 8 cm; Ch,

19 cm), was isolated by chromatography.

Peak XIX: One minor neutral component and a basic peptide, Peptide T-XIX (El, - 3 cm; Ch, 2.5 cm). This peptide was prepared by electrophoresis.

Peak XX: Two basic components; the major peptide, Peptide T-XX (El, - 7 cm; Ch, 32 cm), was isolated by electrophoresis.

Peak XXI: Two basic and one neutral component. Peptide T-XXI-1 (El, 0 cm; Ch, 31 cm), Peptide T-XXI-2 (El, - 5 cm; Ch, 2.5 cm), and Peptide T-XXI-3 (El, - 6 cm; Ch, 1 cm) were prepared by electrophoresis.

Peak XXII: Three components; one neutral and two basic. The major component, Peptide T-XXII-2 (El, - 7.5 cm; Ch, 2 cm) was separated from the minor components, Peptide T-XXII-1 (El, - 1.5 cm; Ch, 14 cm) and Peptide T-XXII-0 (El, 0 cm; Ch, 2 cm) by electrophoresis, while the latter two peptides were isolated by chromatography. Peptide T-XXII-0 gave a positive test for tryptophan. This peptide was not identified in the peptide map of the original digest.

Peak XXIII: Four minor components and two major basic peptides. Peptide T-XXIII-1 (El, - 10 cm; Ch, 3 cm) and Peptide T-XXIII-2 (El, - 8.5 cm; Ch, 12 cm) were isolated by chromatography, followed by electrophoretic separation of the minor components.

Peak XXIV: This fraction contained mainly Peptide T-XXIII-2 and another peptide, Peptide T-XXIV (El, - 6.5 cm; Ch, 0.5 cm); their separation was achieved by chromatography, followed by electrophoresis.

Peak XXV: One major component, Peptide T-XXV (El, - 11.5 cm; Ch, 1.5 cm) was prepared by chromatography.

Peak XXVI: Two basic peptides; Peptide T-XXVI-1 (El, - 2.5 cm; Ch, 1 cm) and Peptide T-XXVI-2 (El, - 5.5 cm; Ch, 15 cm) were isolated by chromatography.

(c) Amino acid composition of the tryptic peptides

Peptides representing 0.05 to 0.1 μmole were hydrolyzed with 1 ml of 6N HCl in evacuated sealed tubes for 24 hours at 110°. The hydrolysates were freed of excess HCl by evaporation in an evacuated desiccator over NaOH pellets, quantitatively transferred to the analyzer column with a small volume (100 μl) of 0.1N HCl, and analyzed. Table VII shows the amino acid composition of the tryptic peptides. No correction was made for hydrolytic losses of amino acids. The values for ammonia were greatly variable and unreliable and, therefore, were not included in the table.

Trace amino acids in a purified peptide did not represent more than 5 to 10% in terms of the stoichiometry of the component amino acids. In most cases, these trace amino acids were Asp, Thr, Ser, Glu, Pro, Gly and Ala, indicating that they might have been incorporated into the peptide preparations from the same source. It is probable that either the paper or the solvent used for preparative purposes was the source of impurities. In general, the amino acid compositions of peptides were in agreement with their chromatographic and electrophoretic movements.

Of the total 130 residues in the parent protein, 119 residues have been accounted for by the tryptic peptides. This represents about 92 per cent of the original polypeptide. Those peptides which contained

both arginine and lysine, or more than one residue of lysine or arginine, have not been included in this calculation. Peptide T-XXII-O, the yield of which was less than 1%, is also excluded from the total. When the number of residues of individual amino acids of the tryptic peptides are added, some amino acids give an excess of what has been assumed for the parent polypeptide. This is true for Thr, Ser, Gly, Met, Ileu and Leu. A situation like this may arise from partial degradation of true tryptic peptides, with the formation of new peptides. Peptides, marked with an asterisk in Table VII, contain more than one basic residue; this suggests that they are produced as a result of incomplete digestion. These peptides might have been partially hydrolyzed with the formation of a greater number of peptides than could be expected from the amino acid composition of the polypeptide. It should be mentioned that the polypeptide was digested with a single charge of enzyme in order to minimize the formation of spurious peptides.

Yields of the tryptic peptides have been calculated from the amino acid analyses of the purified peptides. These values represent only minimum yields. It has been found that preparations of peptides by paper electrophoresis or chromatography often incur losses amounting to 50%. Because of variable losses in different steps of purification, it was not possible to evaluate the actual yield of the tryptic peptides.

TABLE VII

Amino acid composition of the peptides obtained by tryptic hydrolysis of polypeptide 50-52

The composition of each peptide is given as the molar ratios of the amino acids, calculated without correction for hydrolytic destruction. The assumed number of residues is given in parentheses. Values for residues less than 0.01 mole are omitted. The peptides are numbered as in the text.

Amino acid	T-VI	T-VII	T-VIII	T-IX	T-XI	T-XII	T-XIII
Aspartic acid	0.03	1.96(2)	1.11(1)			1.07(1)	
Threonine		0.93(1)	0.99(1)	0.78(1)			
Serine	0.93(1)	1.00(1)	0.97(1)	0.94(1)	1.20(1)	0.91(1)	
Glutamic acid	0.96(1)		1.78(2)	1.08(1)		0.04	
Proline	0.11			2.32(2)	0.95(1)	0.13	
Glycine	0.10	2.06(2)	2.00(2)	1.10(1)	1.13(1)	0.08	
Alanine	1.07(1)		1.97(2)		0.97(1)		
Valine							
Methionine					0.82(1)		
Isoleucine		0.93(1)					
Leucine		2.03(2)	1.13(1)				
Tyrosine							
Phenylalanine							
Lysine	1.04(1)		1.06(1)	0.84(1)			
Histidine							
Arginine		1.00(1)			0.87(1)	1.02(1)	1.00(1)
Total residues	4	10	11	7	6	3	1
Yield	26%	30%	26%	1.5%	5%	7%	38%

continued...

TABLE VII (continued)

Amino acid	T-XIV	T-XV-2	T-XV-3	T-XVI-1	T-XVII	T-XVIII	T-XIX	T-XVI-2
Aspartic acid	0.01	1.20(1)		0.07	0.10	0.05	1.10(1)	
Threonine		1.02(1)			0.02		1.00(1)	
Serine	0.01		1.05(1)	1.84(2)	0.27	0.13	2.08(2)	0.04
Glutamic acid					0.08	0.07	0.12	
Proline	0.81(1)	1.14(1)						
Glycine	0.95(1)	0.09	1.81(2)	1.09(1)	1.06(1)	2.11(2)	0.19	0.90(1)
Alanine	1.03(1)				0.08	0.05	2.93(3)	
Valine		0.84(1)						
Methionine							0.82(1)	
Isoleucine		0.78(1)						
Leucine				2.07(2)		0.95(1)	1.00(1)	1.04(1)
Tyrosine							0.92(1)	
Phenylalanine								
Lysine	1.21(1)		1.13(1)	0.08				1.06(1)
Histidine							1.05(1)	
Arginine		1.02(1)		1.00(1)	0.94(1)	0.96(1)	1.03(1)	
Total residues	4	6	4	6	2	4	12	3
Yield	14%	18%	4%	2%	2%	9%	14%	30%

continued...

TABLE VII (continued)

Amino acid	T-XX	T-XXI-1	T-XXI-2	T-XXI-3	T-XXII-0	* T-XXII-1	* T-XXII-2
Aspartic acid		0.96(1)	0.35	0.06	1.91(2)	1.93(2)	1.13(1)
Threonine		0.09	1.94(2)		0.83(1)	0.62(1)	0.03
Serine	0.05	0.11	1.18(1)	0.94(1)	2.18(2)	1.71(2)	0.10
Glutamic acid		0.09	0.89(1)	0.03	2.50(2)	0.98(1)	0.04
Proline			1.07(1)	0.08	2.12(2)	1.30(1)	1.67(2)
Glycine	0.07	1.11(1)	1.79(2)	1.02(1)	4.24(4)	2.30(2)	0.13
Alanine		1.00(1)	0.24	0.11	3.25(3)	2.50(2)	2.00(2)
Valine							
Methionine							
Isoleucine	0.94(1)						
Leucine		0.05	1.16(1)			0.55	
Tyrosine			0.89(1)			0.55	0.86(1)
Phenylalanine	1.00(1)	1.95(2)				1.13(1)	
Lysine	1.07(1)	0.07	1.08(1)	1.11(1)	0.92(1)	0.97(1)	2.17(2)
Histidine			1.13(1)	0.93(1)	1.20(1)	2.05(2)	1.09(1)
Arginine		0.98(1)	0.55			0.76(1)	0.06
Tryptophan					+ (1)[a]		
Total residues	3	6	11	4	19[b]	15[b]	9[b]
Yield	17%	21%	18%	11%	0.9%	2%	12.5%

a Tryptophan was identified on the paper chromatogram.

* Peptide containing more than one basic residue.

b Not included in the total.

continued...

TABLE VII (concluded)

Amino acid	T-XXIII-1	T-XXIII-2	* T-XXIV	* T-XXV	* T-XXVI-1	T-XXVI-2	Total residues recovered in the tryptic peptides	Total residues in the original protein
Aspartic acid	0.05		0.91(1)	0.06	1.96(2)	0.08	7	9
Threonine					0.09	0.04	7	6
Serine	0.12	1.05(1)	0.99(1)	0.96(1)	0.36	0.13	14	11
Glutamic acid	0.03		0.14	1.08(1)	2.86(3)	0.09	5	9
Proline				0.92(1)	2.21(2)	1.12(1)	7	8
Glycine	0.19	1.09(1)	1.93(2)	0.13	1.72(2)	1.01(1)	20	19
Alanine	0.07		1.45(1)	0.07	1.15(1)	0.09	9	11
Valine					1.33(1)	0.97(1)	2	3
Methionine							2	1
Isoleucine							3	2
Leucine		1.20(1)			0.23		10	8
Tyrosine					0.06		2	3
Phenylalanine					1.97(2)	1.02(1)	4	7
Lysine			0.61(1)	0.06	0.90(1)		9	11
Histidine	0.96(1)		1.27(1)	0.09	1.83(2)	0.88(1)	5	8
Arginine	1.05(1)	0.64(1)	0.91(1)	2.27(2)	1.08(1)	0.99(1)	13	13
Tryptophan								1
Total residues	2	4	8[b]	5[b]	17[b]	6	119	130
Yield	4%	8%	2%	8.5%	15%	10%		

* Peptide containing more than one basic residue.

b Not included in the total.

(d) Amino acid sequences of the tryptic peptides of polypeptide 50-52

Peptide T-VI: (Glu-NH_2-Ala-Ser)-Lys

The Edman degradation was not successful with this peptide. Even after several attempts at the stepwise chemical degradation from the NH_2-terminal, the amino acid composition of the peptide remained unchanged. Peptide T-VI was then digested with carboxypeptidase B for 60 minutes, followed by carboxypeptidase A. Aliquots of the hydrolysate were analyzed by the automatic amino acid analyzer. The moles of amino acids released per mole of the peptide at different times of digestion are given below. In the calculation of the molar ratios of the liberated amino acids, lysine was considered to be completely released after 60 minutes.

90 min: Lys, 0.96; Ser, 0.27; Ala, 0.13; Glu, 0.10.

150 min: Lys, 1.00; Ser, 0.33; Ala, 0.15; Glu, 0.10.

270 min: Lys, 1.00; Ser, 0.37; Ala, 0.17; Glu, 0.12.

Serine appears to be the next residue released. The slow release of serine and alanine by carboxypeptidase A, coupled with the fact that the NH_2-terminal is not reactive, suggests that peptide T-VI is a pyrrolidone carboxylyl peptide. Cyclization of an N-terminal glutamine residue with the formation of a pyrrolidone carboxylic acid residue has been observed in the structure studies of ribonuclease (246,247). An NH_2-terminal glutamine residue cyclizes rapidly at or below pH 3. Since the tryptic digest of polypeptide 50-52 was treated with acid (HCl) to stop the enzyme action and was chromatographed on Dowex 50 in acid conditions (pH 3.1), it appears quite likely that peptide

T-VI was originally a glutaminyl peptide and was converted to a pyrrolidone carboxylyl peptide during isolation.

The peptide was neutral in the original tryptic digest, as was evident from its electrophoretic mobility. After elution from the Dowex 50 column, the peptide did not change its electrophoretic movement. Peptide T-VI would be basic with a glutamine residue, and with a pyrrolidone carboxyl residue it is expected to be neutral. It appears, therefore, that the cyclization of the glutamine residue occurred before the peptide was chromatographed on Dowex 50. If peptide T-VI contains a glutamic acid residue, the peptide should be neutral; but it remains unexplained why the peptide did not react with phenylisothiocyanate. On the other hand, a pyrrolidone carboxyl residue at the NH_2-terminal, because of its structural similarity with proline, may explain the inefficiency of carboxypeptidase A to release serine and alanine from the peptide.

Peptide T-VII: Asp-Thr-Ileu-Leu-Gly-Asp-Ser-Leu-Gly-Arg.

Four stages of the Edman degradation were performed with the following results:

Stage 0: Asp, 1.96; Thr, 0.93; Ser, 1.00; Gly, 2.06; Ileu, 0.93; Leu, 2.03; Arg, 1.00.

Stage 1: Asp, 1.15; Thr, 1.01; Ser, 0.83; Gly, 2.11; Ileu, 0.96; Leu, 1.96; Arg, 1.05.

Stage 2: Asp, 1.02; Thr, 0.31; Ser, 1.13; Gly, 2.18; Ileu, 1.00; Leu, 2.04; Arg, 1.05.

At this stage, the residual peptide was purified on a short column of Dowex 50-X2.

Stage 3: Asp, 1.15; Thr, 0.20; Ser, 1.20; Gly, 1.82; Ileu, 0.20; Leu, 1.84; Arg, 1.00.

Stage 4: Asp, 0.95; Thr, 0.23; Ser, 1.22; Gly, 1.94; Ileu, 0.23; Leu, 1.08; Arg, 0.82.

Peptide T-VII was acidic and, therefore, contained two aspartyl residues.

Chymotryptic digestion:- Peptide T-VII was digested with chymotrypsin in 0.1M ammonium bicarbonate buffer, pH 8.0 for 24 hrs. at 25°. A peptide map of the digest showed the presence of four peptides. The mobilities of these chymotryptic peptides under standard conditions were as follows: Peptide T-VII-C-I (El, + 7 cm; Ch, 12.8 cm), Peptide T-VII-C-II (El, + 4.3 cm; Ch, 19 cm), Peptide T-VII-C-III (El, 0; Ch, 2.8 cm), and Peptide T-VII-C-IV (El, - 4.6 cm; Ch, 4 cm). These peptides were isolated by preparative paper electrophoresis. Peptides T-VII-C-I and T-VII-C-IV were in poor yields. The amino acid composition of Peptide T-VII-C-II was (Asp, Thr, Ileu, Leu, Gly). This peptide arose from the NH_2-terminal of Peptide T-VII.

The Edman degradation of Peptide T-VII-C-III gave the following results:

Stage 0: Asp, 0.96; Ser, 0.93; Gly, 1.20; Leu, 1.03; Arg, 0.86.

Stage 1: Asp, 0.21; Ser, 0.84; Gly, 1.21; Leu, 1.03; Arg, 0.97.

Stage 2: Asp, 0.13; Ser, 0.34; Gly, 1.22; Leu, 0.91; Arg, 0.87.

Stage 3: Asp, 0.21; Ser, 0.30; Gly, 1.20; Leu, 0.40; Arg, 0.60.

The sequence of Peptide T-VII-C-III is, therefore, Asp-Ser-Leu-Gly-Arg. Combining this with the NH_2-terminal sequence of Peptide T-VII, the complete sequence of the peptide can be deduced as Asp-Thr-Ileu-Leu-Gly-Asp-Ser-Leu-Gly-Arg.

The hydrolysis of the Gly-Asp bond in this peptide by chymotrypsin was rather unexpected. Similar unexpected cleavage of a Thr-Gly bond has been observed in cytochrome c (223). The formation of 4 peptides by the chymotryptic digestion of Peptide T-VII suggests that leucyl bonds were also hydrolyzed.

Peptide T-VIII: Gly-Ala-Glu-NH_2-Asp-Ala-Gly-Glu-NH_2-Thr-Leu-Ser-Lys.

Four stages of the Edman degradation were carried out. The results are given below.

Stage 0: Asp, 1.11; Thr, 0.99; Ser, 0.97; Glu, 1.78; Gly, 2.00; Ala, 1.97; Leu, 1.13; Lys, 1.06.

Stage 1: PTH-Glycine was identified in solvent systems A and F.

Stage 2: Asp, 1.05; Thr, 0.80; Ser, 1.09; Glu, 1.97; Gly, 0.99; Ala, 1.27; Leu, 1.01; Lys, 0.99.

Stage 3: Asp, 0.98; Thr, 0.79; Ser, 1.34; Glu, 1.17; Gly, 1.05; Ala, 1.18; Leu, 0.99; Lys, not calculated.

Stage 4: Asp, 0.34; Thr, 0.79; Ser, 0.96; Glu, 0.92; Gly, 1.20; Ala, 0.88; Leu, 0.88; Lys, not calculated.

PTH-Glutamine was identified at the third stage of degradation in solvent systems A and F.

Chymotryptic digestion:- Digestion of Peptide T-VIII with

chymotrypsin produced two peptides. They have been designated Peptide T-VIII-C-I and Peptide T-VIII-C-II. Peptide T-VIII-C-I moved 3 cm and Peptide T-VIII-C-II moved 12 cm from the origin when chromatographed in n-butanol-acetic acid-water (200:30:75, v/v) mixture for 21 hours. These peptides were eluted from the chromatogram with 0.5% acetic acid. The amino acid composition of Peptide T-VIII-C-II was (Asp, 1.16; Thr, 0.90; Glu, 1.91; Gly, 2.09; Ala, 2.13; Leu, 0.92). Peptide T-VIII-C-I contained only serine and lysine, and constituted the C-terminal sequence of Peptide T-VIII. PTH-Serine was identified in the solvent system F, after one step of the Edman degradation of Peptide T-VIII-C-I.

Carboxypeptidase A digestion:- Peptide T-VIII-C-II was digested with carboxypeptidase A. The digestion mixture contained 0.05 µmole peptide in 50 µl of water, 10 µl of 0.4M Tris buffer, pH 8.2, 10 µl of an enzyme preparation containing 7 mg of protein per ml. The total volume was adjusted to 100 µl and the mixture was incubated for 18 hrs. at 40°. The digest, after taking to dryness over H_2SO_4 and NaOH in an evacuated desiccator, was analyzed by the peptide map technique. The liberated amino acids were of the following order: Leu (+4), Thr (+4), Glu-NH_2 (+3), Gly (+2) and Ala (+1). According to the specificity of chymotrypsin, leucine must be the C-terminal. The C-terminal sequence of T-VIII-C-II is, therefore, -Ala-Gly-Glu-NH_2-Thr-Leu.

Peptide T-VIII was neutral at pH 6.4; two of the three acidic amino acid residues of the peptide, therefore, should be amidated. The identification of PTH-glutamine in the Edman degradation and glutamine in the carboxypeptidase digest confirmed this. Both glutamic acid

residues of the peptide were found to be in the amide form. With these available data, the sequence of Peptide T-VIII has been proposed as shown above.

Peptide T-XI: (Gly-Ser-Met-Pro-Ala)-Arg.

This peptide was subjected to four stages of the Edman degradation.

Stage 0: Ser, 1.20; Pro, 0.95; Gly, 1.13; Ala, 0.97; Met, 0.82; Arg, 0.87.

State 1: Ser, 0.89; Pro, 1.10; Gly, 0.58; Ala, 1.08; Met, 0.82; Arg, 1.10.

Stage 2: Ser, 0.60; Pro, 0.69; Gly, 0.60; Ala, 1.16; Met, 0.95; Arg, 0.89.

Stage 3: Ser, 0.61; Pro, 0.89; Gly, 0.70; Ala, 1.16; Met, 0.49; Arg, 0.95.

Stage 4: Ser, 0.55; Pro, 0.40; Gly, 0.67; Ala, 0.96; Met, 0.30; Arg, 1.00.

The results of the stepwise degradations of this peptide cannot be satisfactorily explained. After the first stage of degradation, only 50 per cent of glycine was lost. This was probably due to incomplete cyclization of the PTC-peptide. Purification of the residual peptide was not possible because of the unavailability of the material. Both serine and proline gave low values in the second stage of the degradation. Since proline reappeared in the third stage, it seemed likely that the apparent loss of proline in the second stage was due to errors in its determination. About 50 per cent of the peptide remained unreactive during the degradations, and this was the source of misleading results. It appears that Peptide T-XI contains an N-terminal glycine, though the sequence remains uncertain.

Peptide T-XII: Asp-Ser-Arg

One stage of the Edman degradation established the sequence.

Stage 0: Asp, 1.07; Ser, 0.91; Arg, 1.02.

Stage 1: Asp, 0.18; Ser, 0.91; Arg, 1.09.

This peptide did not move in electrophoresis at pH 6.4. Therefore, the β-COOH group of the aspartic acid residue was free.

Peptide T-XIII: Arg

The amino acid analysis of the peptide yielded only arginine. All of the arginine was removed quantitatively after the first stage of the Edman degradation, indicating that Peptide T-XIII was free arginine.

Peptide T-XIV: Gly-Ala-Pro-Lys

Two stages of the Edman degradation established the sequence of the tetrapeptide.

Stage 0: Pro, 0.81; Gly, 0.95; Ala, 1.03; Lys, 1.21.

Stage 1: Pro, 0.95; Gly, 0.13; Ala, 0.97; Lys, 1.08.

Stage 2: Pro, 1.05; Gly, 0.16; Ala, 0.13; Lys, 0.95.

Peptide T-XV-2: Asp-NH_2-Ileu-Val-Thr-Pro-Arg.

The Edman degradation gave the following results:

Stage 0: Asp, 1.20; Thr, 1.02; Pro, 1.14; Val, 0.84; Ileu, 0.78; Arg, 1.02.

Stage 1: Asp, 0.15; Thr, 1.17; Pro, 1.30; Val, 0.80; Ileu, 0.60; Arg, 1.13.

Stage 2: Asp, 0.10; Thr, 0.91; Pro, 1.14; Val, 0.95; Ileu, 0.13; Arg, 0.99.

Stage 3: Asp, 0.16; Thr, 0.87; Pro, 1.18; Val, 0.28; Ileu, 0.00; Arg, 1.04.

Stage 4: Asp, 0.17; Thr, 0.30; Pro, 1.01; Val, 0.08; Arg, 0.98.

Stage 5: Asp, 0.21; Thr, 0.40; Pro, 0.63; Arg, 1.00

Peptide T-XV-2 moved 3.5 cm towards the cathode in electrophoresis under standard conditions. The peptide, therefore, contains an asparagine residue to account for a net positive charge at pH 6.4.

Peptide T-XV-3: Gly-Ser-Gly-Lys.

Two stages of the Edman degradation were performed.

Stage 0: Ser, 1.05; Gly, 1.81; Lys, 1.13.

Stage 1: Ser, 0.93; Gly, 1.07; Lys, 0.47.

Stage 2: Ser, 0.31; Gly, 1.22; Lys, 0.47.

More than 50 per cent of lysine was lost in the first stage of degradation. Loss of lysine was observed in peptide T-VIII after the third stage. Peptide T-XV-3 behaved differently in this respect. Since lysine is the C-terminal, the sequence of this peptide can be written as Gly-Ser-Gly-Lys.

Peptide T-XVI-1: Gly-Leu-Ser-Leu-Ser-Arg

The Edman degradation gave the following results:

Stage 0: Ser, 1.84; Gly, 1.09; Leu, 2.07; Arg, 1.00.

Stage 1: Ser, 1.74; Gly, 0.36; Leu, 2.14; Arg, 1.15.

Stage 2: Ser, 1.81; Gly, 0.00; Leu, 1.38; Arg, 0.82

Stage 3: Ser, 1.18; Leu, 0.80; Arg, 0.82.

Stage 4: Ser, 1.25; Leu, 0.56; Arg, 0.50.

On the consideration that arginine is the C-terminal, the proposed sequence of the peptide is shown above. At the stage 4 of the Edman degradation, both leucine and arginine were lost almost to the same extent. Similar loss of arginine during the course of degradations was observed with Peptide T-VII-C-III. This was probably owing to progressive accumulation of side-reaction products in the residual peptide, which, during acid hydrolysis, destroyed arginine.

Peptide T-XVI-2: Gly-Leu-Lys

One stage of the Edman degradation established the sequence.

Stage 0: Gly, 0.90; Leu, 1.04; Lys, 1.06.

Stage 1: Gly, 0.30; Leu, 1.13; Lys, 0.87.

Peptide T-XVII: Gly-Arg

The sequence of this peptide was obvious from the specificity of trypsin. One step of the Edman degradation confirmed this.

Stage 0: Gly, 1.06; Arg, 0.94.

Stage 1: Gly, 0.28; Arg, 1.00.

Peptide T-XVIII: Leu-Gly-Gly-Arg

Two stages of the Edman degradation gave the necessary information for deducing the sequence.

Stage 0: Gly, 2.11; Leu, 0.95; Arg, 0.96.

Stage 1: Gly, 2.10; Leu, 0.12; Arg, 0.80.

Stage 2: Gly, 1.08; Leu, 0.08; Arg, 0.92.

Peptide T-XIX: Tyr-Leu-Ala-Ser-(Asp,Thr,Ser,Ala_2,Met,His)-Arg.

Four stages of the Edman degradation were performed.

Stage 0: Asp, 1.10; Thr, 1.00; Ser, 2.08; Ala, 2.98; Met, 0.82; Leu, 1.00; Tyr, 0.92; His, 1.05; Arg, 1.03.

Stage 1: PTH-Tyrosine was identified in the solvent system F.

Stage 2: Asp, 1.08; Thr, 1.02; Ser, 1.65; Ala, 2.89; Met, 1.14; Leu, 0.22; Tyr, 0.00; His, 1.01; Arg, 0.98.

Stage 3: Asp, 1.22; Thr, 1.08; Ser, 1.72; Ala, 2.33; Met, 0.91; Leu, 0.00; His, 0.92; Arg, 0.85.

Stage 4: Asp, 1.09; Thr, 0.90; Ser, 1.29; Ala, 1.98; Met, 0.96; His, 0.88; Arg, 0.90.

The NH_2-terminal sequence of peptide T-XIX is, therefore, NH_2-Tyr-Leu-Ala-Ser. The peptide moved 3 cm towards the cathode under standard conditions of electrophoresis. This basic property of the peptide at pH 6.4 is very likely due to histidine, and the aspartic acid residue appears not to be amidated. There was not enough material for further studies on this peptide.

Peptide T-XX: Ileu-Phe-Lys

Two stages of the Edman degradation established the sequence.

Stage 0: Ileu, 0.94; Phe, 1.00; Lys, 1.08.

Stage 1: Ileu, 0.14; Phe, 1.06; Lys, 0.88.

Stage 2: PTH-Phenylalanine was identified in the solvent system A.

Peptide T-XXI-1: Phe-Phe-Gly-Ala-Asp-Arg.

Four stages of the Edman degradation were performed with the following results:

Stage 0: Asp, 0.96; Gly, 1.10; Ala, 1.00; Phe, 1.95; Arg, 0.98.

Stage 1: Asp, 0.91, Gly, 1.18; Ala, 1.04; Phe, 0.96; Arg, 0.91.

Stage 2: Asp, 1.00; Gly, 1.08; Ala, 1.00; Phe, 0.10; Arg, 0.81.

Stage 3: Asp, 0.96; Gly, 0.55; Ala, 1.13; Phe, 0.00; Arg, 0.89.

Stage 4: Asp, 1.03; Gly, 0.60; Ala, 0.48; Arg, 0.97.

Glycine was not completely removed in the third stage and that was the reason for a high value of alanine in the subsequent stage of degradation. However, the results clearly indicated that next to glycine occurred alanine. The peptide was neutral at pH 6.4, indicating the presence of an aspartic acid residue. The sequence is shown above.

Peptide T-XXI-2: Thr-Thr-(His,Pro,Tyr,Ser,Glu,Gly_2,Leu)-Lys.

The peptide was subjected to four stages of the Edman degradation.

Stage 0: Thr, 1.91; Ser, 1.15; Glu, 0.85; Pro, 1.06; Gly, 1.76; Leu, 1.16; Tyr, 0.89; Lys, 1.07; His, 1.12.

Stage 1: Thr, 1.08; Ser, 1.22; Glu, 0.96; Pro, 1.34; Gly, 1.67; Leu, 0.97; Tyr, 0.78; Lys, 0.89; His, 1.09.

Stage 2: Thr, 0.28; Ser, 1.27; Glu, 0.92; Pro, 1.16; Gly, 1.59; Leu, 1.10; Tyr, 0.87; Lys, 0.80; His, 1.01.

Stage 3: Thr, 0.34; Ser, 1.28; Glu, 0.98; Pro, 0.68; Gly, 1.49; Leu, 0.97; Tyr, 0.60; Lys, 0.48; His, 0.51.

Stage 4: Thr, 0.40; Ser, 1.60; Glu, 0.96; Pro, 0.66; Gly, 1.74; Leu, 1.00; Tyr, 0.20; Lys, 0.55; His, 0.54.

The degradations could not be followed more than two stages. In the third stage losses of proline, histidine and lysine were prominent. A significant loss of tyrosine occurred in the fourth stage. The peptide moved 2.5 cm towards the cathode in electrophoresis under standard conditions. It appears that Peptide T-XXI-2 contains a glutamic acid residue instead of glutamine. The slight movement of the peptide towards the cathode at pH 6.4 is due to the presence of histidine in the molecule.

Peptide T-XXI-3: His-Gly-Ser-Lys

Three stages of the Edman degradation were performed.

Stage 0: Ser, 0.93; Gly, 1.02; Lys, 1.11; His, 0.93.

Stage 1: Ser, 0.89; Gly, 0.89; Lys, 1.20; His, 0.22.

Stage 2: Ser, 0.90; Gly, 0.51; Lys, not determined.

Stage 3: Ser, 0.43; Gly, 0.50; Lys, 1.00; His, 0.22.

The cyclization of PTC-glycine was not complete in the second stage of degradation and, therefore, the value for serine was high in the subsequent degradation. Nevertheless, the course of degradation could be followed. The sequence is given above.

Peptide T-XXIII-1: His-Arg

One stage of the Edman degradation was performed.

Stage 0: His, 0.96; Arg, 1.05.

Stage 1: His, 0.23; Arg, 1.00.

Peptide T-XXVI-2: His-Gly-Phe-Leu-Pro-Arg

Five stages of the Edman degradation gave the following results:

Stage 0: Pro, 1.12; Gly, 1.01; Leu, 0.97; Phe, 1.02; His, 0.88; Arg, 1.00.

Stage 1: Pro, 1.04; Gly, 1.03; Leu, 1.00; Phe, 0.97; His, 0.17; Arg, 0.96.

Stage 2: Pro, 1.12; Gly, 0.48; Leu, 1.06; Phe, 0.85; His, 0.17; Arg, 0.96.

Stage 3: Pro, 1.03; Gly, 0.47; Leu, 1.06; Phe, 0.29; His, 0.17; Arg, 1.07.

Stage 4: Pro, 1.04; Gly, 0.43; Leu, 0.33; Phe, 0.26; His, 0.13; Arg, 0.95.

Stage 5: Pro, 0.03; Gly, 0.50; Leu, 0.19; Phe, 0.15; His, 0.11; Arg, 0.43

The sequence of this peptide is shown above. The glycine value was always higher in subsequent degradations when other amino acids were removed from the peptide almost quantitatively. This might be due to incomplete extraction of PTH-glycine from the solution of the residual peptide.

DISCUSSION

1. Methods - Extraction

Several methods have been used in the past for the extraction of peptide from biological sources. These methods, though different from one another in certain respects, bear a similarity in their general principle, which involves two separate procedures: extraction and precipitation. The tissue powders or homogenates are extracted with an aqueous acid or salt solution and the extract is then treated with an organic solvent to precipitate the proteins. The choice of a precipitant is, however, arbitrary; some of the peptides may also be precipitated along with the proteins. In some special cases, where the solubility of the peptide is known beforehand, the precipitating agent can be used directly during the extraction; for example, ACTH was isolated by using an acid-acetone mixture (132). But this kind of one-step method of extraction cannot be applied, in general, for the isolation of peptides of unknown properties. Therefore, it is necessary to employ a method which offers diversified conditions suitable for the extraction of peptides with varying properties. The percolation method, used in this work, is one such method. In this method, concentrated ethanol is initially used to denature and precipitate the larger proteins. Peptides are then eluted with a gradual increment of water and acid concentrations in ethanol. The maintenance of the ethanol concentration at 70%, while the concentration of acid is being gradually increased, keeps most of the

proteins precipitated, but facilitates the dissolution and extraction of more and more peptides. A whole range of conditions suitable for the extraction of many peptides is thus available.

The percolation procedure is not only a suitable extraction procedure, it is also an elegant method of fractionation. The use of concentrated ethanol in the early stages of percolation of the tissue powders removes residual lipids, so that they do not contaminate the peptides which are percolated later. This is one of many advantages of the percolation method. Extraction of peptides by other methods may contain fatty materials, especially when the peptide source is a lipid-rich tissue. In the percolation method, most of the free amino acids are extracted first because of their solubility in ethanol. According to their solubility in the percolating solvents, peptides themselves are fractionated into groups while being extracted. This is a great help for the subsequent purification of the peptides. Considering all these facts, the percolation method is one of the best procedures available for the extraction and simultaneous fractionation of peptides from biological materials.

In the original method of percolation, NaCl was used as the electrolyte, but in this modified procedure (270) it has been replaced by acetic acid for several reasons. Firstly, acetic acid does not incorporate any non-volatile material in the peptide extracts and, therefore, the problem of desalting does not arise. Secondly, an acid solvent facilitates extraction of peptides -- a great variety of peptides are soluble in acid-ethanol. The acid is also helpful for the dissociation

of peptides from proteins. Thirdly, because of its weak acidity, acetic acid does not rupture peptide bonds. It has been shown that 80% of vasopressin and 87% of oxytocin can be extracted by this method (293). This is evidence for a high degree of efficiency of the percolation method.

While percolation was used for the extraction of peptides from the tissue powder, a different procedure was used for the fresh tissue. Fresh tissues contain proteolytic enzymes, and it is therefore necessary to stop the enzyme action as soon as the tissue is procured. This was done by collecting the tissues directly in 0.1N HCl. Dilute HCl is also a useful solvent for the extraction of peptides. This solvent has been used for the extraction of hormonally active peptides from the hypothalamus and various other tissues. Although dilute hydrochloric acid is one of the universally applicable solvents for extraction of peptides, its use may be dangerous, if proper care is not taken. Hydrochloric acid attacks peptide bonds even when dilute. It has been shown that 0.03M HCl can liberate specifically aspartic acid and also other amino acids from proteins at 105° (236). However, the action of the acid is minimized in the cold. Since dilute HCl extracts free amino acids and small proteins of the tissue along with the peptides, the presence of these amphoteric substances in the extract decreases the effective concentration of the acid by buffering and thereby the probability of hydrolysis of peptide bonds is decreased.

Methods - Purification

The extracted peptides need further purification before they can be characterized. The conventional methods of fractional precipitation used for proteins are not suitable for the fractionation of peptides because of their relatively small size. Fractionation of peptides can be easily achieved by gel filtration. Gels of various cross-linkages are now available for this purpose. Since we were basically interested in small peptides, Sephadex G-25 was chosen for the initial fractionation of the extracts. This gel excludes larger peptides and proteins. The larger peptides, excluded by Sephadex G-25, can be separated from the proteins by filtration through Sephadex G-50. Thus, peptides can be separated into two groups -- one having molecular weights up to 5,000 and the other having molecular weights from 5 to 10,000. These two gels cover the whole peptide range. Besides this crude fractionation, gel filtration also separates peptides of each of these two groups into subgroups of similar molecular weight.

The percolation method allows peptides to be separated depending on their solubility and the gel filtration permits their separation on the basis of molecular size. But still the desired purity may not be attained. There is a possibility that several peptides of similar molecular size may be eluted together upon gel filtration. Their separation is then possible only by the use of theirionic properties. Therefore, ion-exchange chromatography was chosen as the next step of the purification scheme. Cellulose ion-exchangers and Dowex 50 were used for this purpose. The choice of an

anion or cation exchange resin to be employed depended on the ionic behavior of the peptides in electrophoresis.

Peptides of hypothalamic extracts were thus purified by using the properties of their molecular size and charge. Sometimes, a second gel filtration step was introduced after the ion-exchange step. This gave a final check in the purity of the preparation and was also useful for desalting of the peptide.

The problem of desalting has been avoided, as much as possible, by the use of volatile buffers. Gel filtration has been carried out in dilute acetic acid, CM-cellulose chromatography, in ammonium acetate buffers and Dowex 50 chromatography, in pyridine acetate buffers. The desalting process is not only a tedious one, it also results in losses of peptides. In the case of small peptides, e.g., the tryptic peptides, desalting cannot be successfully accomplished by gel filtration. The other methods, available for this purpose, have to be adapted for each peptide and, therefore, cannot be applied universally. Besides heavy losses, desalting procedures may involve partial decomposition of labile peptide bonds.

2. Purity

One of the major peptide fractions of the fresh hypothalamic extract appeared as peak 9 in the Dowex 50 chromatography (Fig. 4). This preparation has been named "peptide 9". Materials of this peak were ninhydrin negative and appeared as a single sharp peak in both Dowex 50 and Sephadex chromatography (Fig. 5); but the preparation was

divided into two components by partition with a chloroform-methanol mixture. One component was soluble (90%) in chloroform-methanol and the other was insoluble (8%). The amino acid composition of the original preparation (Table I) closely resembled the compositions of the components (Table II) taken together, except for some minor differences in the cases of valine and isoleucine. None of the components was ninhydrin positive. It appears that these two compounds either occurred originally as separate entities in the "peptide 9" preparation or have been formed as a result of cleavage of some covalent bonds. When equal weights of these components were analyzed, the peptide content of the insoluble component was found to be several times higher than that of the soluble component. This suggests that a major portion of the non-peptide material is attached to the chloroform-methanol soluble component. This is also evident from its solubility. In the amino acid analyses, the lysine peak appeared earlier than usual and its value was increased by about 30% in the 48-hour hydrolysate of the "peptide 9" preparation. Moreover, only one residue was recovered after the chloroform-methanol fractionation. This raises the question of identity of the "lysine". If it is lysine, there is no obvious explanation for the loss of one residue, while no loss has been encountered with other amino acids.

The distribution of charge and the number of amino acid residues in these two components are so similar that their separation cannot be expected on Dowex 50 or Sephadex. It is probable that these two components were originally present in the "peptide 9" preparation

and were not derived by the cleavage of covalent bonds.

Another major peptide of the fresh hypothalamic extract was peptide C. It was purified by chromatography on CM-cellulose, in which it appeared as the third peak (Fig. 7). By a careful selection of the tubes, it was possible to avoid contamination of the adjacent second peak. Peptide C behaved as a single zone in electrophoresis over a wide range of pH and its isoelectric pH was 8.2 (Fig. 11). The peptide was found to decompose on storage, with the formation of a neutral peptide and a basic component. It was, therefore, necessary to purify the peptide by preparative paper electrophoresis before it could be used for further studies. The decomposition of this peptide was noticeable only after a long period of storage. Within the short period of an experiment, its self-decomposition was insignificant.

The same CM-cellulose column chromatography yielded peptides A and B in the first peak. The neutral peptide A and the acidic peptide B were isolated by paper electrophoresis. Paper electrophoresis may be taken as an efficient analytical tool for detecting heterogeneity There can be little doubt in the homogeneity of these peptides. Peptides A and B were widely separated in electrophoresis and no ambiguity arose in the cutting out of the peptide-containing zones. However, the possibility that two or more peptides may move together without any noticeable difference in their mobilities cannot be ruled out. But it seems improbable in this case, because these peptides formed single zones in electrophoresis both at a higher and at a lower pH.

Polypeptide 50-52 is the major peptide isolated from the per-

colate fractions 50 to 52 of the acetone-dried hypothalamic tissue powder. The homogeneity of this polypeptide can be attested from the facts that it formed a symmetrical peak in the CM-cellulose chromatography (Fig. 16), it moved as a single zone in electrophoresis over the pH range of 6 to 12, with an isoelectric pH at 9.4 (Fig. 19), and it appeared as a single peak on ultracentrifugal sedimentation (Fig. 20). With these available data, it can be said that the preparation is homogeneous. The question whether the polypeptide is absolutely pure cannot be satisfactorily answered. In fact, such a question can be asked of any protein or peptide preparation without a direct answer. However, the observed properties of polypeptide 50-52 show that the major population of its molecules are alike. The polypeptide was not hydrolyzed by leucine aminopeptidase and did not produce any α-DNP-amino acid. These facts may also be added to the evidence for its homogeneity.

3. Molecular Size

An idea of the molecular size of the isolated peptides may emerge from their behavior on gel filtration and from their amino acid compositions. It is known that Sephadex G-25 excludes substances with a molecular weight greater than about 5,000. Similarly, Sephadex G-50 excludes those with a molecular weight greater than 10,000. The exclusion property of the gel, however, depends on the molecular size rather than the molecular weight. It is also guided by other factors, such as swelling of the gel, aromaticity of the substance, its charge

and, above all, the solvent. The solvent used in this work was kept the same in different experiments so that the elution patterns could be compared.

Peptides of the fresh hypothalamic extract, namely, peptides A, B, and C were isolated from the second peak of Sephadex G-50 chromatography (Fig. 6). This peak was retarded, indicating that the contents have molecular weights less than 10,000. The amino acid compositions of the purified peptides and the molecular weights calculated from them also agree with this estimate. Peptide A contains 81 residues (Table IV) with a calculated molecular weight in the order of 9,000. Peptide B contains 15 residues, giving a molecular weight of about 1,500 (Table IV). Peptide C is as large as peptide A, and contains 80 residues (Table III). There are 8 lysine and 6 arginine residues in peptide C. A tryptic hydrolysis of this peptide should theoretically produce 15 peptides, but it yielded 12 peptides (Fig. 12). If one considers that the peptide may contain some bonds which are resistant to trypsin, it is not difficult to conclude that the amino acid composition, calculated from the molar ratios of the component amino acids, represents the actual molecular size, even though the tryptic digestion did not produce the exact number of peptides that were expected. The calculated molecular weight of peptide C is 8,696.

It is noticeable that peptides A, B and C, which constitute peak II of the Sephadex G-50 column (Fig. 6) came from peak II of the Sephadex G-25 column (Fig. 2). The apparent retardation of peptides A and C on Sephadex G-25 indicates that they may have molecular weights

lower than those calculated from their amino acid compositions. When the purified peptide C was passed through the same column of Sephadex G-25, it was not retarded. The apparent retardation of the peptides may be due to the presence of non-peptidic materials in the extract, which may decrease the resolving power of the gel. Peptide B, because of its smaller size, should be retarded more than peptide A or C. The emergence of this peptide in peak II (Fig. 2) is probably a reflection of its acidic character and the absence of aromatic amino acids in the molecule.

The amino acid composition of polypeptide 50-52 gives a calculated molecular weight of 14,300. A sedimentation coefficient of 1.24S gives also the same range. However, the elution behavior of this polypeptide in the Sephadex G-50 gel filtration was unexpected. The retardation of peak III (Fig. 15), which contained the polypeptide, suggests a molecular weight lower than that calculated. On the other hand, chromatography of the polypeptide on Sephadex G-75 with phosphate buffer at pH 7 showed that its exclusion volume was less than that of horse heart cytochrome c (mol. wt. 12,500), which means that the polypeptide is larger in size than cytochrome c.

Polypeptide 50-52 contains one tryptophan residue and the tyrosine-tryptophan ratio, measured by the spectrophotometric method, is 3. The calculated amino acid composition (Table V) confirms this value. Since the number of residues has been calculated from the molar ratios of the constituent amino acids, the ratios can only be multiplied by an integer to approach the actual composition of the polypeptide.

Whether the polypeptide contains double the number of residues or triple, can be decided by examining the number of peptides formed by trypsin hydrolysis. According to the assumed amino acid composition, the polypeptide contains 13 arginine and 11 lysine residues, giving a total of 24 possible sites of attack by trypsin. A complete digestion should, therefore, produce 25 peptides. The polypeptide yielded 27 major tryptic peptides (Fig. 23), which is very close to the theoretical value. Should the molecule be larger by a factor of two, the number of tryptic peptides would have been 50 or more. The isolation of the tryptic peptides showed that 4 of these peptides contained more than one basic residue; in other words, trypsin did not hydrolyze completely all the arginyl and lysyl bonds. An incomplete digestion may produce more peptides than the number expected in a complete digestion. The digestion of the polypeptide with trypsin produced some minor peptides (Fig. 23), which is evidence of relatively slow hydrolysis of some bonds. Therefore, it appears that the assumed amino acid composition of the polypeptide and the molecular weight derived from this composition represent the actual size of the polypeptide molecule.

The number of chymotryptic peptides (Fig. 21) are also compatible with the present composition of the polypeptide. The retardation of this polypeptide on Sephadex G-50 is probably due to its high basicity; its isoelectric pH is 9.4 (Fig. 19). Although the dextran gel is theoretically free of ionic groups, the possibility of the presence of some carboxylic groups cannot be excluded. This may cause apparent retardation of basic peptides.

4. Characteristics of the Hypothalamic Peptides

A comparison of the peptides, isolated either from the fresh hypothalamic tissue extract or from the acetone-dried powders, reveals some striking similarities among them. None of the peptides contains a cystine or a half-cystine residue. Peptides A and C, and polypeptide 50-52 contain one residue of methionine each. The relatively low content of sulfur-containing amino acids in these hypothalamic polypeptides is noticeable. Peptide B, the smallest of those isolated, is acidic and does not contain any basic residues. The neutral peptide A and the basic peptide C are of the same size, but differ in their amino acid compositions.

Peptide C, the major peptide of the fresh tissue, is apparently absent in the acetone powder, in which there appears a larger peptide, namely, polypeptide 50-52. However, these two peptides are similar in that both are basic and contain one residue of methionine and tryptophan. The fact that the major peptide of the fresh tissue was not easily found in the hypothalamic percolate may suggest that this peptide may be derived from a larger peptide similar to polypeptide 50-52. This cannot be settled until the complete sequences of both the peptides are known. The percolating conditions were so mild that little degradation of peptide bonds can be expected. Peptides isolated by this method can therefore be taken as a better approximation of their natural states in the original tissue.

The isolated peptides of the fresh hypothalamic extract are smaller in size than the polypeptide isolated from the percolate fractions

50 to 52. If the "fresh hypothalamic peptides" also occur in the tissue powders, they would have been percolated in the earlier fractions which have not been fractionated. One cannot conclude, therefore, that the peptides isolated from the fresh hypothalamic extract are artifactual decomposition products.

Peptides A and C of the fresh tissue are similar in size. The yield of peptide A is about 25% that of peptide C. As has been mentioned before, peptide C decomposes to form a neutral peptide and a basic component containing mainly ammonia. The possibility exists that peptide A may be derived from peptide C as a result of decomposition. But there is no conclusive evidence that this neutral peptide and peptide A are the same.

Considering all the peptides isolated, it can be concluded that the major peptides of the hypothalamus are very large, having molecular weights in the range of 8-15,000. These peptides do not contain cystine, so they are probably single-chain polypeptides. Peptide C has an N-terminal aspartic acid and a C-terminal leucine, whereas polypeptide 50-52 does not have a free N-terminal and its C-terminal residue is most likely arginine.

Polypeptide 50-52 is highly basic and contains 130 residues. On the criterion of its size, this polypeptide falls in the group of small proteins. Among the known small basic proteins are the histones. Histones, in general, have molecular weights in the range of 10-20,000 (143). This is a class of ill-defined proteins and no pure preparation is known to date. Histones have been classified into groups, like

lysine-rich, arginine-rich and slightly lysine-rich (294). All these groups have free terminals and, in fact, their purity is being assessed by the number of terminal residues. The hypothalamic protein, though basic like histones and slightly arginine-rich, lacks a free N-terminal. It appears, therefore, that this small protein is not a member of the histone group.

5. Amino Acid Sequence

In the determination of the sequence of the tryptic peptides of polypeptide 50-52, use has been made of the methods of proved reliability, such as the Edman degradation and digestion with carboxypeptidases. The Edman degradation is the most useful tool in sequence determinations. The use of an improved method of cyclization, described by Konigsberg and Hill (225), circumvented the earlier difficulties encountered with this procedure (247). By employing the subtractive method of analysis, introduced by Hirs, Moore and Stein (246), it was possible to follow the course of stepwise degradations precisely. Where availability of the substance permitted, the residual peptide, after two or three steps of degradation, was purified. Such a purification of the peptides is essential to interpret subsequent results of degradation, where ambiguity arises. However, the general course of degradations up to four or five steps could be easily followed even without intermittent purification.

Most of the peptides produced by the tryptic digestion of polypeptide 50-52 contained not more than six residues (Table VII). For

a peptide that contains six residues, only four steps of the Edman degradation are required to obtain the necessary information for its complete sequence. From the known specificities of trypsin, the basic residues, lysine and arginine, have been assigned to the carboxy-terminals of the peptides. None of the isolated tryptic peptides lacks a basic residue. It appears that the trypsin preparation, used in this study, was free from chymotryptic contamination.

Larger peptides were hydrolyzed to smaller fragments with chymotrypsin. The stepwise chemical degradation from the NH_2-terminals and the carboxypeptidase degradation from the COOH-terminals of the fragments yielded enough data for their sequence. The assignment of amide groups to the carboxylic amino acid residues depended on the electrophoretic movements of the peptides at pH 6.4.

A loss of lysine residues during the stepwise Edman degradation was noticed. Sometimes this loss amounted to more than 50% after two or three steps of degradation, but was not encountered in each lysine-containing peptide. Similar losses of lysine residues during the Edman degradation have been reported before (220,246).

With the assumption that lysine and arginine residues occur as the C-terminals in the tryptic peptides, there seems to be no serious error in the proposed sequences. Inversion of sequences at the C-terminals, though unusual, is not impossible. However, the C-terminal residue in peptide T-VI was definitely proved to be lysine by carboxy-peptidase B digestion and that of peptide T-VIII was also found to be lysine, after one step of the Edman degradation of the dipeptide, Ser-

Lys, formed by chymotryptic digestion of peptide T-VIII. The possibility that some of the isolated tryptic peptides might have been formed as a result of transpeptidation during the digestion of the polypeptide with trypsin cannot be categorically denied, though evidence for transpeptidation in enzymic hydrolysis is very rare (220, 223). The proof of such transpeptidation is only possible by a comparison of sequences of similar peptide fragments produced by chemical or by other enzymic hydrolysis.

Since most of the peptides studied are small, the probability of errors in sequences deduced by the Edman degradation is low. Nevertheless, when a glycine residue was encountered, the cyclization of the derivative was not always complete and, therefore, the results of subsequent degradations had to be interpreted cautiously. This is particularly evident in peptide T-XI. Due to unavailability of material, it was not possible to purify the peptide during the stepwise degradations, and the sequence proposed remained uncertain. In the case of other glycine-containing peptides, there was no difficulty in identifying the residue lost during subsequent steps of degradation.

The sequences of the tryptic peptides have been summarized in Table VIII. Peptide T-XVII is a dipeptide of glycine and arginine. Its sequence is obvious. Similarly, peptide T-XXIII-1 is a dipeptide of histidine and arginine and, therefore, its sequence will be His-Arg. These assumptions were proven by one-step Edman degradation of the peptides.

Peptide T-VIII is neutral and contains 3 acidic amino acid residues. Two of these acidic residues must be amidated to confer a

TABLE VIII

Sequences of the tryptic peptides of polypeptide 50-52

The sequence determined by the Edman procedure (——→) and by carboxypeptidase digestion (←——) are indicated. The point of chymotryptic hydrolysis (↓) is shown. The regions of doubtful sequence are enclosed in parentheses.

Peptide	Sequence
T-VI	(Glu-NH_2-Ala-Ser)-Lys
T-VII	Asp-Thr-Ileu-Leu-Gly↓Asp-Ser-Leu-Gly-Arg
T-VIII	Gly-Ala-Glu-NH_2-Asp-Ala-Gly-Glu-NH_2-Thr-Leu↓Ser-Lys
T-XI	(Gly-Ser-Met-Pro-Ala)-Arg
T-XII	Asp-Ser-Arg
T-XIII	Arg
T-XIV	Gly-Ala-Pro-Lys
T-XV-2	Asp-NH_2-Ileu-Val-Thr-Pro-Arg
T-XV-3	Gly-Ser-Gly-Lys
T-XVI-1	Gly-Leu-Ser-Leu-Ser-Arg
T-XVI-2	Gly-Leu-Lys
T-XVII	Gly-Arg
T-XVIII	Leu-Gly-Gly-Arg
T-XIX	Tyr-Leu-Ala-Ser-(Asp,Thr,Ser,Ala_2,Met,His)-Arg
T-XX	Ileu-Phe-Lys
T-XXI-1	Phe-Phe-Gly-Ala-Asp-Arg
T-XXI-2	Thr-Thr-(His,Pro,Tyr,Ser,Glu,Gly_2,Leu)-Lys
T-XXI-3	His-Gly-Ser-Lys
T-XXIII-1	His-Arg
T-XXVI-2	His-Gly-Phe-Leu-Pro-Arg

zero charge to the peptide. The assignment of the amide groups was possible by the identification of PTH-glutamine after the third step of the Edman degradation of peptide T-VIII, and of glutamine after carboxypeptidase digestion of peptide T-VIII-C-II, derived from chymotryptic hydrolysis of T-VIII. Peptide T-XV-2 is basic and contains one aspartyl residue; therefore, this must be amidated to have a net positive charge in the peptide.

Peptide T-XIII yielded only arginine on amino acid analysis. After one step of the Edman degradation, the residue was devoid of arginine. This proved that Peptide T-XIII was free arginine and not a dipeptide or tripeptide of arginine. The NH_2-terminal sequence of peptide T-VI is not clear. It appears that Peptide T-VI contained an N-terminal glutamine residue which cyclized during isolation with the formation of a pyrrolidone carboxylyl peptide. Peptide T-XXI-2 showed loss of both histidine and proline residues in the third step of the Edman degradation. These values remained unaltered in the fourth step, while tyrosine was lost by about 35%. The sequence of this region, therefore, could not be satisfactorily deduced.

6. Comparison of Sequences of the Tryptic Peptides with Those of Other Proteins

The sequences of the tryptic peptides of polypeptide 50-52, the major peptide isolated from the tissue powder, do not bear similarity in any great extent to the known sequences of the polypeptide hormones or of the other proteins, such as ribonuclease, cytochrome c, the α- and

β-chains of hemoglobin, and lysozyme. However, a tetrapeptide sequence of Peptide T-VIII is very similar to the sequence of residues 25 to 28 in corticotropin A and that of residues 26 to 29 in β-corticotropin. Peptide T-VIII has Gly-Ala-Glu-NH_2-Asp, whereas, in corticotropins, the sequence is Gly-Ala-Glu-Asp.

There are some dipeptide sequences which are common among the proteins, and also among the tryptic peptides of polypeptide 50-52 and the proteins. This may be nothing more than coincidental. Repetition of a dipeptide sequence, or often, of a larger fragment at different places within the same polypeptide chain appears to be a common characteristic of protein structure. Occurrence of a particular residue repeatedly in a sequential order is also known. There seems to be no general rule for these phenomena. Polypeptide 50-52 is no exception to this. It contains clustering of some residues, for example, Phe-Phe, Thr-Thr and Gly-Gly, and repeating sequences like His-Gly, Leu-Ser and Gly-Arg. The formation of free arginine (T-XIII) by tryptic digestion indicates that there may be a continuous sequence of the basic residues in the polypeptide. An accumulation of the basic residues in certain parts of the peptide chain has been found in corticotropins and in cytochrome c. One characteristic feature of polypeptide 50-52 is that the glycine residues tend to occur on either side of a basic residue. About 50% of the tryptic peptides examined contain an NH_2- terminal glycine. The Gly-Arg sequence has been found thrice.

7. General Discussion

In 1955, Winnick et al. (295) first reported that the hypothalamus is relatively devoid of peptides and also deficient in a number of free amino acids. Their qualitative results remained unconfirmed until this work was undertaken. This study confirms their findings that the hypothalamus does not have a great quantity of peptides; but to the contrary, the presence of almost all the free amino acids was noticed. A quantitative aspect of the peptide content of the hypothalamus is evident from the percolation pattern (Fig. 13).

It should be mentioned here that a correct estimation of the peptide content in a complex tissue extract involves several difficulties. None of the methods available can specifically measure peptides. Each method has its own merits and demerits. The most widely used, and probably the best, is the Folin-Lowry method. Even this method gives variable results when applied to the tissue extracts and different values are obtained with different standards. The ninhydrin method, when used differentially before and after alkaline hydrolysis, is an excellent method for qualitative detection of peptides, but cannot be used where absolute quantitation is necessary.

In this study, however, use has been made of both of these methods. As can be seen from Fig. 13, results obtained by these two methods are similar. Virtually no peptides appeared in the extracts before fraction 50. Fractions 50 onwards contained peptide materials which have been fractionated and purified. These fractions contain proteins rather than peptides. The earliest percolate fractions may

contain some peptides, if so, they are very likely to be conjugated with lipids, like the complex fraction, "peptide 9", isolated from the fresh hypothalamic tissue. So far as the peptide content is concerned, results from both the fresh tissue and the acetone-dried powder are similar and neither of the sources yielded large quantities of peptides.

The major peptides of the fresh hypothalamus have molecular weights around 9,000, while in the tissue powder, there occurs a much larger polypeptide having a molecular weight of about 14,000. These polypeptides may be considered small proteins. Percolation of the posterior pituitary tissue powder, on the contrary, yielded significant quantities of peptides (270). A comparison of the percolation pattern of the posterior pituitary tissue with that of the hypothalamic tissue immediately reveals the difference (Fig. 13a). In the case of the posterior pituitary, most of the peptidic materials were eluted with 70-80% ethanol, whereas in the case of the hypothalamus, very little or no peptides appeared in this region (Fig. 13). It is known that middle sized peptides, molecular weights ranging from 1-5,000, are extracted with 70% ethanol in this percolation method. One of the major peptides of the posterior pituitary tissue, percolated with 70% ethanol, has been purified and shown to contain 48 amino acid residues (296,297). No such peptide was found in the hypothalamic percolates. If hypothalamus had peptides of similar size, one would expect them to be percolated with the comparable solvent. Moreover, a comparison of the total peptide contents of the percolates, as represented by the Lowry values, clearly shows that the peptide content of the hypothalamus is

nearly one-tenth that of the posterior pituitary (Fig. 13a; 270).

In view of the fact that the hypothalamus contains biologically active peptides (84), a fresh tissue preparation had been worked up. No small peptides could be isolated, except for the one described as peptide B. Its molecular weight is about 1,500. This peptide lacks basic residues (Table IV). Its yield was insignificant compared to other larger peptides isolated from the same source. It is obvious that microgram quantities of peptides are sufficient to demonstrate the biological activities, but not enough for isolation, purification and characterization. Various groups of workers have been trying to isolate biologically-active peptides from the hypothalamus, but without great success. One of the main difficulties of this kind of work is the unavailability of the starting material. Again, if the tissues are not processed immediately after collection, the active-peptides may be destroyed either by the tissue proteases or by the chemicals used for the inactivation of the proteolytic enzymes. The tissue preparations used in this study were devoid of ACTH-releasing or pressor activities. The presence of other biological activities was not thoroughly tested. The isolated polypeptides were also inactive in the limited test systems. Polypeptide 50-52 of the hypothalamic percolate has been subjected to sequential study to see if some part of the molecule resembles any of the pituitary hormones, but there appears to be no sequential relation between this protein and the peptide hormones. The function of this protein in the hypothalamus remains obscure. Before testing it thoroughly for all sorts of biological activities, any implication as to its biological significance would be presumptuous.

However, certain things have become clear from this study. The hypothalamus does not store large quantities of peptides, though it may be the site of synthesis of several biologically-active peptides. There is both a qualitative and a quantitative difference in the peptide contents of the hypothalamus and of the posterior pituitary.

8. Suggestions for Future Work on This Problem

All the fractions obtained by percolation of the hypothalamic tissue powder should be systematically analyzed. Since the hypothalamus it not rich in peptides, a large quantity of the starting material will be necessary for the purification and characterization of those peptides which occur in relatively minor amounts.

The isolated protein should be subjected to various test systems for its biological activity. This small protein, apparently, may have some function in the hypothalamus. Although the amino acid sequence of some parts of the protein molecule is known, studies should be undertaken towards the completion of its two-dimensional structure, before it can be convincingly compared with other protein or polypeptide hormones. It is evident from the present work that the hypothalamus contains several large molecular weight peptides. Whether these large molecules are precursors of the polypeptide hormones of the pituitary gland is essential to know.

A concurrent study of the peptides of other parts of the brain should be undertaken. Such studies will show the difference, if any, between the peptides of the hypothalamus and of other parts of the brain. This will also provide further information regarding the

general characteristics of the neural peptides of which very little is known.

SUMMARY

Fresh hog hypothalamic tissue was extracted with dilute hydrochloric acid and three peptides were separated by successive use of gel filtration on Sephadex G-25 and Sephadex G-50, chromatography on CM-cellulose, and paper electrophoresis. One of these peptides, peptide A, contains 81 amino acid residues; another, peptide B, contains 15 residues and the third, peptide C, contains 80 residues with a C-terminal leucine. A complex lipopeptide fraction was also isolated by gel filtration on Sephadex G-25 and chromatography on Dowex 50-X2.

Hog hypothalamus acetone powder was percolated with a discontinuous gradient of water and acetic acid in ethanol. Peptides in the percolates were estimated by the Folin-Lowry and ninhydrin methods. One of the major peptides of the percolates was purified by gel filtration and chromatography on CM-cellulose. This peptide contains 130 amino acid residues. The peptide was hydrolyzed with trypsin and the resulting peptide fragments were isolated and purified by chromatography on Dowex 50-X2, followed by paper electrophoresis and paper chromatography. The amino acid sequence of most of the tryptic peptides were determined by the use of the Edman degradation and carboxypeptidase digestion. The amino acid sequences of the peptide fragments were compared with the known sequences of the proteins and peptides.

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CLAIM TO ORIGINAL WORK OR CONTRIBUTION TO KNOWLEDGE

This is the first investigation aimed at the isolation and characterization of the major peptides of the hog hypothalamus, without limiting the study to the search for a particular biologically active peptide. The hypothalamus was found to contain much smaller quantities of peptidic materials than the posterior lobe of the pituitary gland. Several hitherto unknown polypeptides were isolated and partially characterized.

APPENDIX

Acetone-dried hog brain powder (2.58 kg), excluding the hypothalamus, was percolated under the same conditions as the hypothalamus powder (p. 45). The percolation pattern is shown in Fig. 25. As can be seen from the figure, the whole brain contained small amounts of extractable peptide material. Brain tissue contains about 1/5 the peptide content of the hypothalamus (cf. Fig. 13). The elution patterns of the peptides of these two tissues are also different, suggesting that at least some of the peptides of the brain are distinct from the hypothalamic peptides.

A peptide was purified from the brain percolate fractions 71-79. The purification procedures (Fig. 26) were essentially the same as those used for the hypothalamic peptides. The amino acid composition of the peptide is given in Table IX. The peptide contains 13 amino acid residues, with a calculated molecular weight of 1545. The peptide was retarded on Sephadex G-25. On a Sephadex G-50 column (2.5 x 107 cm), its exclusion volume was 329 ml; the exclusion volume for ACTH, on the same column, was 248 ml. This is evidence that the peptide is much smaller than ACTH. The assumed amino acid composition of the peptide probably represents its actual molecular size.

The peptide was isolated from Peak V (Fig. 26) of the CM-cellulose chromatography. This peak was eluted with 0.5M ammonium acetate buffer at pH 6.4 The basic peptide moved 5.5 cm towards the cathode when subjected to electrophoresis in pyridine-acetate buffer,

pH 6.4 for 150 min. at 750 volts. Under the same conditions, free lysine and arginine moved 6.5 and 7.0 cm respectively. The electrophoretic movement of the peptide indicates that the acidic amino acid residues in the peptide are not amidated. Only 7.0 mg of the peptide was obtained from 2.58 kg of acetone powder of hog brain.

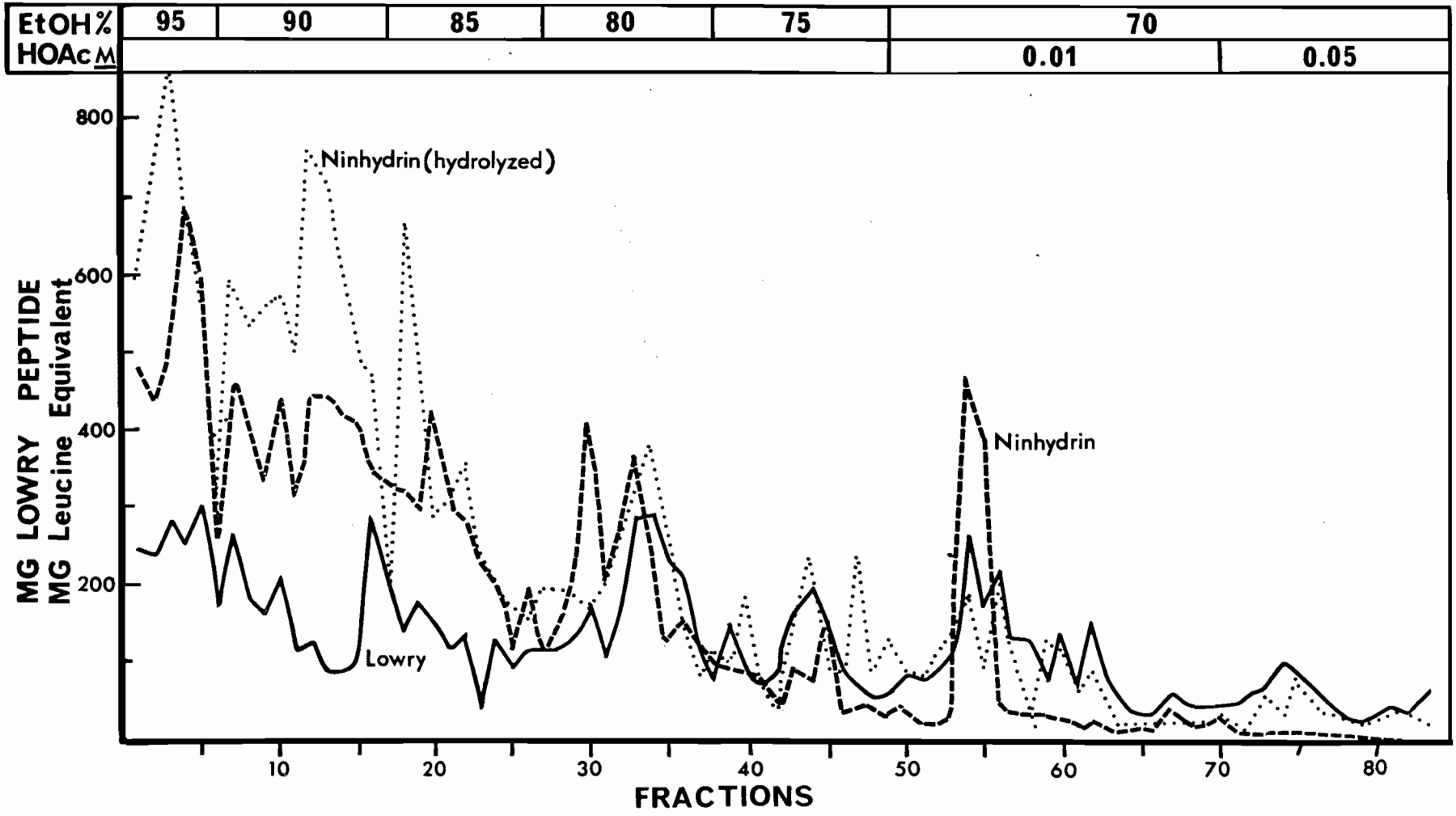

FIG. 25. Percolation of 2.58 kg of hog brain powder (excluding the hypothalamus) with the solvents indicated. Fractions were collected in lots of 900 ml. Aliquots from individual fractions were analyzed by the Lowry method and with ninhydrin before and after alkaline hydrolysis.

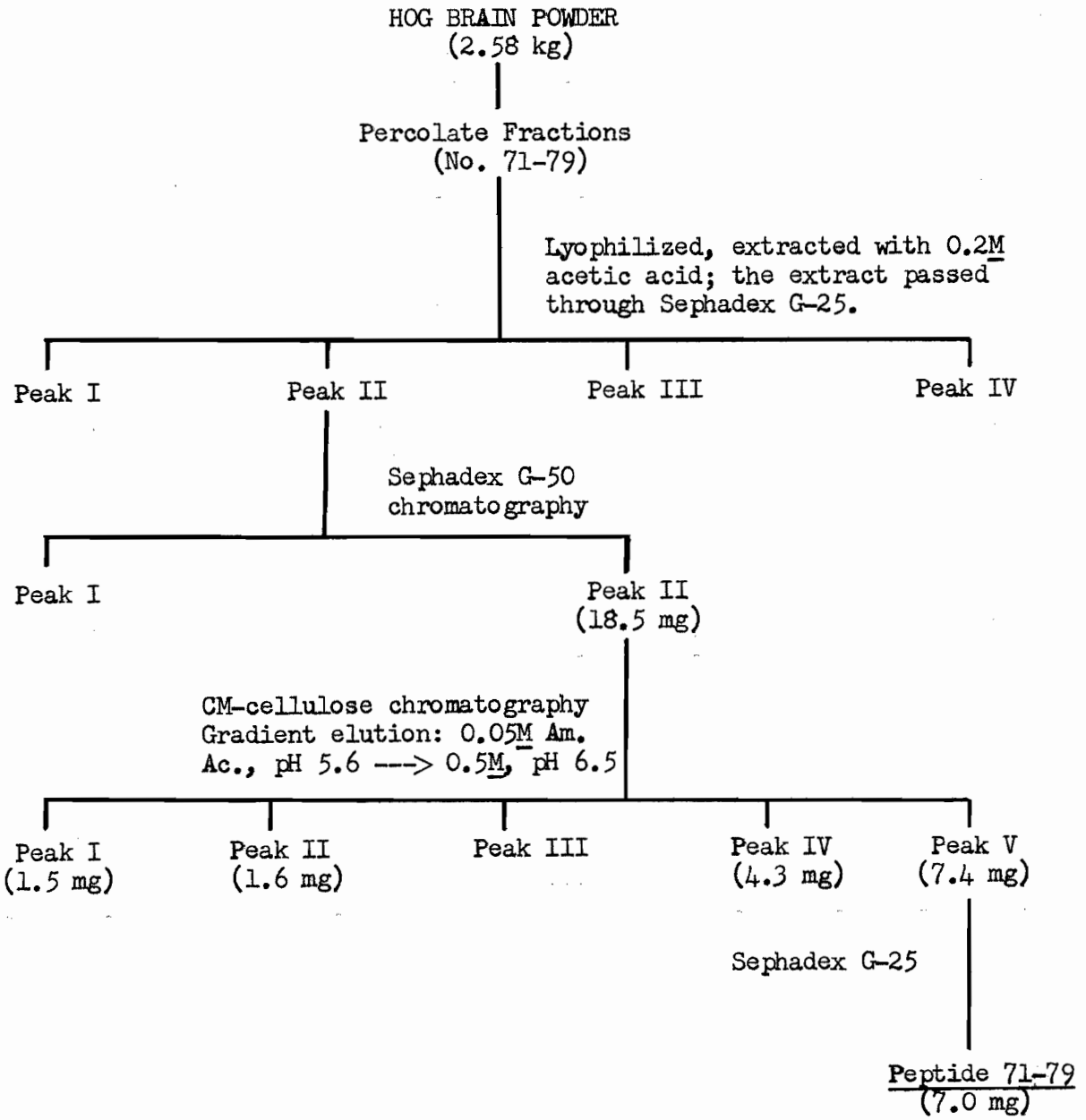

FIG. 26. Flow sheet of the purification of a peptide from the hog brain powder.

TABLE IX

Amino acid composition of the hog brain peptide 71-79

Amino acid	Calculated molar ratio	Nearest integral value
Serine	2.13	2
Glutamic acid	1.68	2
Proline	1.23	1
Glycine	1.13	1
Alanine	1.07	1
Tyrosine	0.90	1
Lysine	1.93	2
Histidine	1.01	1
Arginine	1.94	2
Total		13
Minimum molecular weight		1545