AMINO ACIDS AND PEPTIDES OF POSTERIOR PITUITARY AND HYPOTHALAMUS TISSUES

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INTRODUCTION

In 1917 ABEL AND PINCOFF[1] demonstrated by qualitative chemical tests that posterior pituitary tissue contains high concentrations of "peptones" and "proteoses". Some years later KAMM and co-workers[2] demonstrated the presence of substances belonging to these categories, the hormones ocytocin and vasopressin. From the data of various investigators[3,4], it appears that the concentration of each hormone molecule is approximately 2 mg per g of acetone-desiccated beef posterior lobe, or 0.5 mg per g of fresh tissue**. Very recently melanocyte-stimulating hormone (intermedin) has been prepared from (hog) posterior pituitary acetone powder[5]. Since the specific activity was increased 500 times in purification, the concentration of this polypeptide in the starting material seems comparable to that of ocytocin or vasopressin. Intermedin appears to contain all of the common amino acids, except histidine and *iso*-leucine.

No attempt has been made to measure accurately the total peptide concentration, or to systematically examine posterior pituitary tissue for additional peptides. The present authors, in subjecting crude protein-free extracts of beef and hog posterior glands to paper chromatographic analysis, have found that certain amino acids, not components of the ocytocin or vasopressin molecules, are present in peptidic form. Subsequent experiments revealed an unusually high level of peptides, several times greater than that which could be readily attributable to the known hormones. It appeared of interest to explore and characterize this rich peptide pool. Also, in view of the alleged role of the hypothalamus[6] as the site of synthesis of the polypeptide hormones, this region has likewise been subjected to chemical scrutiny.

EXPERIMENTAL

Materials

Tissues. Fresh beef and pork posterior pituitary glands were kindly supplied by Le Laboratoire de l'Endopancrine, and collected in acetone at —20°. Fresh beef hypothalamus, obtained through the courtesy of Abattoirs de Vaugirard, were dissected into posterior and anterior halves, and utilized at once.

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** One g of acetone-powder, derived from about 4 g of tissue, contains approximately 1000 units of each hormone. The polypeptides in the pure state each have specific activities of the order of 500 units per mg.

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Preparation of extracts. In the case of pork posterior pituitary, two types of protein-free extracts were prepared:

(1) Glands, freed of acetone, were blendorized with 2 parts of 0.5% acetic acid, followed by 7 parts of 95% ethanol. The suspension was heated for several minutes near the boiling point, cooled, and the coagulated protein removed by centrifugation. After standing overnight in the refrigerator, the solution was freed of an additional slight protein precipitate, and then concentrated almost to dryness *in vacuo* to remove the alcohol. It was then reconstituted with the original volume of water, extracted with ether to remove lipids, lyophilized, and the dry residue dissolved (completely) in water to give a clear, almost colorless solution.

(2) The glands were blendorized with 4 parts of 0.5% acetic acid. Then 5 parts of 10% trichloracetic acid (TCA) were added, and the precipitated protein removed by centrifugation. The clear solution was extracted with ether (to remove TCA and lipids), lyophilized, and reconstituted with water.

Only a TCA extract of beef pituitary was employed.

The posterior and anterior portions of beef hypothalamus were (separately) dehydrated and delipidized with acetone. The resulting powders, each representing about 22% of the weight of fresh tissue, were extracted with 20 parts of 5% TCA. The extracts were freed of protein, and extracted with ether.

Preparation of peptide fractions. Two methods were employed, starting with two separate quantities of the stock pork posterior pituitary extract:

(1) The charcoal method developed for posterior tissue[7] was used to adsorb the major part of the peptides, together with small amounts of aromatic amino acids. The subsequently eluted substances were then chromatogramed for 2 days on paper with butanol-formic acid, in order to remove mobile compounds, including the traces of aromatic amino acids. Almost all of the chromatogramed material remained as a single component near the origin, and was extracted with water and concentrated *in vacuo*.

(2) The 4-compartment ionophoretic apparatus of SYNGE[8] was employed[9] (pH 6, 200 volts, 5 hours) to obtain an electrically-neutral fraction, free of vasopressin and intermedin. Subsequently this fraction was subjected to butanol-formic acid chromatography (2 days), the material near the origin extracted with water, rechromatogramed (one day) with pyridine-collidine[7], and the non-mobile fraction again collected. In this way free amino acids and ocytocin were eliminated, as well as any residual vasopressin.

All preparations were stored frozen.

Methods

Determination of conjugated amino acids by ninhydrin colorimetry. Suitable samples of the stock extracts were analysed according to the quantitative procedure of TROLL AND CANNAN[10], before and after acid hydrolysis. The increases in color values were calculated in terms of molar concentrations of conjugated amino acids. Hydrolysis was effected by heating the samples for 15 hours in 5 *N* HCl at 110° in sealed tubes and then removing the acid by evaporating to dryness *in vacuo*.

Determination of peptides by the biuret reaction. The method of GORNALL and co-workers[11] was applied directly to the stock tissue extracts, and the colors evaluated in terms of a reference curve, prepared with crystalline bovine serum albumin.

Ammonia. Samples were hydrolyzed for 2 hours at 100° with 1 *N* HCl, and analyzed by the standard Nessler method.

Qualitative paper chromatography. Acid hydrolysates were analysed with the aid of Whatman No. 1 paper and two solvent systems[12]: butanol-formic acid-H_2O, and phenol—pH 10 buffer. Spots were generally revealed by ninhydrin, but in some cases specific amino acid color reactions[7] were also used.

Semi-quantitative paper chromatography. An accurately-measured amount of a hydrolysate was chromatogramed along a 3 cm-wide vertical section of the paper, using butanol-formic acid solvent (2 days). The exact position of each spot was determined by running identical chromatograms on either side, and then developing these markers with ninhydrin. The individual zones of the central (undeveloped) chromatogram were cut out, and each was placed in a tube containing 3 ml of 0.5% ninhydrin in 90% butanol — 10% pyridine*. Each tube was heated for 2½ minutes at 100° with stirring, cooled, and 0.5 ml of H_2O added. After standing 10 minutes (or longer) at room temperature, with occasional stirring, to facilitate extraction of color from the paper, each tube was read at 570 mμ (440 mμ for proline). The optical densities were referred to standard curves, prepared by chromatograming varying amounts of pure amino acids and determining their molar color values as described above. The results obtained with this procedure are considered accurate to within approximately 10%.

* The TROLL-CANNAN reagents could not be used, since they gave rise to variable ninhydrin colors in the presence of paper, in blank chromatograms.

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RESULTS

Peptide concentrations in tissue extracts

The results are summarized in Table I. It may be seen that the two different methods of extraction gave essentially the same results with hog posterior pituitary tissue. Also the biuret values are comparable to those calculated from the Troll and Cannan ninhydrin method. The former procedure did not include dipeptides; while the latter included ammonia, but not proline.

TABLE I

PEPTIDE CONCENTRATIONS IN PITUITARY AND HYPOTHALAMUS TISSUES

Source of tissue	*Method of precipitating proteins*	*Ninhydrin procedure*				*Biuret method*
		Unhydrolyzed extract	*Extract hydrolyzed with HCl*	*Increase in color value*		*Peptides, mg per 100 g*
		mM per 100 g tissue		*mM per 100 g*	*mg per 100 g**	
Pork posterior pituitary	5% TCA	2.6	10.6	8.0	960	800
	Hot 70% alcohol	2.8	11.2	8.4	1000	850
Beef posterior pituitary	5% TCA	1.6	5.4	3.8	460	470
Beef anterior hypothalamus	5% TCA	1.6	4.4	2.8	—	0
Beef posterior hypothalamus	5% TCA	1.7	4.1	2.4		0

* Assuming an average value of 120 mg per m*M* of amino acid.

The values for beef are only about half as great as those for pork posterior pituitary gland, but it is significant that the peptide level in the bovine tissue is about four times greater than that commonly attributed to the known peptide hormones.

The data for hypothalamus tissue indicate that the biuret reaction is more reliable than the ninhydrin method for detection of polypeptides. Other results to be given presently confirm the view that hypothalamus has no significant peptide pool, and that much of the increased ninhydrin color represents the cleavage of amide groups.

Amides in tissue extracts

Determinations by the Nessler reaction (Table II) indicated rather elevated concentrations of amide groups. The value, 0.55, for beef posterior pituitary is about twice that attributable to the amide ammonia of ocytocin plus vasopressin (assuming 100 mg or 0.1 m*M* of hormones per 100 g tissue). However some of the amide nitrogen was probably due to intermedin[5].

TABLE II

CONCENTRATION OF AMIDE AMMONIA IN PITUITARY AND HYPOTHALAMUS

Tissue	*mM of NH_3 per 100 g tissue*		
	Original extract	*Hydrolyzed extract*	*Ammonia liberated*
Pork posterior pituitary	0.15	1.0	0.85
Beef posterior pituitary	0.05	0.6	0.55
Beef posterior hypothalamus	0.3	1.7	1.4
Beef anterior hypothalamus	0.3	1.8	1.5

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The very high levels in hypothalamus account for more than half of the conjugated ninhydrin-positive substances in this tissue (Table I).

Identification of conjugated amino acids in pork pituitary extract. Paper chromatographic analysis revealed marked changes in pattern following acid hydrolysis. Table III shows that the intensities of the spots were increased in every case. Glutamic acid, and perhaps alanine, were the most abundant free amino acids, but both were also present in conjugated form. All other amino acids were chiefly in peptide combination. Several of these (threonine, serine, valine, alanine, lysine-histidine) are not found in ocytocin or vasopressin, although present in intermedin. Also, quantitative analysis[13] revealed that methionine constituted 0.8% of the total amino acid residues.

TABLE III

CHROMATOGRAPHIC COMPARISON OF UNHYDROLYZED AND ACID-HYDROLYZED EXTRACTS OF PORK POSTERIOR PITUITARY TISSUE

Amino acid	*Relative strength of ninhydrin spots*	
	Original extract	*Hydrolyzed extract*
Threonine*	not visible	very weak
Serine*	not visible	moderate
Proline**	not visible	strong
Valine**	extremely weak	moderate
Phenylalanine**	extremely weak	moderate
Tyrosine**	extremely weak	moderate
Leucine-*iso*leucine**	extremely weak	moderate
Aspartic acid*	very weak	strong
Glycine*	very weak	very strong
Alanine*, **	weak	very strong
Glutamic acid*, **	moderate	very strong
Cystine**	uncertain	moderate
Lysine-histidine**	uncertain	strong
Arginine**	uncertain	strong

* Phenol-pH 10 buffer. ** Butanol-formic acid-H_2O.

Chromatography of hypothalamus extracts. Table IV gives the findings with anterior hypothalamus. Identical results were obtained with the posterior lobe. The chromatograms confirmed the view that this region contains no significant peptide pool. A number of amino acids could not be detected at all, and others (such as glycine, alanine and serine) were in much lower concentrations than in posterior pituitary tissue. Glutamic acid was most abundant, followed by aspartic acid. The substance which gave a strong (brownish-red) spot between arginine and the aspartic-glycine-serine region (butanol-formic acid solvent) with unhydrolyzed extracts, was found to coincide in position with a sample of pure glutamine. Furthermore, this material gave rise to glutamic acid after hydrolysis. Accordingly hypothalamus resembles brain[14,15] in its very high levels of glutamine and glutamic acid.

TABLE IV

CHROMATOGRAPHIC COMPARISON OF UNHYDROLYZED AND HYDROLYZED EXTRACTS OF BEEF ANTERIOR HYPOTHALAMUS

Amino acid	*Relative strength of ninhydrin spots*	
	Unhydrolyzed	*Hydrolyzed*
Threonine*	not visible	not visible
Proline**	not visible	not visible
Valine**	not visible	not visible
Leucine-*iso*leucine**	not visible	not visible
*Phenylalanine***	*not visible*	*not visible*
Tyrosine**	not visible	not visible
Glycine*	not visible	not visible
Serine*	not visible	very weak
Alanine*	very weak	very weak
Arginine**	very weak	weak
Aspartic acid*	moderate	strong
Glutamic acid*, **	strong	very strong
Substance** between arginine and aspartic acid	strong	not visible
Substance** between alanine and tyrosine	weak	weak

* Phenol-pH 10 buffer. ** Butanol-formic acid-H_2O.

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Charcoal-adsorbed peptide fraction

This material represented about half of the total (free plus conjugated) amino acids in the original posterior pituitary extract. When analyzed by the Troll-Cannan method, it gave a 12-fold increase in color value after hydrolysis. Further confirmation of polypeptide structure was provided by paper chromatographic analysis (Table V). At least 13 different amino acids were identified, as well as minor amounts of threonine and methionine. More accurate determinations by the photometric method (Table VI) showed that two-thirds of all the amino acid residues in the adsorbed peptides appeared in four regions: glycine-serine-aspartic acid, threonine-glutamic acid, alanine, and leucine-*iso*leucine.

Neutral peptide fraction

This represented approximately a fourth of the total amino acids in the original extract, and gave an 11-fold increase in color after hydrolysis. Chromatography revealed only six strong, and four weaker spots in the hydrolysate (Table V). Semi-quantitative analysis (Table VI) showed that the neutral preparation differed from the adsorbed fraction in its relatively much lower leucine-*iso*leucine content, and lower percentages of valine and aromatic amino acids. Also it was very rich in glycine, serine being absent. The proportions of acidic to basic amino acids were about the same in the neutral and in the adsorbed preparations.

TABLE V

IDENTIFICATION OF AMINO ACIDS IN CHROMATOGRAMS OF PEPTIDE FRACTIONS

Amino acid identified in hydrolysate	*Adsorbed fraction*			*Neutral fraction*		
	Ninhydrin spot		*Specific qualitative color test*	*Ninhydrin spot*		*Specific qualitative color test*
	Strong	*Weak*		*Strong*	*Weak*	
Glycine	Ph			Ph		
Alanine	Ph, BF			Ph, BF		
Serine	Ph					
Threonine		Ph				
Valine	BF				BF	
Leucine-*iso*leucine	BF				BF	
Phenylalanine	BF				BF	
Tyrosine	BF					
Methionine			x			x
Cystine	BF		x			x
Proline	BF			BF		
Arginine	BF		x	BF		x
Lysine-histidine	BF			BF		*
Aspartic acid	Ph				Ph	
Glutamic acid	Ph, BF			Ph, BF		

BF = 75 % butanol — 15 % formic acid — 10 % H_2O.
Ph = Phenol — pH 10 buffer.
* For histidine.

DISCUSSION

The Troll and Cannan method cannot be used to determine free amino acids accurately, since peptides contribute to the ninhydrin color of unhydrolyzed extracts.

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TABLE VI

RELATIVE PROPORTIONS OF AMINO ACIDS IN PEPTIDE HYDROLYSATES

Amino acid	*% of total residues*	
	Charcoal-adsorbed fraction	*Neutral fraction*
Cystine	4	Uncertain
Lysine + histidine	4	10
Arginine	5	9
Glycine + serine + aspartic acid	16	31
Threonine + glutamic acid	15	19
Alanine	15	12
Proline	6	8
Valine	8	4
Tyrosine	5	Uncertain
Phenylalanine	6	3
Leucine-*iso*leucine	17	2

Nevertheless it is evident that a considerable concentration of free amino acids (such as glutamic) is present in posterior pituitary extracts. The increase in color value after hydrolysis generally gives a good measure of polypeptide concentration, when compared with the Van Slyke manometric-CO_2 method[16]. Furthermore, the agreement of the ninhydrin values with the biuret determinations is satisfactory with pituitary tissue.

The results with the two peptide fractions are, of course, exploratory. The adsorbed material was almost certainly a mixture of several substances, including vasopressin, intermedin, and possibly ocytocin. The neutral fraction was probably free of hormones, and relatively homogeneous.

The abundance of certain amino acids, such as glutamic acid, glycine, and alanine, strongly suggest that unknown peptides rich in these residues, are present in posterior pituitary tissue. These peptides could be metabolically related to the hormones, or might have as yet undisclosed physiological rôles.

The physiological and cytological evidence[4,6] supports the view that ocytocin and vasopressin are formed in the hypothalamus. The quantity of these hormones present in this region is only about one-hundredth of that in the posterior pituitary gland of animals. However, despite the apparent absence of peptides and the deffi-ciency in various free amino acids in protein-free extracts of hypothalamus, it is still possible that the rate of synthesis of the hormones (or their molecular precursors) in hypothalamus is adequate to balance turnover or loss from the posterior pituitary. The high concentrations of glutamic acid and glutamine in hypothalamus correspond to the situation in brain tissue, in which these two compounds account for at least half of the non-protein α-amino nitrogen[15]. There is no present indication that either amino acid has a key role in hormone synthesis.

SUMMARY

Quantitative determinations by both biuret and ninhydrin procedures have shown that beef and pork posterior pituitary glands contain very high concentrations of polypeptides, several times greater than that attributable to known hormones. Amino acid analysis by paper chromato-

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graphic methods support the view that this tissue contains other peptides, in addition to ocytocin, vasopressin and intermedin. Exploratory fractionations of these peptides have been effected by ionophoresis and charcoal-adsorption.

The alleged role of hypothalamus as the site of hormone synthesis was discussed in relation to the finding that this region, unlike posterior pituitary, had no significant peptide pool and was deficient in a number of free amino acids. Hypothalamus like brain, was rich in glutamine and glutamic acid.

RÉSUMÉ

Le dosage des peptides contenus dans la posthypophyse du boeuf et du porc a permis de constater que cette glande possède une grande teneur en peptides, beaucoup plus élevée que celle calculée d'après la richesse en hormones connues (ocytocine, vasopressine, intermédine). Une purification préliminaire de ces peptides a été effectuée à l'aide de l'ionophorèse et de l'adsorption sur charbon.

L'hypothalamus, au contraire, s'est révélé très pauvre en peptides et acides aminés libres, à l'exception de la glutamine et de l'acide glutamique. Le rôle présumé de l'hypothalamus dans la synthèse des hormones a été discuté en fonction de ces résultats.

ZUSAMMENFASSUNG

Quantitative Bestimmung der im Hypophysenhinterlappen des Rindes und des Schweines enthaltenen Peptide, mit Hilfe der Biuret und Ninhydrinmethode, ergab eine sehr hohe Peptinkonzentration, weit höher als die Summe der bekannten Hormone in diesem Organ. Die Analyse dieser Peptidfraktion auf Aminosäuren mit Hilfe chromatographischer Methoden zeigte dass das Hypophysenhinterlappengewebe andere Peptide als Ocytocin, Vasopressin und Intermedin enthält. Versuchsweise Fraktionierung dieser Peptide mit Hilfe von Ionophorese und Adsorption auf Kohle wurde unternommen.

Im Hypothalamusgewebe wurden, im Gegensatz zum Hypophysenhinterlappen, nur sehr kleine Mengen von Peptiden gefunden. Mehrere Aminosäuren konnten auch spürenweise nicht gefunden werden. In diesem Zusammenhang wurde die vermutungsweise wichtige Rolle, welche der Hypothalamus in der Hormonsynthese spielt, erörtert. Hypothalamusgewebe ist, wie Gehirngewebe, an Glutamin und Glutaminsäure reich.

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