13225

A PHARMACOLOGICAL, CHROMATOGRAPHIC, AND CHEMICAL STUDY OF THE NEUROHYPOPHYSIAL PRINCIPLES OF TWO ELASMOBRANCH SPECIES: *RAIA RHINA* AND *SQUALUS ACANTHIAS* (PACIFIC VARIETY)

by

ELIZABETH ANNE HEINICKE

B.Sc.(Hon.), University of Manitoba, 1962

M.Sc., University of Manitoba, 1965

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

in the Department of Zoology

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

July, 1972

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the Head of my Department or by his representatives. It is understood that copying or publication of this thesis for financial ain shall not be allowed without my written permission.

Department of Zoology

The University of British Columbia
Vancouver 8, Canada

Date Sept 21 '72

ABSTRACT

A wide and confusing array of neurohypophysial principles has been attributed to the elasmobranch fish; several of these principles are notably similar, either in their distinguishing characteristics or in the species in which they have been reported to occur. Here, an attempt was made to resolve some of the confusion regarding the exact number and nature of the elasmobranch principles, by a study of the pharmacological, chromatographic, and chemical characteristics of the agents produced by two species: the skate Raia rhina and the dogfish Squalus acanthias (Pacific variety) = Squalus suckleyi.

The neurointermediate lobe extracts of both species contained a major neutral oxytocic fraction and small amounts of a basic antidiuretic principle which appeared to be the widespread agent, arginine vasotocin. This identification was based on direct pharmacological comparison of the purified Squalus principle against synthetic arginine vasotocin. It was the first such characterization of an elasmobranch principle, and possibly of any naturally occurring oxytocin analogue, against its possible synthetic equivalent; earlier comparisons had been made against the customary oxytocin or vasopressin standards.

Neurointermediate lobe extracts of Raia rhina showed an unexpected variation in the extent to which their activity on the isolated rat uterus was potentiated by the presence of magnesium ions in the bathing solution (1.8 fold to 17.5 fold). This could not be correlated with factors such as season or location of the catch; it was shown to result from anomalies in the distinction which different uterine preparations made between the unknown peptide and the oxytocin standard. The potency of Raia extract also varied on the milk-ejection assay, with a corresponding variation in the ratio of milk-ejection activity to rat uterus activity of from 4.4 to 17.5.

Many of the oxytocic principles ascribed to the elasmobranchs have been distinguished by their degree of magnesium potentiation or by the ratio of their milk-ejection activity to their oxytocic activity on the rat uterus. The variability described here for these two values may invalidate such distinctions; in conjunction with other available data, these observations raised the possibility that the many elasmobranch oxytocin-like principles might be reduced to two -- the agent designated as E_1, which did not occur in the two species studied here, and glumitocin, a principle of known structure. Later studies on the neutral fraction of Raia rhina indicated that the agent was glumitocin, but the studies on Squalus suggested the introduction of several new principles to the elasmobranch spectrum.

The identification of the Raia principle as glumitocin was based on direct pharmacological comparison of the agent against synthetic glumitocin, and on chemical analysis of the principle. Direct pharmacological comparison of the Squalus neutral fraction against synthetic glumitocin showed clear differences between the two principles, notably in their degree of magnesium potentiation and in their milk-ejection activities. The Squalus neutral fraction was purified by gel filtration through G-15 Sephadex, by chromatography on Sephadex ion exchangers, and by chromatography on paper. The activity was separated into two distinct fractions on CM Sephadex. These, however, behaved identically throughout numerous comparative procedures; they were subsequently shown to be experimental artifacts, and were recombined. The purified neutral fraction showed an activity of 109 IU/mg, the most active preparation of the Squalus oxytocic agent so far obtained. Amino acid analyses of the purified material suggested that it consists of an equimolar mixture of two peptides, neither of which has been encountered previously in nature.

The work presented here lays a basis for a better understanding of the elasmobranch neurohypophysial principles, but the details of the distribution and structure of these peptides must await yet more extensive studies.

TABLE OF CONTENTS

Page

ABSTRACT. ii

TABLE OF CONTENTS v

LIST OF TABLES. xiii

LIST OF FIGURES xv

ACKNOWLEDGEMENT xvii

Chapter 1 - INTRODUCTION. 1

I. THE COMPARATIVE BACKGOUND TO THE STUDY OF THE ELASMOBRANCH NEUROHYPOPHYSIAL PRINCIPLES. 1

A. The Vertebrate Hypothalamo-Neurohypophysial System. 1

1. Anatomical and Histological Considerations. 1

2. The Nature of the Neurohypophysial Principles. 5

a. The structure of the known principles . 5

b. The biological activities of the peptides. 7

c. The phylogenetic distribution of the peptides. 8

B. The Elasmobranch Hypothalamo-Neurohypophysial System. 12

1. Anatomical and Histological Considerations. 12

2. The Nature of the Elasmobranch Neurohypophysial Principles 20

a. Evidence for neurohypophysial activity in the elasmobranch pituitary 20

Page

b. Evidence supporting the existence of an elasmobranch antidiuretic principle. 25

c. Evidence supporting the existence of several elasmobranch oxytocic principles 26

i. Differences between the oxytocic principles, based on relative milk-ejection activities. 27

ii. Differences between the oxytocic principles, based on magnesium potentiations. 28

iii. Differences between the oxytocic principles, based on chromatographic behavior. 29

(1) The resolution of two oxytocic principles within a single species. 29

(a) Species in which E_1 and E_2 have different magnesium potentiations 30

(b) Species in which E_1 and E_2 have similar magnesium potentiations 31

(c) Species in which neither E_1 nor E_2 is potentiated by magnesium ions 32

(2) The resolution of a single oxytocic fraction within each species 32

II. THE STATEMENT OF THE PROBLEM. 38

Chapter 2 - MATERIALS AND METHODS. 40

A. Collection and Storage of the Glands 40

B. Extraction of the Tissues and Storage of the Extract. 42

C. Methods of Biological Assay. 42

Page

1. Rat Uterus Assay 43

2. Rabbit Milk-Ejection Assay 44

3. Frog Bladder Assay 45

4. Antidiuretic Assay 47

5. Activity Ratios, Used for the Comparison of Different Assay Methods 49

D. Non-Biological Measurements. 51

1. Measurement of Protein or Peptide Content. . 51

2. Measurement of Conductivity. 53

3. Inactivation with Sodium Thioglycollate. . . 53

4. Paper Chromatography 53

5. Isoelectric Focusing 55

E. Purification Procedures. 56

1. Gel Filtration 56

2. Filtration Through Ion Retardation Resin . . 58

3. Flash Evaporation. 59

4. Ion Exchange Chromatography. 59

a. Diethylaminoethyl (DEAE) Sephadex. . . . 60

b. Carboxymethyl (CM) Sephadex. 61

c. Sulfoethyl (SE) Sephadex 65

5. Lyophilyzation (Freeze Drying) 66

6. Hydrolysis and Amino Acid Analysis 67

Chapter 3 RESULTS. 69

I. STUDIES OF THE NEUROHYPOPHYSIAL PRINCIPLES OF THE SKATE, RAIA RHINA. 69

A. Introduction 69

Page

B. Pharmacological and Chromatographic Studies of the Neurointermediate Lobe Extracts 70

1. Biological Activities and Activity Ratios of the Crude Extract 70

2. Studies of the Magnesium Potentiation of the Extracts 71

a. The range of magnesium potentiations in twelve crude extracts 71

b. Paper chromatography of extracts with different magnesium potentiation values 74

c. Joint assays of extracts with different magnesium potentiation values. 78

d. The influence of the estrous cycle on magnesium potentiation values. 81

3. Comparison of Crude Extract with Synthetic Glumitocin 82

a. Assays of crude extract against glumitocin 82

b. Assays of crude extracts and glumitocin against oxytocin. 87

C. The Purification of the Extract. 89

1. Extraction of the Glands, and Purification of the Extract by Gel Filtration 89

2. Passage of the Extract through Ion Retardation Resin and DEAE Sephadex. 91

3. Chromatography of the Principles on CM Sephadex 92

a. The passage through the column 92

b. The basic fraction 95

c. Rechromatography of the combined neutral fraction on CM Sephadex. 96

Page

4. Amino Acid Analysis of the Oxytocic Principle. 98

D. Summary of the Studies on the Neurohypophysial Principles of Raia rhina 102

II. STUDIES OF THE NEUROHYPOPHYSIAL PRINCIPLES OF THE DOGFISH, SQUALUS ACANTHIAS, (PACIFIC VARIETY). . . 104

A. Introduction 104

B. Pharmacological and Chromatographic Studies of the Neurointermediate Lobe Extracts 105

1. Biological Activities and Activity Ratios of the Crude Extracts. 105

2. Studies of Acetone-Extractable Biological Activities. 108

C. The Purification of the Extract. 113

1. Extraction of the Glands 116

2. Preliminary Purification on Sephadex G-15. . . 116

a. The extent of purification 116

b. The minor oxytocic peak. 125

c. Paper chromatography of the Sephadex G-15 eluate 125

3. Passage of the Extract through Ion Retardation Resin. 129

4. Passage of the Extract through DEAE Sephadex 129

5. Chromatography on CM Sephadex. 130

a. The passage through the column 130

b. The chemical analysis of the two neutral peaks. 131

c. The pharmacological analysis of the basic peak 135

Page

6. Purification of the Two Neutral Fractions on SE Sephadex. 137

7. Comparative Studies of the Two Neutral Fractions 141

a. The comparison of the pharmacological activities of the neutral peaks, by assay against synthetic oxytocin (Syntocinon). 144

b. The comparison of the pharmacological activities of the neutral peaks, by assay of one fraction against the other 144

c. Paper chromatography of the two fractions 146

d. Isoelectric focusing of the two fractions 148

e. The comparison of the behavior of the two fractions on SE Sephadex. 148

f. The comparison of the amino acid composition of the two fractions. 151

g. Rechromatography of fraction 1 on CM Sephadex. 151

8. The Nature of the Single Neutral Fraction . . 155

a. Pharmacological studies of the neutral fraction. 155

b. Amino acid analyses of the neutral fraction. 158

i. The analysis of "fraction 2" after purification on SE Sephadex 158

ii. The analysis of the combined fractions 159

iii. The analysis of the combined fractions after paper chromatography. 162

Page

iv. An interpretation of the analyses of the neutral fraction 164

D. Summary of the Studies on the Neurohypophysial Principles of Squalus acanthias. 165

Chapter 4 - DISCUSSION 168

I. STUDIES OF THE NEUROHYPOPHYSIAL PRINCIPLES OF THE SKATE, RAIA RHINA. 168

1. The Variability in the Apparent Biological Potency of Crude Extracts and of Glumitocin. 168

2. The Identity of the Neurohypophysial Principles of Raia rhina 176

II. STUDIES OF THE NEUROHYPOPHYSIAL PRINCIPLES OF THE DOGFISH, SQUALUS ACANTHIAS (PACIFIC VARIETY) 178

1. The Identity of the Basic Principle of Squalus acanthias. 178

2. Considerations of the Identity of the Neutral Principle of Squalus acanthias 179

a. Behavior of the neutral fraction on CM Sephadex. 179

b. Chromatographic and pharmacological characteristics of the neutral principle. 180

c. Amino acid analyses of the neutral fraction 185

d. Theoretical considerations and speculations of possible structure 190

Chapter 5 - SUMMARY. 200

BIBLIOGRAPHY 207

APPENDICES

I. Source of the Neurointermediate Glands used in this Study 220

Page

II. Standards used in Biological Assays. 222

III. Solutions used in Biological Assays. 223

IV. Solutions used in Non-Biological Measurements 226

BIOGRAPHICAL INFORMATION . . . . . .

LIST OF TABLES

Table		Page
I	Amino Acid Sequences of the Known Neurohypophysial Principles.	6
II	The Phylogenetic Distribution of the Known Neurohypophysial Peptides	13
III	Distinguishing Characteristics of the Elasmobranch Oxytocic Principles.	33
IV	Oxytocic Activities, with and without Magnesium, of Crude Neurointermediate Lobe Extracts of Raia rhina; and their Magnesium Potentiations	73
V	Magnesium Potentiations of Pairs of Raia rhina Neurointermediate Lobe Extracts during Independent and Joint Rat Uterus Assays	79
VI	Activities and Activity Ratios of Crude Raia rhina Neurointermediate Lobe Extract, Assayed Against Synthetic Glumitocin.	83
VII	A Comparison of the Activity Ratios Found for Raia rhina Neurointermediate Lobe Extract when Assayed against Synthetic Glumitocin, to the Theoretical Activity Ratios Expected from Mixtures of Glumitocin and Small Amounts of Basic Peptide	85
VIII	Activities and Activity Ratios of Raia rhina Neurointermediate Lobe Extract and of Synthetic Glumitocin, Assayed Against Synthetic Oxytocin (Syntocinon)	88
IX	Amino Acid Analyses of the Purified Neutral Principle from Raia rhina	99
X	The Activities and Activity Ratios of Crude Neurointermediate Lobe Extracts of Squalus acanthias	106

Table		Page
XI	The Oxytocic Activity Extracted from Two Samples of Squalus acanthias Neurointermediate Lobes by the Acetone Used for Collection	110
XII	The Magnesium Potentiation and Chromatographic Behavior of a Crude Extract of Squalus acanthias Neurointermediate Lobes, and of the Activity Recovered from the Acetone in which the Glands were Collected	112
XIII	The Filtration of Squalus acanthias and Raia rhina Neurointermediate Lobe Extract Through Sephadex G-15	124
XIV	The Chromatography of Squalus acanthias and Raia rhina Neurohypophysial Principles on CM Sephadex, Following Purification on Sephadex G-15 and DEAE Sephadex	134
XV	Amino Acid Analyses of Squalus acanthias Neutral Fraction 1 and Neutral Fraction 2 at Two Stages of Purification	136
XVI	The Activities and Activity Ratios of the Basic Neurohypophysial Principle from Squalus acanthias	138
XVII	The Activities and Activity Ratios of the Two Neutral Fractions[a] from Squalus acanthias	145
XVIII	The Activities and Activity Ratios of Squalus acanthias Neutral Fraction 2[a], Assayed against Neutral Fraction 1[a]	147
XIX	The Activities and Activity Ratios of the Oxytocin-like Fraction[a] from Squalus acanthias, Assayed against Synthetic Glumitocin	157
XX	Amino Acid Analyses of the Purified Neutral Fraction from Squalus acanthias	160
XXI	Amino Acid Analysis of the Unhydrolyzed, Purified Neutral Fraction[a] from Squalus acanthias	161
XXII	Amino Acid Analysis of the Hydrochloric Acid Used for Hydrolyses[a]	163

LIST OF FIGURES

Figure		Page
1	The cat, schematic longitudinal section of the pituitary gland.	4
2	Squalus acanthias, median diagrammatic section of the pituitary gland	15
3	Descending paper chromatograms of three crude neurointermediate lobe extracts of Raia rhina, on Whatman 3MM paper, in n-butanol:acetic acid:water = 4:1:5, at room temperature.	76
4a	Chromatogram of the partially purified neurointermediate lobe extract of Raia rhina on a 35 cm x 0.9 cm column of CM Sephadex. . . .	94
4b	Chromatogram of combined fraction 1 and fraction 2 of Raia rhina (purified on Sephadex G-15 and on CM Sephadex - see Fig. 4a, above) on a second 35 cm x 0.9 cm column of CM Sephadex.	94
5	Descending paper chromatograms of Squalus acanthias neurointermediate lobe extract, at various stages of purification, on Whatman 3MM paper, in n-butanol:acetic acid:water = 4:1:5, at room temperature	115
6a	General outline of the procedures followed during the purification of the active principles from the neurointermediate lobe (NIL) of Squalus acanthias (Pacific variety) . .	118
6b	Detailed outline of the procedures followed during the purification of the active principles from the neurointermediate lobe (NIL) of Squalus acanthias (Pacific variety) . .	120

Figure | Page

7 Chromatogram of 10 ml of crude Squalus acanthias neurointermediate lobe extract on a 90 cm x 2.4 cm column of Sephadex G-15. . . 123

8 Descending paper chromatogram of Squalus acanthias neurointermediate lobe extract (Preparation 2, after partial purification on Sephadex G-15) on Whatman 3MM paper, in n-butanol:acetic acid:water = 4:1:5, at room temperature 128

9 Chromatogram of partially purified neurointermediate lobe extract of Squalus acanthias (Preparation 3; Sephadex G-15-purified) on a 35 cm x 0.9 cm column of CM Sephadex. 133

10a Chromatogram of Squalus acanthias neutral fraction 1a (fraction 1 from Preparations 2 and 3, combined; CM Sephadex-purified) on a 35 cm x 0.9 cm column of SE Sephadex 143

10b Chromatogram of Squalus acanthias neutral fraction 2a (fraction 2 from Preparations 2 and 3, combined; CM Sephadex-purified) on a 35 cm x 0.9 cm column of SE Sephadex 143

11 Isoelectric focusing of a mixture of Squalus acanthias neutral fraction 1 and neutral fraction 2 (Preparation 3, Sephadex G-15- and CM Sephadex-purified) in a pH range of 7-9 at 800 volts for 25 hours. 150

12a Chromatogram of partially purified neurointermediate lobe extract of Squalus acanthias (Preparation 4; Sephadex G-15-purified) on a 35 cm x 0.9 cm column of CM Sephadex. 154

12b Chromatogram of Squalus acanthias fraction 1 (Preparation 4; purified on Sephadex G-15 and on CM Sephadex - see Fig. 12a, above - and also on SE Sephadex) on a second 35 cm x 0.9 cm column of CM Sephadex 154

ACKNOWLEDGEMENTS

The author wishes to express her sincere gratitude to Dr. A. M. Perks of the Zoology Department for his supervision of, and encouragement during, this investigation.

She would like to thank Mr. V. Swiatkiewicz for his help in collecting the glands. She wishes to thank Dr. G. Somero and Mr. H. Behrisch of the Zoology Department for carrying out the isoelectric determinations. She is grateful to Dr. G. Dixon of the Biochemistry Department and to Dr. O. Parkes of the Pharmacology Department for the amino acid analyses which were performed in their laboratories.

She is also indebted to the National Research Council for financial assistance in the form of an N. R. C. Studentship for the years 1965-68, and to the H. R. MacMillan family for an H. R. MacMillan Family Fellowship for the year 1968-69.

Chapter 1

INTRODUCTION

I. THE COMPARATIVE BACKGROUND TO THE STUDY OF THE ELASMOBRANCH NEUROHYPOPHYSIAL PRINCIPLES

This study deals with the active principles produced by the hypothalamo-neurohypophysial system of certain elasmobranch species. Although the elasmobranch system is basically similar to that found in other vertebrates, there are some important differences. Therefore the general vertebrate system will be outlined before a more detailed description of the elasmobranch system is presented.

A. The Vertebrate Hypothalamo-Neurohypophysial System

1. Anatomical and Histological Considerations

The vertebrate hypothalamo-neurohypophysial system consists of aggregations of secretory nerve cells in the hypothalamic region of the brain, of nerve tracts formed by the axons of these nerve cells, and of their terminations in the pituitary gland - a region known as the neurohypophysis (Bargmann and Scharrer, 1951; Sloper, 1966).

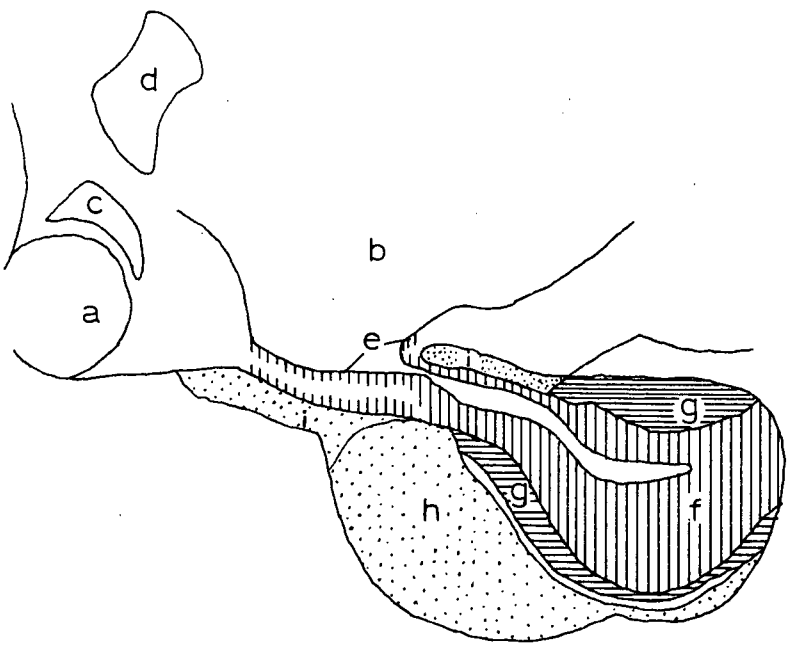
The hypothalamic nuclei which form a part of this system consist of the paired supraoptic and paraventricular nuclei found in reptiles, birds, and mammals, and of the

homologous preoptic nuclei of the cyclostomes, elasmobranchs, teleosts, and amphibians (Bargmann and Scharrer, 1951; Sloper, 1966). Other nerve cells, such as those of the lateral tuberal nuclei, may also be involved in the system (Christ, 1966; Sloper, 1966).

The axons of the secretory nerve cells form the hypothalamo-hypophysial nerve tracts, which pass into the specialized region of the infundibular floor which forms the neurohypophysis (Bargmann and Scharrer, 1951; Christ, 1966; Sloper, 1966). In dipnoan fishes, amphibians, reptiles, birds, and mammals the rostral portion of the neurohypophysis contains a specialized region, the median eminence, from which small portal blood vessels pass to the adenohypophysis (Sloper, 1966). Caudal to the median eminence the neurohypophysis thickens to form the infundibular process (Fig. 1). The intervening portion of the neurohypophysis, which is difficult to distinguish in many species, is the infundibular stem (Sloper, 1966). The parts of the neurohypophysis which do not include the median eminence are known as the pars nervosa (Gorbman and Bern, 1962), and will be referred to as such in this thesis.

The nerves of the hypothalamo-hypophysial tracts terminate in the median eminence and infundibular process (reviewed in Christ, 1966; Green, 1966; Sloper, 1966), and it is generally accepted that the secretion produced by the neurosecretory cells is released from these nerve endings (Sloper, 1966), probably into the blood vessels.

Figure 1. The cat, schematic longitudinal section of the pituitary gland. From Russell (1965; Fig. 1). a: optic chiasma, b: third ventricle, c: supraoptic nucleus, d: paraventricular nucleus, e: median eminence, f: pars nervosa (e, f: neurohypophysis), g: pars intermedia, h: pars distalis, i: pars tuberalis (g, h, i: adenohypophysis).


d
c
b
a
e
g
i
h
g
f

The study presented here is concerned with the nature of the secretion which is released from the infundibular process (pars nervosa). It can be traced throughout the neurosecretory system as a stainable colloid when viewed by light microscopy, or as smaller neurosecretory granules when seen by electron microscopy; it has been associated with the biological activity (vasopressor, antidiuretic, and oxytocic) which has been found in all parts of the system (Bargmann, 1960). In part, at least, it consists of the active neurohypophysial principles in association with a carrier protein, "neurophysin" (Chauvet et al., 1960a). It is stored in nerve endings in the infundibular process, from which it is released into the blood stream (Bargmann and Scharrer, 1951).

2. The Nature of the Neurohypophysial Principles

a. The structure of the known principles

All the neurohypophysial principles so far isolated and analyzed are octapeptides. Their structures are listed in Table I. It can be seen that all the peptides have the same basic structure, with differences between their molecules occurring in amino acid positions 3, 4, and 8 (see Sawyer, 1968). However the two vasopressins and arginine vasotocin are chemically basic peptides while the others are more nearly neutral. This difference is important and useful in the isolation and purification of the different principles.

Table I

Amino Acid Sequences of the Known Neurohypophysial Principles

Arginine vasopressin (1, 2)[a]

Cys. Tyr. Phe. Glu(NH_2). Asp(NH_2). Cys. Pro. Arg. Gly(NH_2)
1 2 3 4 5 6 7 8 9

Lysine vasopressin (2, 3)

Cys. Tyr. Phe. Glu(NH_2). Asp(NH_2). Cys. Pro. Lys. Gly(NH_2)

Arginine vasotocin (4, 5, 6, 7, 8, 9)

Cys. Tyr. Ileu. Glu(NH_2). Asp(NH_2). Cys. Pro. Arg. Gly(NH_2)

Oxytocin (10, 11, 12)

Cys. Tyr. Ileu. Glu(NH_2). Asp(NH_2). Cys. Pro. Leu. Gly(NH_2)

Mesotocin (8-ileu oxytocin) (13)

Cys. Tyr. Ileu. Glu(NH_2). Asp(NH_2). Cys. Pro. Ileu. Gly(NH_2)

Isotocin (4-ser, 8-ileu oxytocin) (9, 14)

Cys. Tyr. Ileu. Ser .Asp(NH_2). Cys. Pro. Ileu. Gly(NH_2)

Glumitocin (4-ser, 8-glu(NH_2) oxytocin) (15)

Cys. Tyr. Ileu. Ser .Asp(NH_2). Cys. Pro. Glu(NH_2). Gly(NH_2)

[a]References: 1: Acher and Chauvet (1953), 2: du Vigneaud, Ressler, and Trippett (1953), 3: Popenoe et al. (1952), 4: Katsoyannis (1958), 5: Chauvet et al. (1960b), 6: Acher et al. (1960), 7: Rasmussen and Craig (1961), 8: Heller and Pickering (1961), 9: Wilson and Smith (1969), 10: du Vigneaud, Ressler, Swan, Roberts, Katsoyannis, and Gordon (1953), 11: du Vigneaud, Lawler, and Popenoe (1953), 12: du Vigneaud (1956), 13: Acher et al. (1964), 14: Acher et al. (1962), 15: Acher et al. (1965).

b. The biological activities of the peptides

Biological activities characteristic of these peptides have been known for many years. Oliver and Schäfer (1895) first observed that the injection of pituitary extracts into the dog caused a rapid rise in blood pressure. A decade later, Dale (1906) found that pituitary extract from the ox not only produced a rise of blood pressure in the pithed early-pregnant cat but also caused *in vivo* contractions of the uterus. He later (1909) observed a similar oxytocic effect of ox posterior lobe (= pars nervosa + pars intermedia) extract on the uterus of the cat, dog, guinea pig, rat and rabbit, both *in vivo* and *in vitro*. The galactobolic or milk-ejection effect of posterior lobe extracts was seen by Ott and Scott (1910), who found that intravenous injection of the extracts into the lactating goat was followed rapidly by a great increase in the rate of milk let-down. The avian vasodepressor effect was observed first by Paton and Watson (1912). They found that extracts injected intravenously into the duck, at a dose level which would raise the blood pressure of the rabbit and cat, caused a decrease in the blood pressure of the bird. Posterior lobe extracts were also found to cause antidiuretic effects in mammals (von den Velden, 1913). In a presumably analagous manner, they resulted in water uptake in whole amphibians (Heller, 1941a), and in the transport of water across the amphibian bladder (Bentley, 1958; Sawyer, 1960). More recently, a contraction

of the hen oviduct has been observed, both *in vivo* and *in vitro*, in response to neurohypophysial peptides (Munsick *et al.*, 1960).

Although the various neurohypophysial peptides share a number of biological actions, different peptides exhibit certain effects more strongly than others. On the basis of their strongest biological activities the peptides can be roughly classified as either oxytocic or vasopressor. This classification corresponds in a general way to the classification on the basis of charge. The neutral peptides (oxytocin, mesotocin, isotocin, glumitocin) have strong uterotonic (oxytocic) and milk-ejection potencies, and relatively little pressor or antidiuretic effects. On the other hand the basic peptides show high pressor and antidiuretic activities. Of these basic vasopressor principles, arginine and lysine vasopressin have little rat uterus or milk-ejection potency; however, arginine vasotocin, which contains structural elements of both oxytocin and arginine vasopressin, has roughly equal oxytocic and pressor activity (see Heller, 1963; Sawyer, 1965; Sawyer *et al.*, 1970).

c. The phylogenetic distribution of the peptides

In general, each class of vertebrate produces one or more of both the basic and the neutral peptides. The cyclostomes, in which no neutral peptide has yet been found, are the only exception. The following account of the distribution of the neurohypophysial hormones is based on considerations presented by Sawyer (1968).

Oxytocin is the neutral peptide produced by all mammals so far investigated. Most mammals also produce arginine vasopressin, but a few produce lysine vasopressin instead, and some species appear to produce both. Lysine vasopressin appears in some species of the suborder Suina, which includes pigs, peccaries, and the hippopotamus. Many of these species produce both arginine and lysine vasopressin as well as oxytocin. When this is the case, some individuals within a given species may produce only one of the vasopressins, while other individuals of the same species appear to secrete both (Ferguson, 1969). Only the domestic hog (Sus) and the bushpig (Potamochoerus porcus) produce lysine vasopressin exclusively (Sawyer, 1968; Ferguson and Pickering, 1969). Apart from the Suina, lysine vasopressin has also been reported in the mouse, Mus musculus, Peru strain (Stewart, 1968). In addition to oxytocin and vasopressin, some mammalian foetuses, those of the sheep (Ovis aries) and of the seal (Callorhinus ursinus), appear to produce arginine vasotocin in small amounts (Vizsolyi and Perks, 1969).

Birds produce oxytocin and arginine vasotocin. Chauvet et al. (1960b) identified arginine vasopressin as well as arginine vasotocin in pituitary extracts of the chicken (Gallus domesticus); but other workers (Munsick et al., 1960; Heller and Pickering, 1961; Munsick, 1964) could find no pharmacological evidence for its presence in the same species. Furthermore, Munsick (1964) was able to distinguish the avian

principles from arginine vasopressin by column chromatography. He suggested that the arginine vasopressin observed by Chauvet et al. was introduced into the chicken extract on the mammalian neurophysin that was used in the isolation of the avian principles.

Arginine vasotocin and mesotocin (8-isoleucine oxytocin) have been found in reptiles (Munsick, 1966; Acher et al., 1969). In addition, Pickering (1967) found that the neutral fraction of the cobra (Naja naja) consisted of a mixture of mesotocin and oxytocin.

Amphibians also secrete arginine vasotocin and mesotocin (Acher et al., 1960; 1964; Follett and Heller, 1964b). However one species, Rana pipiens, has been found to produce oxytocin rather than mesotocin (Munsick, 1966).

The available evidence indicates that arginine vasotocin, mesotocin and oxytocin also occur among the lungfish (Follett and Heller, 1964b; Pickering and McWatters, 1966; Sawyer, 1969).

Among the ray-finned fish, arginine vasotocin has been identified in the Teleostei (Heller and Pickering, 1961; Rasmussen and Craig, 1961; Acher et al., 1961; Wilson and Smith, 1969) and there is evidence that it is produced also by the Holostei and Chondrostei (Follett and Heller, 1964a). The neutral principle, isotocin (4-serine, 8-isoleucine oxytocin) has been identified in the Teleostei (Acher et al.,

1962; Wilson and Smith, 1969) and it is thought to occur among the Holostei (Follett and Heller, 1964a). An oxytocin-like fraction occurs in the Chondrostei, but its identity has not been determined (Follett and Heller, 1964a). The primitive bichir, Polypterus senegalis, appears to produce the same isotocin and arginine vasotocin as the higher actinopterygians (Sawyer, 1969).

Among the cartilaginous fish, the holocephalian Hydrolagus collei (ratfish) has been found to produce two principles which resemble arginine vasotocin and oxytocin in their chromatographic and pharmacological characteristics, and in their amino acid compositions (Sawyer, 1965; Sawyer et al., 1967; Pickering and Heller, 1969). However there is also some chromatographic evidence that the oxytocic fraction of this species and of two other holocephalians, Chimaera monstrosa (rabbit fish), and Callorhynchus sp., consists of two distinct oxytocic principles (Heller and Roy, 1965 b; 1967; Roy, 1969). Neither has yet been identified. The number and nature of the principles produced by the elasmobranchs is uncertain. These will be discussed in a later section.

Arginine vasotocin has been found in the cyclostomes, but so far there is no evidence for the presence of a neutral principle (Sawyer, 1968; Rurak and Perks, unpublished observations).

Table II summarizes the known phylogenetic distribution of the neurohypophysial peptides.

B. The Elasmobranch Hypothalamo-Neurohypophysial System

1. Anatomical and Histological Considerations

The elasmobranch pituitary is notably different from the well-known mammalian pattern, and so it will be described in detail. It is shown diagramatically in Fig. 2.

The adenohypophysis consists of three lobes; the intermediate lobe, the anterior lobe, and the ventral lobe (Meurling, 1967a). The intermediate lobe, or pars intermedia, at the posterior end of the adenohypophysis, consists of irregular epithelial cell columns separated by a dense system of blood sinuses. The anterior lobe continues rostral from the intermediate lobe. The ventral lobe is isolated in the base of the cranium, and may or may not retain a connection with the anterior lobe.

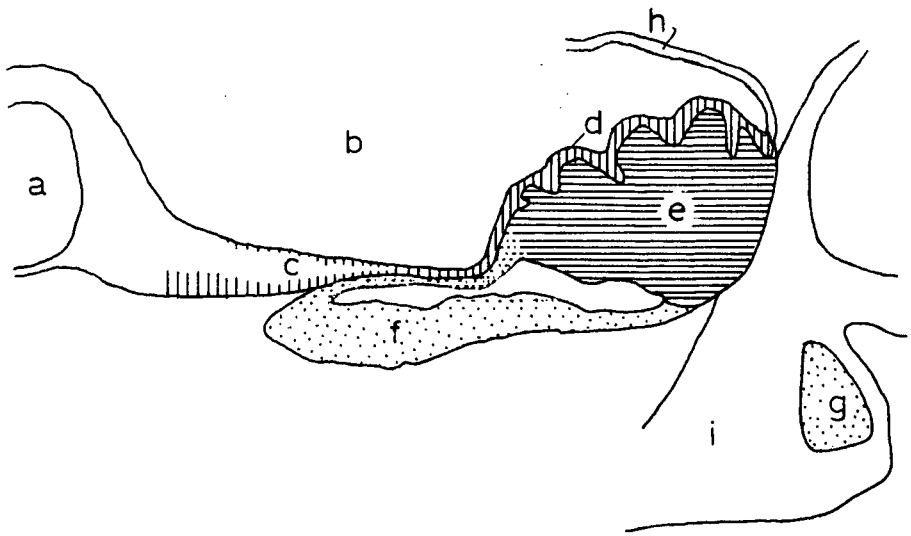
In elasmobranchs, the neurohypophysis is not readily apparent, and for some time its presence was doubted. Herring (1908a; 1911; 1913) could find no differentiation of the pituitary of the skate, Raia batis, into adenohypophysis and neurohypophysis. He found that during the development of the gland there is no evagination of the infundibulum to form a pars nervosa as occurs in higher forms. However he did notice that the lamina of nervous tissue which lies in the infundibular floor above the pituitary "passes into" the

Table II

The Phylogenetic Distribution of the Known Neurohypophysial Peptides

Vertebrate type	Neurohypophysial peptide						
	Arginine vasopressin	Lysine vasopressin	Arginine vasotocin	Oxytocin	Meto-tocin	Iso-tocin	Glumi-tocin
Mammals	+	+	+	+			
Birds	+(?)		+	+			
Reptiles			+	+	+		
Amphibians			+	+	+		
Bony fish							
lungfish			+	+	+		
ray-finned fish			+			+	
bichir			+			+	
Cartilaginous fish							
holocephalians			+	+			
elasmobranchs			+				+
Cyclostomes			+				

Figure 2. Squalus acanthias, median diagrammatic section of the pituitary gland. From Meurling (1962; Fig. 1 and 1967a; Fig. 2). a: optic chiasma, b: third ventricle, c: median eminence, d: pars nervosa (c, d: neurohypophysis), e: pars intermedia (d, e: neurointermediate lobe), f: anterior lobe, g: ventral lobe (e, f, g: adenohypophysis), h: saccus vasculosus, i: cranium.


h
d
b
a
e
c
f
g
i

tissue of the pituitary.

Other workers also observed nervous tissue in the elasmobranch adenohypophysis. Sterzi (1909) and Baumgartner (1915), both working on the dogfish, Squalus acanthias, found numerous large solid bundles of nerve fibers passing into the tissue of the pars intermedia from the floor of the brain above. The bundles were separated from the pars intermedia by a connective tissue membrane. Then in 1925 Hogben and de Beer observed a small but definite mass of fibers leaving the infundibular floor in the skate (sp?). In this case the fibers ramified among the cells of the pars intermedia. Hogben and de Beer suggested that this diffuse structure might be a type of pars nervosa.

The existence of the elasmobranch pars nervosa was firmly established in 1952 by Scharrer, who was able to show a complete hypothalamo-neurohypophysial system in the dogfish Scyllium stellare by means of serial sections, stained with Gomori's chromhaematoxylin-phloxin stain for neurosecretory granules. He demonstrated a preoptic nucleus, whose cells contained neurosecretory granules. A fiber tract, also laden with neurosecretory granules, led from the preoptic nucleus into the pars intermedia of the adenohypophysis, where there were also clear accumulations of neurosecretory material. He thereby demonstrated that a pars nervosa is indeed present in Scyllium. However, it is a diffuse structure, and consists of the sum of all the secretion-laden nerve endings of the

preoptico-hypophysial tract, intimately intermingled with the cells of the pars intermedia. Together the pars nervosa and pars intermedia form the neurointermediate lobe (NIL) of the elasmobranch pituitary gland (Perks, 1960).

In 1962, Braak found a slightly modified form of neurohypophysial structure in the shark, Spinax niger. He demonstrated the system by means of serial sections, stained for neurosecretory material by Gomori's aldehyde-fuchsin technique. Braak showed that the large neurons of the preoptic nucleus were impregnated with powdery neurosecretory granules, and he was able to trace the preoptico-hypophysial tract throughout its length. He found that the pars nervosa of this species, in contrast to that of Scyllium, formed a relatively thin tissue layer on the dorsal surface of the pars intermedia. A blood vessel network separated the neural tissue from the pars intermedia, and the secretion-laden nerve endings of the preoptico-hypophysial tract terminated on the basement membrane of this network. Finger-like evaginations of the infundibular floor and the underlying pars nervosa penetrated the pars intermedia, and together they formed the neurointermediate lobe.

Braak further confirmed the structure of the hypothalamo-neurohypophysial system with a whole-mount staining and embedding technique. Whole brains of Spinax niger were selectively stained with Humberstone's Victoria Blue solution, a modification of Adams and Sloper's performic acid-alcian

blue stain for neurosecretion. The brains were then embedded in transparent benzyl-benzoate methyl-salicylate. By this technique the entire morphology of the system could be observed at a glance and its spatial relationships appreciated. Again the pars nervosa was found to be a discrete structure, although indented into the pars intermedia.

Meurling (1962) performed a detailed histological study of the pituitary of the spiny dogfish Squalus acanthias, one of the species investigated in the present study. In this species the secretion-laden nerve endings were also separated from the pars intermedia by a connective tissue membrane, and were confined to the infundibular floor. This was in agreement with the findings of Sterzi and Baumgartner in the same species. The pars nervosa of Squalus, like that of Spinax, sends thick processes into the pars intermedia, and these processes consist of foldings of the entire infundibular floor. Meurling did notice a small degree of nerve penetration from neural to intermediate tissue, usually in places where the separating connective tissue membrane was absent.

As the above account illustrates, the pars nervosa component of the elasmobranch neurointermediate lobe may occur in two main histologically discernable forms (Meurling, 1962). These are firstly the diffuse type described by Hogben, de Beer, and Scharrer in the skate (sp?) and in

Scyllium stellare , and secondly the discrete type observed by Sterzi, Baumgartner, and Braak in Squalus acanthias and Spinax niger. The discrete type of pars nervosa remains as a distinct part of the brain wall, which sends projections into the dorsal surface of the pars intermedia but remains separated from it by a largely continuous membrane. Although some neurosecretory axons may pass through gaps in the membrane, the intermingling with the pars intermedia is slight (Meurling, 1962; 1967d). Other elasmobranchs with a pars nervosa of similar form to that of Squalus acanthias are Etmopterus spinax = Spinax niger (Braak, 1962; Meurling, 1963), Squatina squatina (Mellinger, 1966), and the following sharks, which are considered to be relatively primitive - Heptanchus, Hexanchus, Heterodontus = the Port Jackson shark, and Chlamydoselachus (Stendell, 1913; 1914; Norris, 1941; Colbert, 1961).

In the diffuse type of pars nervosa there is no limiting membrane and the nerve fibers of the hypothalamo-hypophysial tract penetrate and intermingle with the tissue of the pars intermedia (Meurling, 1962; 1967d). In Scyllium stellare, Scyllium canicula and Mustelus mustelus there is a strong intermingling of neurosecretory and intermedia components, but a distinct pars nervosa layer may still be recognized in the infundibular floor (Scharrer, 1952; Bargmann, 1955; Meurling, 1962; 1967a; Perks, 1969). In Pristiurus melanostomus and in the skates and rays (Dasyatis marinus,

Trygon pastinaca = Dasyatis pastinaca, Torpedo ocellata, Raia batis, R. oxyrhynchus, R. fullonica, R. radiata, and R. clavata) there is complete fusion between neural and intermediate elements and one cannot discern a pars nervosa as a discrete morphological entity (Bargmann, 1955; Meurling, 1962; 1967a; 1970; Polenov and Belenky, 1965; Mellinger, 1966; Perks, 1969).

Although the diffuse and discrete types of pars nervosa may be found in such species as the skates (ex. Raia clavata) and the dogfish Squalus acanthias, respectively, other species may show some degree of intermediate form in their structures. However, physiological studies might well concentrate on the two extreme types mentioned here.

2. The Nature of the Elasmobranch Neurohypophysial Principles

a. Evidence for neurohypophysial activity in the elasmobranch pituitary

The activity of elasmobranch neurohypophysial extracts, as measured by standard bioassay techniques, is very low. Therefore the demonstration of oxytocic and antidiuretic principles in these fish was especially difficult.

In his remarkable pioneer work, Herring (1908a; 1908b) was unable to find that pituitary extracts of the skate, Raia batis, had any effect on the blood pressure or urine flow of anaesthetized cats, and he concluded that the active principles found in the pars nervosa and pars intermedia of mammals,

birds, and teleosts were not present in the elasmobranchs. However in 1913 he found that a Raia batis extract caused an immediate and well marked milk-ejection response in the lactating cat. Later (1915) he observed that pituitary extracts of the skate, Raia clavata, caused contraction of the isolated virgin rat uterus. However, he attributed this activity to the chromophobe cells of the pars intermedia tissue.

Other biological activities characteristic of neurohypophysial extracts were gradually attributed to the elasmobranch pituitary. Hogben (1925) found an avine (duck) blood depressor response to skate (sp?) extracts; and Hogben and de Beer (1925) demonstrated a contraction of the guinea pig uterus, again using skate (sp?) pituitary extract. Later, Heller (1941a) showed that extracts of both skate (sp?) and dogfish (sp?) pituitary exerted an antidiuretic effect in the rabbit.

On the other hand, Waring and Landgrebe (1950) failed in "exhaustive" efforts to demonstrate rat vasopressor or guinea pig uterus responses to elasmobranch extracts, and ascribed previous workers' findings to the presence of histamine. However, as pointed out by Hogben and de Beer (1925), histamine actually relaxes the rat uterus, and so it could not be responsible for the contractions observed by Herring in the rat.

In 1959 Maetz *et al.* not only confirmed oxytocic activity (contraction of the isolated rat uterus) in the elasmobranchs *Hexanchus griseus* and *Scyliorhinus caniculus*, but also demonstrated yet another property, a natriferic effect. They observed that pituitary extracts of these species brought about the active transport of Na^+ across the skin of the amphibian *Rana esculenta*, an effect also exhibited by very low concentrations of teleost and amphibian neurohypophysial extracts. In the same year, Sawyer (1959) also confirmed the presence of neurohypophysial activity in the pituitary of the dogfish, *Squalus acanthias*.

During the same period of research, Perks *et al.* (1960; Perks and Dodd, 1963a; 1963b) subjected neurointermediate lobe, saccus vasculosus, hypothalamus, "indifferent brain", and ventral lobe extracts of several elasmobranch species (*Squalus acanthias*, *Scyliorhinus caniculus*, *Raia clavata*, and *Raia batis*) to a series of bioassays, which included the isolated rat uterus, rabbit milk-ejection, rat vasopressor, and rat antidiuretic estimations. They demonstrated that rat uterus, milk-ejection, and antidiuretic activities were indeed present in all four species, and furthermore that these activities were preferentially located in the neurointermediate lobe. No vasopressor activity was detected. In fact, a slight vasodepressor effect was noted, but Herring (1915) had found this effect to be due to contaminants which could be removed from the extracts by

treatment with absolute alcohol.

Confirmation that these neurointermediate lobe activities were in fact due to neurohypophysial principles lay in the observations that they were destroyed by treatment with sodium thioglycollate, which reduces the S-S bond of cystine (van Dyke, 1942), with sodium hydroxide and by ultraviolet light (Dudley, 1920). It is important that histamine, suggested by Waring and Landgrebe to be responsible for the elasmobranch "neurohypophysial" activity, is resistant to sodium hydroxide treatment (Dudley, 1920).

Perks and Dodd found that the milk-ejection, oxytocic, and antidiuretic potencies of the elasmobranch neurointermediate lobe extracts, when they were assayed against mammalian principles, were generally in the ratio 3:1:0.05. This activity profile suggested that the elasmobranchs do not produce a vasopressor-antidiuretic principle, but that they do elaborate an oxytocic principle which is different from oxytocin itself. This principle would have a small intrinsic antidiuretic activity, and its milk-ejection activity would be greater than its oxytocic (rat uterus) effect.

The elasmobranch activity profile was extended to four further species, Dasyatis sabina (stingray), Sphyrna mokarran (hammerhead shark), Eulania milberti (brown shark), and Carcharinus leucas (bull shark), and was confirmed for

the dogfish Squalus acanthias (Perks, 1966). Perks also found that the oxytocic agent from these species behaved chromatographically like oxytocin, which was in agreement with a similarity between their molecules.

Sawyer et al. (1961) confirmed and extended the pharmacological profile of the oxytocic agent of Squalus acanthias. By administering large doses they were able to demonstrate a weak rat vasopressor activity, although this was partially obscured by the rat vasodepressor effect noted by Herring (1915) and by Perks and Dodd (1963a). They showed that Squalus neurointermediate lobe extracts caused contraction of the hen oviduct, brought about water transport across the isolated frog bladder, and exerted a fowl vasodepressor effect. In addition they measured the oxytocic activity of the Squalus extracts after the addition of magnesium ions to the medium in which the rat uterine horn was suspended. The presence of magnesium ions augments the response of the rat uterus to neurohypophysial peptides, but the increase is greater for oxytocin analogues than for oxytocin itself (Munsick, 1960). Therefore the potency of an analogue measured against an oxytocin standard in the presence of magnesium is greater than its potency when the assay is performed without magnesium. The apparent increase in potency due to the presence of magnesium ions may be defined as follows (Sawyer, 1967):

Magnesium (Mg^{++}) potentiation = R_{Mg} =

$$\frac{\text{activity on rat uterus in presence of 0.5mM } Mg^{++}}{\text{activity on rat uterus in absence of } Mg^{++}}$$

The R_{Mg} of the two Squalus extracts investigated by Sawyer et al. (1961) were 1.27 and 2.03. A magnesium potentiation of approximately 2 for this species was later confirmed by Sawyer (R_{Mg} = 2.6; 1967) and by Swiatkiewicz (R_{Mg} = 1.2 - 3.2; 1968).

b. Evidence supporting the existence of an elasmobranch antidiuretic principle

While most vertebrates possess an antidiuretic-vasopressor peptide as well as an oxytocic principle, the results of the early investigations did not support the presence of such an antidiuretic fraction among the elasmobranchs (Perks et al., 1960; Perks and Dodd, 1963a; Sawyer et al., 1959; Heller and Pickering, 1961) and it appeared that elasmobranchs elaborated an oxytocic fraction only. However later work, published during the course of the present study, and outlined below, indicated that an arginine vasotocin-like principle was present, although in very small amounts.

Acher and his group (Acher et al., 1965; 1967; Chauvet et al., 1965) found in four species of skate (Raia clavata, R. batis, R. naevus, and R. fullonica) that in addition to the main oxytocic fraction there was a second very small fraction which possessed pressor activity equal to, or

greater than, its oxytocic activity, and which had hydro-osmotic activity in addition. This fraction behaved like arginine vasotocin both during elution chromatography from Amberlite CG-50 columns and during paper chromatoelectrophoresis.

Sawyer (1965; 1967) found that the dogfish, Squalus acanthias, elaborated a second small oxytocic fraction which possessed only about 1.2 - 1.4% of the oxytocic activity of the main fraction, but which accounted for almost all of the total antidiuretic and frog-bladder activity. This small fraction could be separated from the main activity either by elution chromatography on CM cellulose or on CM Sephadex columns, or by paper chromatography. In both cases its behavior was the same as that of arginine vasotocin. The relative pharmacological activities of this fraction (antidiuretic activity, frog-bladder activity, and rat uterus activity in the presence or absence of Mg^{++}) also matched those of synthetic arginine vasotocin.

c. Evidence supporting the existence of several elasmobranch oxytocic principles

Soon after it had been established that the elasmobranch oxytocic agent was not oxytocin itself, it began to appear that it represented not one but several neutral principles. However, it was not clear whether each elasmobranch species elaborated a single neutral principle which varied between species, or whether each species produced a mixture of peptides, and that the proportions of the peptides

in the mixture varied.

The different principles could be distinguished by their chromatographic and pharmacological characteristics in the following three ways:

i. By differences in relative milk-ejection activities.

ii. By differences in magnesium potentiation.

iii. By differences in chromatographic behavior.

Each of these methods of differentiation will be discussed separately, below.

i. Differences between the oxytocic principles, based on relative milk-ejection activities.

As mentioned previously, the general ratio of milk-ejection activity to rat uterus activity of the elasmobranch neurohypophysial extracts is approximately 3:1. This has been found for at least eight species (Perks and Dodd, 1963a; Perks, 1966; Sawyer et al., 1961; Sawyer, 1967). Surprisingly, upon purifying the oxytocic principle of the ray Raia ocellata, Perks and Sawyer (1965) found that the relative milk-ejection activity of both the crude extract and the purified principle was much higher than expected. The ratio of milk-ejection to rat uterus activity of the crude extract was 10.3±1.1 and that of the purified peptide was 8.5±1.0. These two ratios were not significantly different, but contrasted with those of all species previously investigated. The magnesium potentiation, and the ratio of antidiuretic to rat uterus activity,

were of the expected magnitude, but the relative frog-bladder activity of the purified R.ocellata principle was much lower than that found previously for crude Squalus acanthias extracts (Sawyer et al., 1961). However, this difference may simply have reflected the presence of the vasotocin-like principle in the crude Squalus preparation. If this is so, then the only clear way in which the R. ocellata principle differed from that of other elasmobranchs was in its high milk-ejection potency.

This difference led to the classification (Sawyer, 1964) of elasmobranch oxytocic principles into EOP I (elasmobranch oxytocic principle I) and EOP II (elasmobranch oxytocic principle II). EOP I, found in the majority of elasmobranchs, had a ratio of milk-ejection to rat uterus activity of approximately 3. EOP II, occurring in R.ocellata, had a ratio of from 8 to 10 (see Table III).

The R.ocellata purified peptide, EOP II, was subjected to amino acid analysis and found to consist of cystine, tyrosine, serine, glutamic acid, aspartic acid, proline, isoleucine, and glycine in approximately equimolar amounts (Perks and Sawyer, 1965).

ii. Differences between the oxytocic principles, based on magnesium potentiations

Acher et al. (1965) purified the major active fraction of the thornback ray, Raia clavata, and by enzymic degradation determined its amino acid sequence to be that of 4-serine,

8-glutamine oxytocin, which they called "glumitocin". The same amino acid composition and chromatoelectrophoretic behavior was found for the major active fraction of R. batis (Chauvet et al., 1965), R. naevus and R. fullonica (Acher et al., 1967), which suggested that they also contained glumitocin.

Glumitocin has the same amino acid composition as EOP II, but differs in its magnesium potentiation. The R_{Mg} of glumitocin from R. clavata and R. batis was found to be 10, the highest value reported at that time for any naturally occurring oxytocin analogue. In contrast, a lower potentiation of approximately 2 had been found for EOP II, and also for EOP I. Another of the postulated elasmobranch principles, E_1 (see Section iii, below) had been characterized by a high magnesium potentiation value of 6 in some species, although the value was lower in several other species, and similar to that found for EOP I and EOP II. Clearly, at this time considerations of the magnesium potentiations of elasmobranch neurohypophysial principles were complex and difficult to interpret.

iii. Differences between the oxytocic principles, based on chromatographic behavior

(1) The resolution of two oxytocic principles within a single species

Descending paper chromatograms of crude extracts of two dogfish, Squalus acanthias and Scyliorhinus caniculus,

developed in n-butanol: acetic acid: water = 4:1:5 were found to yield two areas of oxytocic activity (Heller and Pickering, 1961). During the time of the present study, this observation was extended to an additional nine species (Heller and Roy, 1965a; 1965b; 1967; Roy, 1969). One of the fractions ran with an R_F of approximately 0.5 to 0.8, the same R_F as oxytocin in this solvent system. The other fraction ran more slowly, with an R_F of approximately 0.3 to 0.5. The two fractions were designated as E_1, the slow-moving moiety, and as E_2, the fast-moving principle. Both E_1 and E_2 were labile to sodium thioglycollate, an indication that both fractions represented true neurohypophysial principles.

E_1 and E_2 have been further distinguished by their pharmacological properties, mainly by their magnesium potentiation. On this basis the species studied may be divided into three groups, which will be discussed in turn:

(a) Species in which E_1 and E_2 have different magnesium potentiations

In this case the fast-moving E_2 was characterized by a potentiation of approximately 2 and the slow-moving E_1 showed a higher value of approximately 6. Such a difference was found in the spotted dogfish Scyliorhinus caniculus (Heller and Roy, 1965b; Roy, 1969), the guitar fish Rhinobatis rhinobatis (Roy, 1969) and the thornback ray Raia clavata (Heller and Roy, 1965a; Roy, 1969).

The pharmacological difference between E_1 and E_2 was further substantiated by differences in natriferic and antidiuretic potency. Natriferic activity was found in the fast-moving fraction, E_2, of Scyliorhinus and Raia, but none could be detected in the slow-moving fraction (Heller and Roy, 1965a; Roy, 1969). The antidiuretic activity of E_1 relative to its oxytocic activity was greater than that of E_2 (Roy, 1969). However, since the values given were only approximations based on a few assay responses, the significance of the result is difficult to assess. No significant difference was found in the guinea pig milk-ejection activities of the two fractions relative to their oxytocic activities (Roy, 1969).

(b) Species in which E_1 and E_2 have similar magnesium potentiations

The potentiations of both fractions were approximately 2. This was found in the bull shark Carcharinus leucas (Roy, 1969) and in the lemon shark Negaprion brevirostris (Heller and Roy, 1965b; Roy, 1969).

In addition to the similarity in their magnesium potentiations, the two fractions showed relative guinea pig milk-ejection activities which were not significantly different. The natriferic activities were not tested. In both species, only the relative antidiuretic activity of the slow fraction was found to be greater than that of the fast-moving moiety (Roy, 1969). Again, however, these were only approximate assays, and the results may not be significant.

Therefore in the case of Carcharinus and Negaprion the only characteristic which distinguishes clearly between E_1 and E_2 is their chromatographic mobility.

(c) Species in which neither E_1 nor E_2 is potentiated by magnesium ions

This included a primitive elasmobranch, the cow shark Hexanchus griseus, as well as three holocephalians, the rabbit fish Chimaera monstrosa, the ratfish Hydrolagus collei, and Callorhynchus sp. (Heller and Roy, 1965b; 1967; Roy, 1969). The high antidiuretic activity of E_1 in Hexanchus has been attributed to contamination of the slow fraction by arginine vasotocin (Roy, 1969) and the natriferic and guinea pig milk-ejection activities of the two fractions in these species have not been investigated. Again in this case the only clear difference between E_1 and E_2 is in their chromatographic mobility.

(2) The resolution of a single oxytocic fraction within each species

The chromatographic differentiation of the elasmobranch oxytocic agent into two fractions, E_1 and E_2, by Heller and his group has not been observed by other workers. Perks (1966) subjected five species, the dogfish Squalus acanthias, the stingray Dasyatis sabina, the hammerhead shark Sphyrna mokarran, the brown shark Eulania milberti, and the bull shark Carcharinus leucas, to chromatography on both Whatman 3MM paper and glass paper impregnated with silicic acid. A variety of solvent systems was used,

Table III

Distinguishing Characteristics of the Elasmobranch Oxytocic Principles

Principle	Species	Paper chromatography R_F[a]	Pharmacological ratios R_{ME}	Pharmacological ratios R_{Mg}	Reference[b]
EOP I	Squalus acanthias	0.5-0.8	1.5-2.6	1.2-3.2	1,2,3,4,5,6,7
	Scyliorhinus caniculus	-	3.0,4.5	-	2
	Raia clavata	-	3.5	-	2
	Raia batis	-	4.5	-	2
	Dasyatis sabina	0.6-0.8	3.4	-	5
	Sphyrna mokarran	0.4-0.6	2.6	-	5
	Eulania milberti	0.5-0.7	3.8	-	5
	Carcharinus leucas	0.5-0.7[c]	4.2	-	5
EOP II	Raia ocellata	-	8.5,10.3	2.6	3
Glumitocin	Raia clavata	-	-	10	8,9
	Raia batis	-	-	10	9
	Raia naevus	-	-	10	10
	Raia fullonica	-	-	10	10
$E_1(R_{Mg}=6)$	Scyliorhinus caniculus	0.25-0.50[c]	-	5.5,6.3	11,13,14
	Raia clavata	0.04-0.40[c]	-	6.1	12,14
	Rhinobatis rhinobatis	0.35-0.60[c]	-	5.1	14
$E_1(R_{Mg}=2)$	Carcharinus leucas	0.30-0.50[c]	-	1.8	14
	Negaprion brevirostris	0.35-0.45	-	2.9,3.0	13,14
$E_1(R_{Mg}=?)$	Squatina squatina	0.10-0.30	-	-	14
	Squalus acanthias	0.35-0.45	-	-	11

Table III continued

Principle	Species	Paper chromatography R_F[a]	Pharmacological ratios R_{ME}	Pharmacological ratios R_{Mg}	Reference[b]
$E_1(R_{Mg}=1)$	Hexanchus griseus	0.10-0.20	-	0.95,1.3	14
$E_2(R_{Mg}=2)$	Scyliorhinus caniculus	0.50-0.90[c]	-	2.5	11,13,14
	Raia clavata	0.35-0.80[c]	-	2.1	12,14
	Rhinobatis rhinobatis	0.70-0.90[c]	-	2.0	14
	Carcharinus leucas	0.65-0.90[c]	-	1.9	14
	Negaprion brevirostris	0.5 -0.8[c]	-	2.2,2.6	13,14
	Squatina squatina	0.45-0.90	-	2.0	14
$E_2(R_{Mg}=?)$	Squalus acanthias	0.5 -0.6	-	-	11
$E_2(R_{Mg}=1)$	Hexanchus griseus	0.45-0.90[c]	-	1.0,0.85	14

[a]Abbreviations used: R_F = the ratio of the distance from the origin to the location of the oxytocic principle/distance from the origin to the solvent front, on a paper chromatogram developed in n-butanol:acetic acid:water = 4:1:5. R_{ME} = ratio of rabbit milk-ejection activity/activity on the rat uterus (Mg^{++} absent). R_{Mg} = magnesium potentiation (see text, page 25).

[b]References: 1: Sawyer et al. (1961), 2: Perks and Dodd (1963a), 3: Perks and Sawyer (1965), 4: Sawyer (1965), 5: Perks (1966), 6: Sawyer (1967), 7: Swiatkiewicz (1968), 8: Acher et al. (1965), 9: Chauvet et al. (1965), 10: Acher et al. (1967), 11: Heller and Pickering (1961), 12: Heller and Roy (1965a), 13: Heller and Roy (1965b), 14: Roy (1969).

[c]Entire range, more than one chromatogram.

including the n-butanol:acetic acid:water = 4:1:5 system of Heller, Pickering, and Roy. In all cases the oxytocic activity ran as one fraction, with an R_F similar to that of oxytocin, or to the fast-running E_2 principle of Heller. It is interesting that two of the species, Squalus acanthias and Carcharinus leucas, were used by both Perks and Heller with different results. It may be noted that Perks had chromatogrammed crude neurointermediate lobe extracts while Heller and his co-workers deproteinized at least some of their extracts with trichloracetic acid. Adsorption of the active principles onto protein may have prevented Perks' earlier extracts from resolving. However in later experiments Perks (personal communication, 1970) was still unable to achieve resolution of Squalus extracts into slow- and fast-moving components, even after preliminary treatment of the extracts with trichloracetic acid (TCA).

Swiatkiewicz (1968) ran repeated chromatograms of Squalus acanthias extracts, with steadily increasing loads, in the solvent system n-butanol:acetic acid:water = 4:1:5; each time he found only one active oxytocic fraction. Again, the magnesium potentiation was always approximately 2 (R_{Mg}=1.2-3.2). Although his extracts had not been treated with TCA, some of them had been deproteinized by gel filtration through Sephadex G-15, and therefore they were quantitatively comparable to Heller's extracts in their total protein content.

Using the same solvent system, Sawyer (1967) ran paper chromatograms of the active principles of the same species, Squalus acanthias, at three stages in their purification. These three stages were:

1) after deproteinization by treatment with TCA
2) after gel filtration through Sephadex G-25
3) after ion exchange chromatography on CM Sephadex, a cation exchanger.

The TCA-treated and Sephadex G-25-purified extract yielded a major oxytocic fraction at R_F 0.5-0.8 or 0.5-0.9, and a much smaller arginine vasotocin-like fraction (see section b above, page 23) at R_F 0.3-0.4. Following elution of the principles from the CM Sephadex, this small fraction had been separated away, and all the remaining oxytocic activity was found to run on paper in one fraction, again at R_F 0.5-0.8. This activity may well have corresponded to the fast-running principle, E_2, but again there was no evidence for the presence of the E_1 moiety.

Only one oxytocin-like principle was found in the species which elaborate either EOP II or glumitocin. In the purification and amino acid analysis of the Raia ocellata principle, EOP II (see section c, i, page 25), Perks and Sawyer (1965) found evidence of only one peptide, and Acher and his co-workers (1965; 1967) found only one oxytocin-like peptide (see section c, ii, page 26), with a trace of a vasotocin-like principle (see section b, page 23), in their

purification and analysis of Raia clavata, R. batis, R. fullonica, and R. naevus. It is noteworthy that R. clavata is one of the species in which Heller found a division into E_1 and E_2, where these principles showed magnesium potentiations of 6 and 2, respectively.

There is no immediate explanation for the inconsistency in the appearance of the principles E_1 and E_2. It may be that their occurrence varies with season or with other factors, not yet understood (Perks, 1966). There is a possibility that E_1 might be arginine vasotocin, which also runs slowly on paper in the solvent system used; however, this is offset by the following considerations:

1. In chromatograms of some of the species (Scyliorhinus caniculus, Carcharinus leucas, Negaprion brevirostris, and Hydrolagus collei) a third discrete oxytocic area of very little activity was found running even more slowly than E_1 (Roy, 1969).

2. Neither of the two relatively faster-running components of Scyliorhinus (that is, E_1 and E_2) was inactivated by trypsin, although synthetic arginine vasotocin was completely inactivated (Roy, 1969).

3. The ratio of antidiuretic activity to rat uterus activity is greater for arginine vasotocin that for the slow-running E_1 principle (Roy, 1969).

It is clear that the number and nature of the elasmobranch oxytocin-like principles is uncertain and confused.

EOP I, EOP II, and glumitocin represent differences between species, while E_1 and E_2, on the other hand, are both produced within a single species. However, the fractions E_1 and E_2 may themselves vary between species. In addition, there has been some overlap between those species observed to produce either EOP I or glumitocin, and those found to produce the mixture of E_1 and E_2. Table III summarizes the distribution and distinguishing characteristics of the different elasmobranch oxytocic principles. It is clear that further studies are greatly needed.

II. THE STATEMENT OF THE PROBLEM

The elasmobranch neurohypophysial principles, E_1, E_2, EOP I, and EOP II, had already been reported at the beginning of this study; glumitocin was analyzed shortly after this work began, and traces of an arginine vasotocin-like principle were observed in several species. The variety of neurohypophysial principles among the elasmobranchs appeared greater than that which occurs in any other vertebrate class, and their exact number and nature was uncertain. In the work reported here an effort was made towards resolving this confusion, both by pharmacological studies on crude extracts and by the preparation of purified principles which could be subjected to more rigorous pharmacological, chromatographic, and chemical analysis. Two species were studied: the skate, Raia rhina (Jordan and Gilbert, 1880) and the dogfish, Squalus acanthias L.

(Pacific variety) = Squalus suckleyi (Gerard, 1854). These represented the two extreme types of elasmobranch pituitary structure; skates had been found to contain the diffuse form of the pars nervosa (Meurling, 1967a), and the dogfish showed the discrete, and perhaps primitive, form of this pituitary region (Meurling, 1962).

Chapter 2

MATERIALS AND METHODS

A. Collection and Storage of the Glands

The pituitary neurointermediate lobes from two species of elasmobranch, Raia rhina (the longnose skate) and Squalus acanthias, Pacific variety, (the spiny dogfish), were obtained by trawl fishing off the coast of British Columbia and Washington State, between 1966 and 1968. The locations and dates of the various catches are listed in Appendix I.

The fish were dissected in living condition on board ship. The walls and roof of the braincase were removed to reveal the brain. The optic nerves, and the smaller nerves and blood vessels which attached to the brain, were severed. The connection between the anterior lobe of the pituitary and the ventral lobe, which is embedded in the floor of the braincase, was cut. The brain was then pulled back from its anterior end, and the pituitary gland on its ventral surface was exposed. The anterior lobe was peeled away, and the neurointermediate lobe was cut free from the underlying saccus vasculosus, and removed. Glands obtained in this way were treated by one of two methods:

1. Neurointermediate lobes dried in acetone

The lobes were placed in 25 ml glass collecting bottles containing acetone which had previously been dried over $CaCl_2$ and filtered through Whatman #1 paper. Usually the glands of a particular species taken in one trawl were placed in the same collecting bottle. Twenty minutes after dissection the acetone was poured off and replaced with fresh acetone. This was repeated at half hour intervals for the next two hours, and again after twenty-four and forty-eight hours. The glands were then dried on an agate mortar over gentle heat (32°C) to remove the last traces of acetone. For long term storage in the cold (4°C), the glands were either kept in a vacuum dessicator over P_2O_5, or were sealed into glass tubes.

2. Lyophilyzed neurointermediate lobes

Tissues collected during 1968 were lyophilyzed. Freshly dissected glands were placed in 5 ml polyethylene screw cap vials and frozen on dry ice. The frozen neurointermediate lobes were placed in a Virtis lyophilyzer, evacuated to 0.01 mm Hg pressure. After twenty-four hours the tissues were removed and stored at 4°C, either in a dessicator over P_2O_5 or in sealed glass tubes.

The method by which each sample of glands was treated is listed in Appendix I.

B. Extraction of the Tissues and Storage of the Extract

The dried tissues were usually extracted at a concentration of 40 mg dry tissue/ml in an aqueous 0.25% solution of acetic acid. Samples #2 and #3 of Squalus acantias, and samples #1 to #12 of Raia rhina (Appendix I) were extracted in a 0.25% solution of acetic acid which contained 0.9% NaCl.

Before extraction, the tissues were ground for approximately five minutes in the appropriate volume of 0.25% acetic acid, or in 0.25% acetic acid with 0.9% NaCl. This process was carried out using a 10 or 50 ml glass extractor (Thomas) fitted with a teflon grinder (Thomas) and driven by an electric motor (Fultork). Then the extractor tube was plugged with cotton and placed in a boiling water bath for three minutes. The tube was allowed to cool, and the extract was filtered through Whatman #1 paper. If large volumes of extract were involved, filtration was speeded with a Buchner funnel. The filtered extracts were stored for short periods of time in screw cap vials in the cold (4°C), without apparent loss of activity. However extracts which were stored for periods of several weeks or more were frozen immediately, and kept at -18°C.

C. Methods of Biological Assay

A number of methods of assay, based on the different biological activities of neurohypophysial peptides, were

used to assess the potencies of the elasmobranch principles. These are outlined below:-

1. Rat Uterus Assay

This assay was performed on the isolated rat uterus as outlined by Holton (1948) and modified by Munsick (1960). Female Wistar albino rats, weighing 180 to 220 gm and in natural estrous condition were used. The rat was stunned by a blow on the head and the blood vessels of the neck were severed. The uterus, ovaries, and vagina were removed and placed in bathing solution (composition given in Appendix III). The two uterine horns were separated. One of the horns was attached to a muscle hook and supported vertically, ovary upwards, in a 5 ml cylindrical muscle bath filled with bathing solution. The ovary was attached by a silk thread to the pointer of a smoked drum kymograph. The tissue was aerated with 5% CO_2 in air or oxygen throughout the assay. The muscle bath was connected at its base to a two liter reservoir of bathing solution, which was also aerated with 5% CO_2 in air or oxygen, so that fresh solution could be flushed through the muscle bath between each dose of peptide. The muscle bath and reservoir were contained in a 21 liter water bath maintained at 32°C by a Bronwill Circulating Motor (Will Corp.).

Assays were performed against synthetic standards: oxytocin (Syntocinon), glumitocin, or arginine vasotocin

(see Appendix II), which were freshly diluted with water for each assay. Unknowns were diluted with water and neutralized with aqueous 2 N NaOH or, later, with solid $NaHCO_3$. The standards and unknowns were always given in volumes of 0.20 ml or less.

The doses of unknown and standard were delivered into the muscle bath at five minute intervals, following the "four point" statistical procedure of Holton (1948). The potency and the fiducial limits of the potency, expressed at the 95% confidence level, were found by the method of Holton (1948). Limits were calculated only if an assay of three or more groups of four responses was obtained. Responses were measured as the height in mm of the record of the uterine contraction.

2. Rabbit Milk-Ejection Assay

The effect of the unknown in causing ejection of milk from a lactating rabbit was measured by this assay. In general the method followed was that of van Dyke et al. (1955).

The rabbit was removed from her young the evening before the assay. An hour before the assay she was given an intraperitoneal injection of 0.65 ml/kg body weight of "Dial" anaesthetic (composition given in Appendix III). When the animal was unconscious, the marginal vein of one of its ears was cannulated with a 22 gauge tuberculin

needle attached to a 15 cm length of PE 50 polyethylene tubing (Clay Adams); the whole cannula was filled with an aqueous 0.9% solution of NaCl. The tip of a teat was removed. One of the exposed mammary ducts was cannulated with a blunt 20 gauge tuberculin needle connected by PE 60 polyethylene tubing to a venous transducer (Statham P23BB). The cannula, transducer, and tubing contained a 1% aqueous solution of sodium citrate.

The assays were performed against synthetic oxytocin (Syntocinon) or synthetic glumitocin (see Appendix II) which were freshly diluted with aqueous 0.9% NaCl solution for each assay. The unknowns were diluted with 0.9% NaCl solution and neutralized with aqueous 2 N NaOH or solid $NaHCO_3$ before assay. The standards and unknowns were injected into the ear cannula in volumes of up to 0.20 ml, and each dose was followed immediately by a 0.20 ml wash of 0.9% NaCl solution. The doses were given at intervals of six to ten minutes. The "four point" statistical procedure of Holton (1948) was followed. After each injection the rise in intramammary pressure was recorded on a Beckman Type RS Dynograph (Beckman Instruments). Responses were measured as the height of the record in mm above the base line.

3. Frog Bladder Assay

The effect of the unknown in causing an increase in the passage of water across the isolated frog bladder wall

was measured. The bladder is particularly sensitive to arginine vasotocin, and the assay is a valuable tool for detecting the presence of this peptide. The method used was that of Sawyer (1960; and personal communication to Dr. A. M. Perks).

Male bullfrogs (Rana catesbiana) were stunned by a blow on the head. The urinary bladder was dissected free and placed in Ringer's solution (composition given in Appendix III). The two lobes of the bladder were cut apart. The neck of each half-bladder was tied with a silk thread around the flared end of a hollow glass cannula (length 9.4 cm; diameter 0.9 cm) and the bladder was filled with 5 ml of distilled water. Each bladder and cannula was suspended by means of the cannula's side arms in a tissue bath (11 cm high and 2.7 cm in diameter) which contained 25 ml of Ringer's solution. The bladders were aerated with compressed air throughout the assay. Assays were performed at room temperature.

The bladders were weighed at 15 minute intervals until a constant weight loss occurred over three successive periods. A dose of standard or unknown was added to the outer bath and the bladder was weighed after 15, 30, and 45 minutes. It was then transferred to fresh Ringer's and allowed to return to its resting rate of weight loss before another dose of peptide was given. Assays were performed

against synthetic oxytocin (Syntocinon) or synthetic arginine vasotocin (see Appendix II). Syntocinon was used undiluted; arginine vasotocin was freshly diluted with water for each assay. Unknowns were neutralized with aqueous 2 N NaOH or solid $NaHCO_3$ and delivered into the Ringer's solution undiluted. Dose volumes were always 0.20 ml or less. Doses were given by the "four point" statistical procedure of Holton (1948). One group of four responses was obtained on each half-bladder. Responses were measured as the average of the weight losses in the second and third 15 minute intervals after the dose was given minus the average of the weight losses in the two periods before the dose was given.

4. Antidiuretic Assay

The ability to cause an antidiuresis in the anaesthetized rat was used as a measure of potency. In general, the method followed was that of Jeffers et al. (1942), as modified by Dicker (1953).

A male Wistar albino rat of approximately 250 gm was anaesthetized with 5 ml/100 gm body weight of 12% ethanol in water, given through a stomach tube (#8 French urethral catheter). Two percent aqueous xylocaine hydrochloride (Astra Pharmaceuticals) was injected under the skin of the neck, and one of the jugular veins was cannulated with a 20 cm length of PE 10 polyethylene tubing filled with 0.9% NaCl solution. A tracheal cannula of PE 50 polyethylene tubing was inserted. Xylocaine hydrochloride was injected under

the skin above the urinary bladder and the bladder was cannulated with a 15 cm length of PE 200 polyethylene tubing. The penis was tied off. The rat was brought to 108% of its original weight with an aqueous solution of 1.5% ethanol and 0.05% NaCl, given by stomach tube. Throughout the assay, 2 ml of this solution were given as each 2 ml of urine were produced. After the rat had come to a constant rate of urine flow, injections of standard and unknown were begun.

Assays were performed against a mixture of arginine and lysine vasopressin (Pitressin) or against the synthetic peptides, arginine vasotocin and glumitocin (see Appendix II). These were freshly diluted with aqueous 0.9% NaCl solution for each assay. Unknowns were diluted with 0.9% NaCl solution and neutralized with aqueous 2 N NaOH or solid $NaHCO_3$. The standards and unknowns were injected into the jugular cannula in volumes of 0.20 ml or less, and each dose was followed immediately by a wash of 0.20 ml of aqueous 0.9% NaCl solution. The "four point" procedure of Holton (1948) was followed.

The drops of urine were directed over a drop recording unit (Palmer). The arm of an attached timer (Palmer) moved upwards on a kymograph drum at a constant speed between the drops and fell whenever a drop was produced. Thus the recording gave a direct picture of the antidiuretic effect of an injection. The responses were quantitated by the

following measurements:-

$$\text{The antidiuretic response} = \frac{\text{height of record during response}}{\text{height of record during resting flow}}$$

Where: 1) the height during resting flow = the average height in mm of the tracings made in the three minutes immediately preceeding the injection;

and 2) the height during the response = the average height in mm of the 20 to 40 tracings which were centered around the peak of the response. The number of tracings measured for each response within a group of four was constant; however the number varied between assays in accordance with the characteristics of each preparation.

5. Activity Ratios, Used for the Comparison of Different Assay Methods

The difference in potency, or activity, found for an unknown by two different methods of assay was expressed by the following series of ratios:

$$R_{Mg}, \text{ or } D_{Mg} = \frac{\text{activity on the rat uterus, } Mg^{++} \text{ present}}{\text{activity on the rat uterus, no } Mg^{++}}$$

$$R_{ME}, \text{ or } D_{ME} = \frac{\text{activity on the milk-ejection assay}}{\text{activity on the rat uterus, no } Mg^{++}}$$

$$R_{ADH}, \text{ or } D_{ADH} = \frac{\text{activity on the antidiuretic assay}}{\text{activity on the rat uterus, no } Mg^{++}}$$

$$R_{FB}, \text{ or } D_{FB} = \frac{\text{activity on the frog bladder assay}}{\text{activity on the rat uterus, no } Mg^{++}}$$

Where: "R" refers to the ratios of assays performed against oxytocin (Syntocinon) and vasopressin (Pitressin) standards, as above.

and "D" refers to the ratios of assays performed against other peptides, as indicated in the appropriate text.

The fiducial limits of these ratios, expressed at the 95% confidence level, were found by the following formula, supplied by Butler, statistics consultant to the Nuffield Institute for Medical Research, Oxford University (personal communication to Perks).

$$\text{Limits of } M_1 - M_2 = M_1 - M_2 \pm t\sqrt{S_{M_1}^{\,2} + S_{M_2}^{\,2}}$$

Where: subscripts 1 and 2 refer to assays 1 and 2

M = log R, and R = ratio of the dose of unknown to standard

S_M = standard error of M, calculated by the method of Holton (1948)

t = Student's t, corresponding at the 5% level of significance to F degrees of freedom, where F is calculated by the method of Brownlee (1965):

$$F = \frac{\left[S_{M_1}^2 + S_{M_2}^2\right]^2}{\frac{S_{M_1}^2}{n_1} + \frac{S_{M_2}^2}{n_2}}$$

where: F = combined degrees of freedom for the two assays

and n = degrees of freedom in each assay, calculated by Holton (1948).

Since the antilogs of the limits of $M_1 - M_2$ = limits of R_1/R_2, and since $R_1k_1 = P_1$ and $R_2k_2 = P_2$

where P = potency found for the unknown in each assay

and k = constant = $\frac{\text{dose of standard (dilution of unknown)}}{\text{volume of unknown}}$

then, (limits of R_1/R_2) (k_1/k_2) = limits of P_1/P_2 .

D. Non-Biological Measurements

1. Measurement of Protein or Peptide Content

The purity of the neurohypophysial peptide solutions

was estimated by the ratio of their biological activity to their peptide content ("specific activity"). Protein or peptide content was measured by the method of Lowry et al. (1951).

The unknowns were measured against bovine serum albumin (Armour). A series of aqueous solutions of the standard, ranging from 25 µg/ml to 500 µg/ml, were prepared freshly from a stock solution which was made at 0.05 gm/ml. Unknowns were diluted with water to the appropriate concentration.

One ml of alkaline copper solution (see Appendix IV) was added to 0.20 ml of each standard and unknown, and to a water blank. The solutions were mixed well and allowed to react for ten minutes at room temperature. Then 0.10 ml phosphomolybdic-phosphotungstic acid reagent (see Appendix IV) was added to each and mixed immediately on a Genie-Vortex Stirrer (Fisher Scientific). The samples were left at room temperature for one hour, during which time a blue colour developed. The optical density of the unknowns and standards was measured at λ = 750 mµ on a Unicam Spectrophotometer SP 600, modified to accommodate small volumes. A standard curve was drawn and the peptide concentration of the unknowns was read from it.

2. Measurement of Conductivity

Specific conductivity measurements were used for the rapid approximation of salt concentrations during the ion exchange chromatography procedures. The conductivity of the solutions was measured directly on a Radiometer Conductivity Meter Type CDM 2d, and was expressed as the specific conductivity, mho/cm.

3. Inactivation with Sodium Thioglycollate

Neurohypophysial peptides lose their biological activity after incubation with sodium thioglycollate at a neutral pH (van Dyke et al., 1942). Unknowns were tested by the method of Vogt (1953). One part of 0.1 N sodium thioglycollate was added to nine parts of the test solution. The mixture was neutralized with 2 N NaOH and left at room temperature for one hour. The extent of inactivation was measured by assay on the rat uterus.

4. Paper Chromatography

The elasmobranch principles were subjected to paper chromatography for partial isolation and purification, and for the comparison of their chromatographic behavior with that of known peptides.

Descending chromatograms were run on Whatman 3MM paper, 52 cm by 22 cm, as described by Perks (1966). The samples were applied 2 inches below the anti-siphon rod. Two or three origins, each 2.5 cm long, were spaced across

the chromatogram between outer margins of 4.0 cm. Samples ranging from 20 to 1,100 mU rat uterus activity and from 0.02 ml to 0.67 ml in volume were applied to a given origin. During most experiments, one or two standards of known chromatographic behavior - such as synthetic oxytocin (Syntocinon), arginine/lysine vasopressin (Pitressin), or synthetic arginine vasotocin - were run next to the unknown. The solvent system which was used was n-butanol:acetic acid: water = 4:1:5 (Heller and Pickering, 1961). The lower solvent phase was placed at the bottom of the chromatography tank. Equilibration time was two hours; at midpoint the chromatogram was positioned in the tank. The upper solvent phase was run into the solvent trough and the chromatogram was developed for twelve hours at room temperature (approximately 22°C).

After development, the paper was dried under a stream of cool air. Below each origin a longitudinal strip was cut; it ran from the origin to the solvent front, and extended 0.5 cm on each side of the origin. Each strip was cut into ten equal sections. Each section was placed in 1 ml of an aqueous 0.25% solution of acetic acid or in 1 ml of 0.25% acetic acid containing 0.9% NaCl, and left in the cold for one hour. The fluid content was expressed manually from each section and mixed with that which remained in the beaker. The eluates were neutralized with aqueous 2 N NaOH or solid $NaHCO_3$, and assayed on the appropriate preparation

to locate the active peptides. Recovery of the active principles from the paper usually approximated 33%.

On one occasion, one large sample (3.64 IU rat uterus activity, no Mg^{++} present) of Squalus extract was subjected to paper chromatography in the above solvent system, as a final step after its purification by gel filtration and ion exchange chromatography. In this case the origin was 3 cm across and the width of the eluted strip was 5 cm. It was divided into twenty sections, and these were eluted three times into 2 ml of 0.25% acetic acid, in order to recover as much activity as possible.

5. Isoelectric Focusing

Two purified elasmobranch preparations were subjected to isoelectric focusing (Svensson, 1961; 1962a; 1962b; Vesterberg and Svensson, 1966). By this procedure substances whose isoelectric points differ by as little as 0.02 pH units can be separated.

An LKB 8101 electrofocusing column (LKB Produkter), of 110 ml capacity, was used according to the instructions of the manufacturer. A series of ampholyte-sucrose fractions of increasing density was prepared (see Appendix IV). The peptide sample was incorporated into one of the middle fractions; sample volumes were approximately 1 ml. The ampholyte-sucrose fractions were poured into the electrofocusing column in order of decreasing density; they were

preceeded into the column by a cathode solution (see Appendix IV) and followed by an anode solution (see Appendix IV). Runs were made at 300 volts for 48 hours (ampholyte pH range 3-10) or at 800 volts for 25 hours (ampholyte pH range 7-9). The column was drained at a rate of 1.7 ml/min. Fractions of 0.75 ml or 1.0 ml were collected in an LKB Fraction Collector (LKB Produkter) and assayed on the rat uterus in order to locate the position of the unknown peptide in the newly established pH gradient.

E. Purification Procedures

1. Gel Filtration

The first step in the purification of the neurohypophysial peptides was their separation from the large protein molecules present in the crude extracts. To achieve this, the extracts were filtered through a column of Sephadex G-15 (Pharmacia Fine Chemicals), a gel which separates molecules on the basis of size.

The gel was prepared in 0.2 M acetic acid, which was also used as the eluant. 150 gm of Sephadex G-15 (Pharmacia), Lot T08507, was added to 2 liters of 0.2 M acetic acid. The mixture was stirred for 5 minutes and left to settle for one hour. The supernatant, containing the "fines", was decanted. This was repeated six times, including one overnight stirring period, using 2 liters of 0.2 M acetic acid each time. A large funnel was fitted into the top of a glass chromatography

column (Kontes; height 100 cm; inner diameter 2.4 cm) and the column and part of the funnel were filled with 0.2 M acetic acid. Aliquots of the swollen gel were added to the funnel and allowed to settle slowly, with continuous stirring, into the column. After the gel had built up to a height of 3 cm the stopcock was opened slightly, and left open for the remainder of the building period. The column was built to a height of 90 cm, and a circle of Whatman #1 paper was placed over the gel surface.

Thirteen samples of crude extract were passed through the column at room temperature (approximately 22°C). Between uses, the column was washed with 0.2 M acetic acid and stored in the cold (4°C). In order to obtain better separation of the neurohypophysial peptides from the larger proteins, the size of the samples applied to the column was restricted to 10 ml. However, in later experiments the sample volumes were increased to as much as 35 ml in order to reduce the dilution of the peptides during their passage through the gel. Before the sample was applied, the acetic acid in the column was drained down to the surface of the gel. The extract was carefully added dropwise onto the top of the gel, and the fluid was allowed to run slowly into the surface. 5 ml of 0.2 M acetic acid were then applied in the same way and allowed to run into the gel. The column was filled to the top with 0.2 M acetic acid and connected through polyethylene tubing to a reservoir of 0.2 M acetic acid. The

rate of flow of the eluant through the column was set at 0.3 ml/min. The eluate was led through PE 260 polyethylene tubing up to a fraction collector located on a level with the top of the column. The size of the fractions varied from 2 to 5 ml during different runs. The fractions were tested, as required, for rat uterus activity, Lowry peptide concentration, and for conductivity.

2. Filtration Through Ion Retardation Resin

After gel filtration, several of the peptide solutions were passed through an ion retardation resin which removed some of their salt content and so facilitated the adsorption of the peptides onto subsequent Sephadex cation exchangers. Biorad Ion Retardation Resin AG11A8, Lot 5867, was used.

Burettes (volume 25 ml; inner diameter 0.9 cm) were used as columns. For each column, 20 gm of resin was taken up in 50 ml water and poured into the column to a height of 40 cm, with the stopcock slightly open. A wash of 200 ml of 0.2 M acetic acid was run through the resin. This was followed by sufficient water (approximately 1 liter) to reduce the specific conductivity of the eluate to 0.05 mmho/cm. The column was stored in the cold.

The sample was passed through the resin at room temperature, followed by water until no more biological activity could be detected in the eluate. Flow rate was

1.2 ml/min. The recovery of activity was complete. The used resin was discarded.

3. Flash Evaporation

The acetic acid content of the eluates of the Sephadex G-15 and the ion retardation columns interfered with the adsorption of the peptides onto the subsequent ion exchange gels. Therefore it was partially removed from the solutions by flash evaporation.

A horizontal flash evaporator (Bodine Electric) of 500 ml capacity was used. The flask containing the solution to be evaporated was rotated in a water bath maintained at 27°C. The condensor was at first bathed in a stream of cold water but in later experiments a bath of methanol and dry ice was used. The condensor was connected to either a water aspirator or, through a trap, to a Duoseal Vacuum pump (Welch Scientific). When the pump was used, a pressure of 0.1 mm Hg was maintained in the system.

The solution was evaporated just to the point of dryness and the residue, containing the neurohypophysial peptides, was taken up in distilled water. Between 66% and 100% of the oxytocic activity was recovered.

4. Ion Exchange Chromatography

After gel filtration the peptides were further purified by chromatography on columns of Sephadex ion exchangers (Pharmacia). Three gels were used. They were diethylamino-

ethyl (DEAE) Sephadex, carboxymethyl (CM) Sephadex, and sulfoethyl (SE) Sephadex. The neurohypophysial peptides were not retained by the positively charged groups of the DEAE Sephadex. Nevertheless some necessary purification was achieved because of the adsorption onto the gel of negatively charged impurities, some of which have been shown to interfere with later procedures (Perks and Sawyer, 1965). However the peptides were adsorbed and concentrated by the negatively charged CM and SE exchangers. Each exchanger will be discussed separately below.

a. Diethylaminoethyl (DEAE) Sephadex

DEAE Sephadex, A-25 coarse, Lot T04751, was used throughout. The gel was prepared according to the manufacturer's instructions: 15 gm of dry gel was swollen in 200 ml of distilled water for one hour with stirring, allowed to settle, and the water and "fines" were poured off. This was repeated twice. The gel was transferred to a Buchner funnel and washed under suction with:

1) 100 ml of 0.5 N HCl, followed by 1 liter distilled water
2) 100 ml of 0.5 N NaOH, followed by 1 liter distilled water
3) 100 ml of 0.5 N CH_3COOH, followed by 1 liter distilled water
4) 100 ml of 0.2 M NH_4acetate (pH 7.0).

The exchanger was transferred to a beaker and successively equilibrated with 0.2 M, 0.02 M, and 0.002 M solutions of NH_4acetate (pH 7.0). The NH_4acetate buffers had been

prepared by titration of acetic acid solutions at the desired molarity with concentrated NH_4OH until the pH reached 7.0 (Radiometer, pH meter type PHM 22).

Burettes (volume 25 ml; inner diameter 0.9 cm) were used as columns. These were set up and built as described for the Sephadex G-15 column, except the stopcock was closed throughout the building period. The columns were built in 0.002 M NH_4acetate (pH 7.0) to a height of 35 cm. Then 0.002 M NH_4acetate (pH 7.0, specific conductivity 0.21 mmho/cm) was run through the gel until the specific conductivity of the eluate measured 0.21 mmho/cm. The columns were stored at 4°C.

A few samples were run through the columns in the cold (4°C), but most runs were made at room temperature (approximately 22°C). The sample was adjusted to a specific conductivity of 0.21 mmho/cm and pH of 7.0 by dilution with distilled water and the dropwise addition of NH_4OH. A preliminary wash of 50 ml of 0.002 M NH_4acetate buffer was run through the column. The sample was then run into the gel, followed by another 20 ml of the buffer. The flow rate through the column was set between 0.5 and 1.0 ml/min. Between 80% and 100% of the biological activity was recovered in the eluate. The used exchanger was discarded.

b. Carboxymethyl (CM) Sephadex

Two different lots of CM Sephadex exchanger were

used: C-25 medium, Lot T06071, and C-25, bead type, Lot 8852. Both were prepared in the same way: 15 gm of dry gel was swollen in distilled water as described for DEAE Sephadex, transferred to a Buchner funnel and washed under suction with:

1) 100 ml of 0.5 N NaOH, followed by 1 liter distilled water
2) 100 ml of 0.5 N HCl, followed by 1 liter distilled water
3) 100 ml of 0.5 N NH_4OH, followed by 1 liter distilled water
4) 100 ml of 0.2 M NH_4acetate (pH 5.0).

The exchanger was transferred to a beaker and successively equilibrated with 0.2 M, 0.02 M, and 0.002 M solutions of NH_4acetate (pH 5.0). At least five changes of the 0.002 M buffer were required. The NH_4acetate buffers had been prepared by adding concentrated NH_4OH dropwise and with continuous stirring to solutions of acetic acid at the desired molarity until the pH reached 5.0 (Radiometer, pH meter type PHM 22).

The columns were built in 25 ml burettes as described for the DEAE Sephadex, except that 0.002 M NH_4 acetate (pH 5.0) was used. After a column was built, 0.002 M NH_4acetate (pH 5.0, specific conductivity 0.16 mmho/cm) was run through until the specific conductivity of the eluate approached 0.16 mmho/cm. The columns were stored in the cold (4°C).

A few runs were made in the cold (4°C), but most samples were applied to the gel at room temperature (approximately 22°C). The sample, which had just been passed through DEAE Sephadex, was adjusted to a specific conductivity of 0.16 mmho/cm and pH of 5.0 by dilution with distilled water and the dropwise addition of acetic acid. The gel was washed through with 50 ml of 0.002 M NH_4acetate (pH 5.0) and then the sample was applied. When the last of the sample solution had passed into the gel, 1 ml of 0.002 M buffer was added to the top of the gel and allowed to run in. This was repeated twice and then the column was filled to the top with buffer.

The peptides were eluted by passing NH_4acetate buffer of increasing ionic strength through the gel. This was done through a mixing chamber, a 45 ml stoppered glass vial on a magnetic stirrer, which was located between the buffer reservoir and the top of the column below. Initially the system was filled with the dilute buffer in which the column had been built. This was replaced in the upper reservoir by a more concentrated buffer and so the concentration of buffer entering the column gradually increased from that of the dilute buffer to that of the more concentrated buffer. The neutral peptides were eluted off on this first gradient. Then the reservoir was filled with an even more concentrated buffer, and the basic peptide was eluted along the resulting second gradient.

The concentration of buffer necessary to desorb the neutral peptides differed with each of the two lots of CM Sephadex used, and this caused considerable technical difficulty in the earlier stages of the investigation. Further, a higher concentration was employed to elute the neutral peptide of the sakte (Raia rhina) than was used for the neutral fraction of the dogfish (Squalus acanthias). The neutral fraction of Squalus was eluted on a gradient between 0.002 M NH_4acetate (pH 5.0, specific conductivity 0.16 mmho/cm) and either 0.05 M NH_4acetate (pH 5.0, specific conductivity 3.4 mmho/cm), when CM Sephadex Lot T06071 was used, or 0.10 M NH_4acetate (pH 5.0, specific conductivity 6.2 mmho/cm) in the case of Lot 8852. The basic fraction was eluted from both lots of gel on a second gradient leading up to 0.2 M NH_4acetate (pH 7.0, specific conductivity 16.4 mmho/cm). The neutral fraction of Raia was eluted from the gel of Lot 8852 on a gradient between 0.002 M NH_4acetate (pH 5.0) and 0.15 M NH_4acetate (pH 5.0, specific conductivity 9.2 mmho/cm). The basic fraction was eluted on a second gradient leading up to 0.2 M NH_4acetate (pH 7.0). The neutral fraction of Raia was re-applied to CM Sephadex Lot 8852 and eluted on a gradient between 0.022 M NH_4acetate (pH 5.0, specific conductivity 1.7 mmho/cm) and 0.2 M NH_4acetate (pH 5.0, specific conductivity 12.3 mmho/cm).

The flow rate through the column was limited by the resistance of the gel; in all experiments the value was

0.3 ml/min. The eluate was collected in fractions of between 3.5 and 4.8 ml. The location of the neurohypophysial peptides was determined by rat uterus and antidiuretic assay. Each column was used only once.

c. Sulfoethyl (SE) Sephadex

One sample of exchanger, Lot T06773, was used throughout. 10 gm of dry gel was swollen in distilled water as described for DEAE Sephadex, transferred to a Buchner funnel and washed under suction with 0.5 N solutions of NaOH, HCl, and NH_4OH, and with 100 ml of 2 M NH_4formate (pH 2.45). The gel was transferred to a beaker and successively equilibrated with 2 M and then with 0.14 M NH_4formate (pH 2.45).

2 M and 1.5 M NH_4formate buffers were made by adding concentrated NH_4OH dropwise and with continuous stirring to solutions of 2 M and 1.5 M formic acid until the pH reached 2.45. The 0.14 M buffer was prepared from the 2 M buffer as follows: A solution of 2 M NH_4formate was diluted with water to a concentration of 0.05 M. The pH, which had risen upon dilution, was lowered to 2.45 by addition of a measured volume of formic acid. The final formate concentration of the buffer was calculated and found to be 0.14 M.

The columns were built in 0.14 M NH_4formate in 25 ml burettes as described for DEAE Sephadex. After a column was built it was equilibrated with 0.14 M NH_4formate (pH 2.45, specific conductivity 1.9 mmho/cm) until the specific con-

ductivity of the eluate had fallen to 1.9 mmho/cm. The columns were stored at 4°C.

The samples which were applied to this gel contained only neutral neurohypophysial peptides. All were applied and eluted at room temperature (approximately 22°C). They were adjusted to specific conductivity of 1.9 mmho/cm and pH 2.45 by dilution with distilled water and the dropwise addition of formic acid, and run into the gel in the manner described for CM Sephadex. The flow rate during loading was set at between 0.35 and 0.55 ml/min.

The peptides were eluted on an NH_4 formate concentration gradient, in the manner described for CM Sephadex; the range used was from 0.14 M to 1.5 M buffer (pH 2.45, specific conductivities 1.9 and 15.0 mmho/cm, respectively). The elution flow rate was set at between 0.7 and 0.9 ml/min and the size of the fractions collected was 3.4 ml. The location of the principles in the eluate was detected by the rat uterus assay. Each column was used only once.

5. Lyophilyzation (Freeze Drying)

Partial elimination of the buffer salts from the eluates of the CM and SE exchangers was effected by lyophilyzing the solutions to dryness on a Virtis lyophilyzer evacuated to 0.01 mm Hg pressure. For more complete removal of the NH_4 acetate or NH_4 formate the dried residues were taken up in water and lyophilyzed several times.

6. Hydrolysis and Amino Acid Analysis

The lyophilyzed samples were taken up in approximately 3 ml of distilled water and transferred to 5 ml Pyrex tear-drying bulbs (Virtis). Each was again lyophilyzed to dryness. 4.0 ml of triple distilled HCl was added to the bulb. Liquified phenol, 0.04 ml, was also added to the early samples to minimize the breakdown of tyrosine (Dixon, personal communication) but this was found unsatisfactory for sulfur-containing compounds and was omitted from the later samples.

N_2 gas was bubbled through the sample for ten minutes as a brisk stream of fine bubbles; after this time the sample was frozen by immersion of the bulb in liquid N_2. The bulb was then attached to a Duoseal vacuum pump (Welch Scientific) operating at 0.01 mm Hg pressure, evacuated for 30 minutes, and sealed with a flame while under vacuum.

The sealed bulb was placed in a hot air oven at 107°C and left for 18 hours. After hydrolysis the contents were transferred to a 5 or 10 ml pear shaped flash-evaporation flask. The sample was placed in the freezer for 15 minutes, attached while still cold to a flash evaporator, and taken to dryness over a water bath maintained at 30-35°C. 2 or 4 ml of refrigerated water were added to the flask and the evaporation was repeated. The addition of water and subsequent evaporation was repeated several times, until there was no smell of HCl in the sample. The hydrolyzed samples

were stored dry in the freezer (-18°C).

The hydrolysates and two control samples were analyzed for amino acid content on a Beckman model 120 Amino Acid Analyzer or on a Biocal BC-200 Amino Acid Analyzer. The rat uterus activity of the peptide in the samples which were subsequently hydrolyzed and analyzed ranged from 600 to 2880 mU.

Chapter 3

RESULTS

I. STUDIES OF THE NEUROHYPOPHYSIAL PRINCIPLES OF THE SKATE, *RAIA RHINA*

A. Introduction

The skate, *Raia rhina*, was studied in order to extend the work on the elasmobranch neurohypophysis to a new Pacific species. This species was of interest because it belonged to a genus whose various members had already been reported to produce the full range of elasmobranch principles - EOP I, EOP II, E_1, E_2, and glumitocin (Table III, page 33). Further, the principles produced by the diffuse type of pars nervosa found in the skates (Meurling, 1967a) might form an interesting contrast with those produced by the discrete type of neurohypophysis present in *Squalus acanthias*, the principal species studied here (Meurling, 1962).

In addition, Manning's synthesis of glumitocin (Manning *et al.*, 1968) during the course of this work meant that a direct pharmacological comparison could be made between the skate neurohypophysial principle and a standard of possibly identical molecular structure.

B. Pharmacological and Chromatographic Studies of the Neurointermediate Lobe Extracts

1. Biological Activities and Activity Ratios of the Crude Extract

Fifteen glands (sample 6, see Appendix I) were extracted at 40 mg dried tissue/ml. They were assayed against synthetic oxytocin (Syntocinon, Sandoz) for rat uterus and milk-ejection activity, and against vasopressin (Pitressin, Parke Davis) for antidiuretic activity. The activity ratios found for this species were compared to those of other elasmobranchs.

The glands were found to have a rat uterus activity of 2.0 ± 0.3 mU/mg dried tissue. This level of activity was low even for elasmobranchs (Perks and Dodd, 1963a), although similar low levels occur in certain species of large sharks (Perks, 1966). The milk-ejection activity was 8.7 mU/mg and the antidiuretic activity was 0.03 mU/mg. The ratios of the activities, ME (milk-ejection): RU (rat uterus): ADH (antidiuretic), were 4.4:1:0.02. This was in agreement with values found for most other elasmobranchs, including two skates, R. clavata and R. batis; the values characterized the R. rhina principle as the EOP I type, which is distinguished by an R_{ME} of approximately 3, rather than as the principle designated as EOP II, in which the R_{ME} is approximately 10 (Perks and Dodd, 1963a; Sawyer, 1964; Perks, 1966).

2. Studies of the Magnesium Potentiation of the Extracts

a. The range of magnesium potentiations in twelve crude extracts

It was possible that the R. rhina extract could contain a mixture of several neutral peptides, for there had been conflicting reports on the nature and number of principles which constituted the EOP I fraction in several other elasmobranch species. Heller and Pickering (1961), Heller and Roy (1965a), and Roy (1969) reported that two oxytocic principles could be separated by paper chromatography from extracts of Squalus acanthias (spiny dogfish), Carcharinus leucas (bull shark), and Raia clavata (thornback ray), three species which gave a pharmacological spectrum of the EOP I type (Perks, 1966). These agents were designated as E_1 and E_2: E_1, the slow-running principle, was further characterized in some species by the high degree to which its activity on the rat uterus was potentiated by magnesium ions, whilst in contrast, E_2, the fast-running principle, was only moderately magnesium potentiated (see Table III, page 33). Other investigators (Perks, 1966; Sawyer, 1967; Swiatkiewicz, 1968) were unable to confirm these results. They found that chromatograms of Squalus and Carcharinus, developed in the same solvent system - n-butanol:acetic acid:water = 4:1:5 - revealed only one, fast-running principle.

A possible reason for the discrepancy between the results of different authors may have been that at some times one particular principle occurred alone, whilst at other times various intermediate mixtures of the two were present - mixtures dependent on unknown factors such as sex, season, or location. Since in addition to their chromatographic resolution, E_1 and E_2 were also distinguished by their different degrees of magnesium potentiation, it was possible that the presence of two such principles in varying proportions might have been detected by a study of the variability in the magnesium potentiations of crude neurointermediate lobe extracts.

An attempt was made to determine whether there was such a variability in the magnesium potentiations of twelve extracts of Raia rhina pituitaries, obtained in different trawls at various locations. The neurointermediate lobes from samples 1-12 (Appendix I) were extracted and assayed for oxytocic activity on the rat uterus both in the presence and absence of magnesium ions. Their activities and the resulting magnesium potentiations are listed in Table IV. The potentiations varied from 1.8 ± 0.4 to 17.5 ± 4.4, and could have supported the existence of various mixtures of two peptides. However the range was unexpectedly wide. The known potentiations of E_1 and E_2 differed only from 2 to 6 (Table III, page 33), and the highest potentiations which had been reported for neurophypophysial principles were

Table IV

Oxytocic Activities, with and without Magnesium, of Crude Neurointermediate Lobe Extracts of Raia rhina; and their Magnesium Potentiations

Sample number[a]	Rat uterus activity[b] (mU/mg dried tissue)		Magnesium potentiation[c]
	without Mg^{++}	with Mg^{++}	
1	2.2 ± 0.3	4.0 ± 0.7	1.8 ± 0.4
2	1.2 ± 0.05	3.2 ± 0.2	2.7 ± 0.2
3	1.7 ± 0.1	5.7 ± 1.0	3.5 ± 0.6
4	2.7 ± 0.9	9.7 ± 3.9	3.6 ± 1.8
5	1.7 ± 0.2	6.1 ± 0.8	3.7 ± 0.7
6	2.0 ± 0.4	10.9 ± 1.7	5.6 ± 1.2
7	0.94 ± 0.08	5.3 ± 0.8	5.6 ± 1.0
8	1.3 ± 0.5	7.8 ± 1.4	6.1 ± 2.6
9	0.91 ± 0.11	7.0 ± 0.7	7.6 ± 1.1
10	1.4 ± 0.2	17.6 ± 3.7	12.4 ± 2.8
11	2.2 ± 0.4	37.5 ± 5.8	16.8 ± 3.4
12	0.92 ± 0.18	16.1 ± 2.9	17.5 ± 4.4

[a] As in Appendix I.

[b] Assays calculated by the 4-point method of Holton (1948); whenever three or more groups were obtained the fiducial limits are expressed, P = 0.05.

[c] Magnesium potentiation =

$$\frac{\text{rat uterus activity, in presence of 0.5 mM } Mg^{++}}{\text{rat uterus activity, in absence of } Mg^{++}}$$

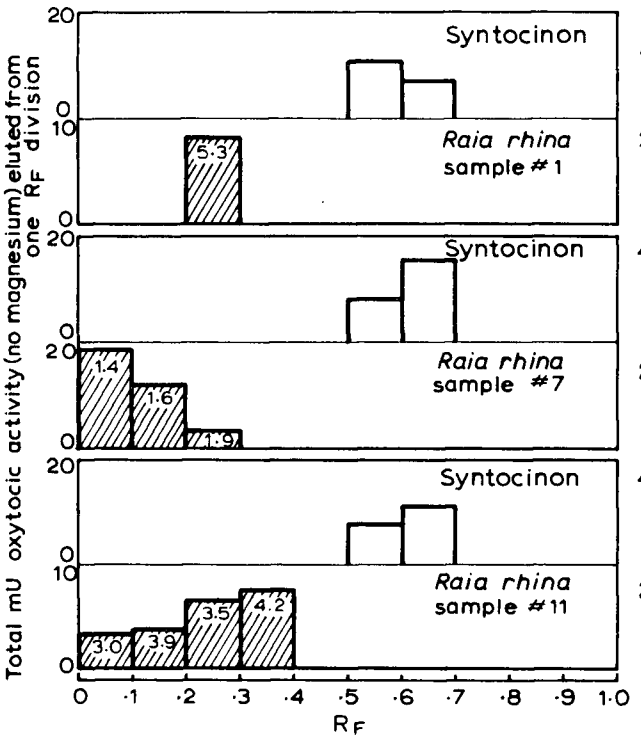
R_{Mg}=10 for glumitocin (Acher et al., 1965) and R_{Mg}=10.8 for the synthetic analogue, 3-norvaline oxytocin (Krejčí and Poláček, 1968). Despite the surprising range of the potentiations obtained for the extracts, the existence of variable mixtures remained a possibility. This could be checked by chromatographic separation of the principles.

b. Paper chromatography of extracts with different magnesium potentiation values

An attempt was made to show by paper chromatography that the different potentiations which had been found for the extracts in the previous experiment reflected differing peptide mixtures, and that each extreme in potentiation reflected a preponderance of either the slow-running or the fast-running peptide. Accordingly, samples 1, 7, and 11 (R_{Mg}=1.8, 5.6, and 16.8, respectively) were subjected to paper chromatography in n-butanol:acetic acid:water = 4:1:5.

Although there had been marked differences between the magnesium potentiations of the three extracts, they all behaved in a similar manner upon chromatography (Fig. 3). In each case a single slow-running peak of oxytocic activity was found, and the front did not extend further forward than an R_F of 0.4. It is probable that this unexpectedly slow migration resulted from the large amounts of inert material which were applied to the origin; from 0.22 to 0.53 ml of extract, which represented from 8.8 to 21.2 mg of dry tissue and contained approximately 1 to 3 mg of Lowry peptide, had

Figure 3. Descending paper chromatograms of three crude neurointermediate lobe extracts of Raia rhina, on Whatman 3MM paper, in n-butanol:acetic acid: water = 4:1:5, at room temperature. White columns = synthetic oxytocin (Syntocinon, Sandoz); hatched columns = oxytocic substances of Raia rhina. Numbers within the columns indicate the magnesium potentiations of the eluates. Loads are stated as mU oxytocic activity = mU rat uterus activity in the absence of Mg^{++}.



	Oxytocic activity (mU)	LOAD Equivalent extracted dry tissue (mg)	Magnesium potentiation	RECOVERY
Syntocinon	40	—	—	43%
Raia rhina sample #1	20	9·2	1·8	41%
Syntocinon	40	—	—	57%
Raia rhina sample #7	20	21·3	5·6	170%
Syntocinon	40	—	—	46%
Raia rhina sample #11	20	9·0	16·8	106%

been applied to each origin.

The recovery of activity from two of the chromatograms (samples 7 and 11) was unexpectedly high, 106% and 170% of the activity which had been applied. A similar phenomenon had previously been observed for chromatograms of two other elasmobranchs, Squalus acanthias and Scyliorhinus caniculus (Pickering, personal communication to Perks, 1960). The recovery of the synthetic oxytocin standard (Syntocinon) from the Raia rhina chromatograms was 43% to 57%.

Although each extract ran as a single oxytocic fraction, it was possible that a peptide mixture might have been partially resolved within the superficially continuous peak - especially as the proper development of the chromatogram may have been inhibited by the high level of inert material in the extracts. An attempt was made to show this by assessing the magnesium potentiation of the oxytocic agent in each R_F unit. The results are indicated in Fig. 3. It was found that the oxytocic peaks were uniformly potentiated throughout their length; there was no indication of the separation of a highly potentiated principle from a less potentiated principle, even on the chromatogram of sample 7, where the intermediate value of the magnesium potentiation might have suggested the presence of a peptide mixture. However, it was surprising to find that the potentiations of the eluates were different from those of the original crude extracts.

This might have suggested the chromatographic removal of an inhibitory or an activating substance from the extract, but the changes in potentiation of the eluates were not all in a consistent direction. The R_{Mg} for samples 7 and 11 had decreased from 5.6 to 1.6 and from 16.8 to 3.7, respectively, while sample 1 had increased from 1.8 to 5.3.

c. Joint assays of extracts with different magnesium potentiation values

The variations which were observed in the potentiations of the three extracts before and after chromatography, and in the potentiations of the series of twelve extracts, may have been due to differences between the uteri on which they were assayed. To investigate this possibility, samples which had been found to have widely different potentiations (Table IV) were reassayed, together, on the same uteri. Samples 1 and 11 were assayed "jointly" both in the presence and absence of magnesium, as were samples 7 and 11 and samples 3 and 10.

In all three cases the two "joint" potentiations were found to be similar (Table V). The potentiations of samples 1 and 11 converged from the wide values of 1.8 ± 0.4 and 16.8 ± 3.4, respectively, to 4.8 and 4.1 ± 1.1. The potentiations of samples 7 and 11, which were 5.6 ± 1.0 and 16.8 ± 3.4 on independent assays, became 2.3 ± 0.9 and 2.5 on joint assay; and the values for samples 3 and 10, originally 3.5 ± 0.6 and 12.4 ± 2.8, were found to be 6.7 ± 1.7 and 8.4.

Table V

Magnesium Potentiations of Pairs of Raia rhina Neurointermediate Lobe Extracts during Independent and Joint Rat Uterus Assays

Experiment	Sample number	Independent determination[a]			Joint determination		
		Activities[b]		Potentiation	Activities		Potentiation
		Assay	mU/mg dried tissue	R_{Mg}	Assay	mU/mg dried tissue	R_{Mg}
1	1	RU[c]	2.2 ± 0.3		RU	2.4	
		RU+Mg	4.0 ± 0.7		RU+Mg	11.5 ± 2.5	
				1.8 ± 0.4			4.8
	11	RU	2.2 ± 0.4		RU	3.1 ± 0.7	
		RU+Mg	37.5 ± 5.8		RU+Mg	12.6 ± 2.8	
				16.8 ± 3.4			4.1 ± 1.1
2	7	RU	0.94± 0.08		RU	1.8 ± 0.6	
		RU+Mg	5.3 ± 0.8		RU+Mg	4.1 ± 1.6	
				5.6 ± 1.0			2.3 ± 0.9
	11	RU	2.2 ± 0.4		RU	1.6	
		RU+Mg	37.5 ± 5.8		RU+Mg	4.1	
				16.8 ± 3.4			2.5
3	3	RU	1.7 ± 0.1		RU	1.3 ± 0.2	
		RU+Mg	5.7 ± 1.0		RU+Mg	8.4 ± 1.9	
				3.5 ± 0.6			6.7 ± 1.7
	10	RU	1.4 ± 0.2		RU	1.3	
		RU+Mg	17.6 ± 3.7		RU+Mg	11.2 ± 4.9	
				12.4 ± 2.8			8.4

Table V. continued

[a]As in Table IV.

[b]Assays calculated by the 4-point method of Holton (1948); whenever three or more groups were obtained the fiducial limits are expressed, P = 0.05.

[c]Abbreviations used: RU = assay performed on rat uterus, in absence of magnesium ion. RU+Mg = assay performed on rat uterus, in presence of 0.5 mM magnesium ion. R_{Mg} = magnesium potentiation, as in Table IV.

These changes in potentiation reflected changes in the potency of the extracts on both types of assay (see Table V); they could not be ascribed solely to inconsistencies in the assay values produced in the presence of Mg^{++}, nor in its absence.

d. The influence of the estrous cycle on magnesium potentiation values

The responses of different uteri to the same principle might be influenced by their stage in the estrous cycle. The uteri used in these experiments had all been taken from rats in the estrous state, as determined by vaginal smear, but no attempt had been made to distinguish between early and late estrous. Therefore sample 6 was reassayed, both with and without magnesium, on the two horns of a uterus taken from a rat in early estrous, and then again on the horns of a uterus in late estrous. While the potency of the extract differed significantly from early estrous to late estrous when the assays were performed in the presence of magnesium ions, the two resulting potentiations were not significantly different from each other, and both were well within the range of values observed earlier (1.8 ± 0.4 to 17.5 ± 4.4). The potencies found on the early estrous preparation were 3.2 ± 0.8 mU/mg dried tissue without magnesium and 25.9 ± 6.6 mU/mg when magnesium was present. On the late estrous preparation the potencies were 2.6 ± 0.4 mU/mg without magnesium and 14.2 ± 2.3 mU/mg with magnesium. The resulting potentiations were 8.2 ± 3.0 and 5.5 ± 1.3 for early and late estrous, respectively. Therefore these brief

experiments suggest that the difference between uteri in early and in late estrous cannot account for the full range of potentiations which were observed. Only further extensive work could establish any more subtle relationships which might exist, and it was considered more important to make more direct attacks on the chemistry and pharmacology of the skate principles at this stage.

3. Comparison of Crude Extract with Synthetic Glumitocin

Glumitocin, the principle occurring in several species of skate (Acher et al., 1965; 1967; Chauvet et al., 1965), was synthesized during the course of this study (Manning et al., 1968). This made it possible to compare the Raia rhina principle to a synthetic peptide with which it might well be identical.

a. Assays of crude extract against glumitocin

Thirty-two neurointermediate lobes from sample 22 (Appendix I) were extracted and assayed against synthetic glumitocin for rat uterus activity both with and without magnesium, for milk-ejection activity, and for antidiuretic activity. The results are shown in Table VI, and the ratios of the activities are expressed as D_{Mg}, D_{ME}, and D_{ADH} to indicate that the assays were performed against a standard (in this case, synthetic glumitocin) other than oxytocin (Syntocinon) or vasopressin (Pitressin).

Table VI

Activities and Activity Ratios of Crude *Raia rhina* Neurointermediate Lobe Extract, Assayed Against Synthetic Glumitocin

Sample number[a]	Activities[b] (ng glumitocin/mg dried tissue)				Activity ratios		
	RU[c]	RU+Mg	ME	ADH	D_{Mg}	D_{ME}	D_{ADH}
22 (32 NIL)	294 ± 39	233 ± 25	1)[d]362 2) 413 ± 107	1640	0.79 ± 0.14	1) 1.2 2) 1.4 ± 0.4	5.6

[a]As in Appendix I.

[b]Assays calculated by the 4-point method of Holton (1948); whenever three or more groups were obtained the fiducial limits are expressed, P = 0.05.

[c]Abbreviations used: RU = activity on rat uterus, in absence of magnesium ions. RU+Mg = activity on rat uterus, in presence of 0.5 mM Mg^{++}. ME = activity on rabbit milk-ejection assay. ADH = activity on rat antidiuretic assay. Activity ratios, D_{Mg}, D_{ME}, and D_{ADH}, are the ratios of the activity found on each assay (RU+Mg, ME, and ADH) to the activity found on the rat uterus assay (RU); where D indicates that the assays were performed against a peptide other than the normal standards.

[d]Two separate determinations were made for milk-ejection activity.

The potencies of the extract on the three assays used primarily for neutral principles (rat uterus with magnesium, rat uterus without magnesium, and milk-ejection) were similar; the magnesium potentiation was eliminated when glumitocin replaced oxytocin as a standard, and the two activity ratios, D_{Mg} and D_{ME}, did not differ significantly from one. This was consistent with the identity of the Raia rhina principle with glumitocin. However the extract was much more active than glumitocin on the antidiuretic assay. This could be explained by the presence in the crude extract of a second peptide, which had a much greater antidiuretic activity than that of glumitocin. During subsequent purification of the active principles a basic fraction having 209 mU of antidiuretic activity was separated away from the neutral fraction of 1008 mU rat uterus activity. The basic fraction was not characterized further, but was tentatively regarded as arginine vasotocin (Section C3b, page 954). Table VII shows the activity ratios that would be expected from a mixture of glumitocin (1008 mU rat uterus activity, no Mg^{++} present) with arginine vasotocin (209 mU antidiuretic activity). The experimental value of $D_{ADH} = 5.6$ agreed well with the theoretical value of $D_{ADH} = 5.0$, whilst the observed values for D_{Mg} and D_{ME} remained consistent with the expected values. Calculation shows that the data could also apply to the presence of glumitocin with either of the two other naturally occurring basic peptides, arginine vasopressin or lysine

Table VII

A Comparison of the Activity Ratios found for Raia rhina Neurointermediate Lobe Extract when Assayed against Synthetic Glumitocin, to the Theoretical Activity Ratios Expected from Mixtures of Glumitocin and Small Amounts of Basic Peptide

Type of data	Peptide mixture	Activity ratios		
		D_{Mg}[a]	D_{ME}	D_{ADH}
Experimental	Raia rhina, crude extract[b]	0.79 ±0.14	1) 1.2 2) 1.4±0.4	5.6
Theoretical	Glumitocin with[c] arginine vasotocin	0.92[d]	1)[e] 0.93 2) 0.92	5.0
	Glumitocin with arginine vasopressin	1.00	1) 1.00 2) 1.00	5.5
	Glumitocin with lysine vasopressin	1.00	1) 1.00 2) 1.00	5.4
	Glumitocin with 8-lysine oxytocin	0.68	1) 0.71 2) 0.66	3.3

[a] Abbreviations as in Table VI.

[b] Activity ratios for crude extract are also given in Table VI.

[c] Theoretical peptide mixtures consist of glumitocin (1008 mU rat uterus activity without Mg^{++} present) and basic peptide (209 mU rat antidiuretic activity); where the basic peptide is one of arginine vasotocin (8-arginine oxytocin), arginine vasopressin (3-phe, 8-arg oxytocin), lysine vasopressin (3-phe, 8-lys oxytocin), or 8-lysine oxytocin.

[d] Theoretical activity ratios were calculated from the following data. References are indicated in brackets and listed below.

Arginine vasotocin: R_{Mg}=1.9(a), R_{ME}=2.2(a), R_{ADH}=2.0(b).

Arginine vasopressin: R_{Mg}=2.3(a), R_{ME}=5.7(a), R_{ADH}=44(a).

Lysine vasopressin: R_{Mg}=2.5(a), R_{ME}=10.2(a), R_{ADH}=33(a).

8-lysine oxytocin: R_{Mg}=1.9(a), R_{ME}=2.3(a), R_{ADH}=0.31(a).

Table VII continued

Glumitocin: R_{Mg}=9.5(c), R_{ME}=(1) 8.2(c), and (2) 15.6(c), R_{ADH}=0.046(d).

References: (a) Sawyer (1965), (b) Sawyer et al. (1961), (c) this thesis, Table VIII, (d) Sawyer et al. (1969).

[e]The two theoretical values of D_{ME} listed for each peptide mixture correspond to the two values found for the R_{ME} of synthetic glumitocin on two separate assays (see Table VIII), and also to the two values of D_{ME} found for the crude extract on the same two assays (Table VI).

vasopressin, or to a mixture of glumitocin with the purely synthetic basic peptide, 8-lysine oxytocin (Table VII). However the presence of these basic principles is unlikely. The vasopressins have been found to be confined to the mammals, and perhaps the birds; 8-lysine oxytocin has low antidiuretic activity, and has not been found in nature.

b. Assays of crude extracts and glumitocin against oxytocin

During each of the preceeding rat uterus and milk-ejection assays, one group of responses was obtained which included the responses to a high and a low dose of synthetic oxytocin (Syntocinon). The activities of the extract and of glumitocin were estimated relative to this more usual standard, but the assays were not sufficiently extended to give values for the errors of these estimates. The activities and their ratios are listed in Table VIII.

It can be seen that two different milk-ejection potencies were found for both the crude extract and glumitocin, even though their activities relative to each other were similar on the two assays (Table VI). This is particularly notable, since both assays were performed on the same rabbit at a twelve day interval; despite this the potency found for both unknowns was 100% greater on the second assay. Since the second assay suggested an increase in potency, the results cannot be explained by deterioration of the unknowns. The standards were prepared freshly.

Table VIII

Activities and Activity Ratios of *Raia rhina* Neuro-intermediate Lobe Extract and of Synthetic Glumitocin, Assayed against Synthetic Oxytocin (Syntocinon)

Principle	Activities[a] (mU/mg dried tissue) or (mU/mg glumitocin)			Activity ratios	
	RU[b]	RU+Mg	ME	R_{Mg}	R_{ME}
Extract of *Raia rhina*	2.7	16.3	1)[c] 22.5 2) 47.3	6.0	1) 8.3 2) 17.5
Glumitocin	7602	72,446	1) 62,196 2) 118,860	9.5	1) 8.2 2) 15.6

[a]All values were obtained by the 4-point method of Holton (1948); only single groups were obtained during these experiments which were designed to compare the extract with glumitocin, and therefore fiducial limits could not be calculated.

[b]Abbreviations as in Table VI;, except the activity ratios, R_{Mg} and R_{ME}; where R indicates that the assays were performed against a normal standard (synthetic oxytocin).

[c]Two milk-ejection assays were performed (see Table VII, footnote [e]).

The corresponding ratios of milk-ejection to rat uterus activity (R_{ME}) reflected these differences in milk-ejection potency with values of 8.2 and 15.6 for glumitocin, and 8.3 and 17.5 for the extract. Earlier in this investigation a third value of R_{ME} = 4.4 had also been found for an extract from this species (Section B1, page 70). Variation in the ratio of milk-ejection activity to rat uterus activity had not previously been reported for any neurohypophysial principle, but the values found here, which ranged from 4.4 to 17.5 for crude extracts of R. rhina, and from 8.2 to 15.6 for glumitocin, demonstrate again the danger inherent in comparing assay values obtained on different preparations, when the unknown and standard are of different molecular structure.

C. The Purification of the Extract

Pharmacological studies of the extract of Raia rhina (Section B3, page 82) indicated that it contained glumitocin. An attempt was made to purify and analyze the principle so that its amino acid composition could be compared to the known composition of glumitocin.

1. Extraction of the Glands, and Purification of the Extract by Gel Filtration

For purification procedures, the glands from samples 13 to 24 (Appendix I) were pooled, with the exception of the 32 glands which were extracted for comparison with

glumitocin (Section B3, page 82). The 220 dried neurointermediate lobes, weighing 1917 mg, were extracted in 48 ml of 0.25% acetic acid and filtered on a Buchner funnel. The residue which remained after filtration was re-extracted in 15 ml of 0.25% acetic acid and refiltered. A total of 57 ml extract was recovered. The oxytocic activity (rat uterus, without magnesium) of the extract was low, 36.8 ± 6.9 mU/ml, or 1.1 mU/mg dried tissue. Its Lowry peptide content was 5,100 µg/ml and the ratio of oxytocic activity to Lowry peptide was 7.2 mU/mg. The total activity in the extract was 2098 mU. To this was added 8 ml of extract remaining from previous experiments and containing 392 mU oxytocic activity, to give a total of 2490 mU of oxytocic activity.

The extract was passed through a 90 cm x 2.4 cm column of Sephadex G-15 gel (in 0.2 M acetic acid) in two lots of 30 and 35 ml each. The large sample size was adopted in order to minimize the dilution of the extract during its passage through the column. The results of the two runs are summarized in Table XIII, page 124). The active eluates from the two runs were combined in a volume of 284 ml. This had an oxytocic activity of 7.2 mU/ml and a Lowry peptide content of 266 µg/ml. The total activity recovered was 2,045 mU or 82% of that applied to the column. Approximately 77% of the original Lowry peptide had been separated away from the neurohypophysial principles, and the ratio of oxytocic

activity to Lowry peptide in the eluate was 27 mU/mg, a 3.8 fold purification. The sample was diluted to 4.4 times its original volume.

2. Passage of the Extract through Ion Retardation Resin and DEAE Sephadex

The active eluate from the Sephadex G-15 was passed through a 40 cm x 0.9 cm column of Biorad Ion Retardation Resin AG11A8, which removed some of the salt content from the eluate and so facilitated the adsorption of the active principles onto a subsequent cation exchanger. The acetic acid (0.2 M, specific conductivity 0.71 mmho/cm), by which the peptides had been eluted from the Sephadex G-15, was not removed by this procedure. Nevertheless the specific conductivity of the peptide solution was reduced to 0.97 mmho/cm from 1.50 mmho/cm. There was no loss of oxytocic activity.

The eluate from the ion retardation resin was adjusted to pH 7.0 and specific conductivity 0.21 mmho/cm by dropwise addition of NH_4OH and dilution with water to a final volume fo 4500 ml. It was passed through a 35 cm x 0.9 cm column of DEAE Sephadex, an anion exchanger, which had been equilibrated with 0.002 M NH_4acetate (pH 7.0). The active principles were not adsorbed onto the gel. However this step had been found necessary for the subsequent adsorption of the oxytocic principle of the skate Raia

ocellata onto CM Sephadex (Perks and Sawyer, 1965). No loss of oxytocic activity was expected, but the level of activity in the eluate was too low to be assessed.

3. Chromatography of the Principles on CM Sephadex

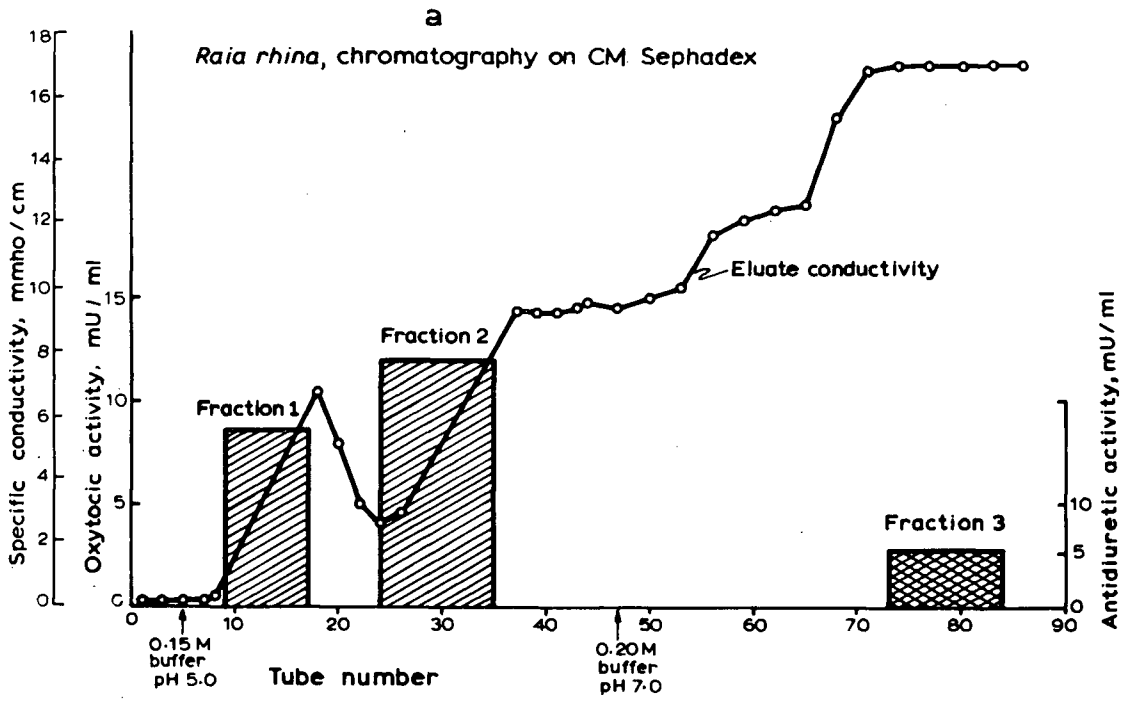
a. The passage through the column

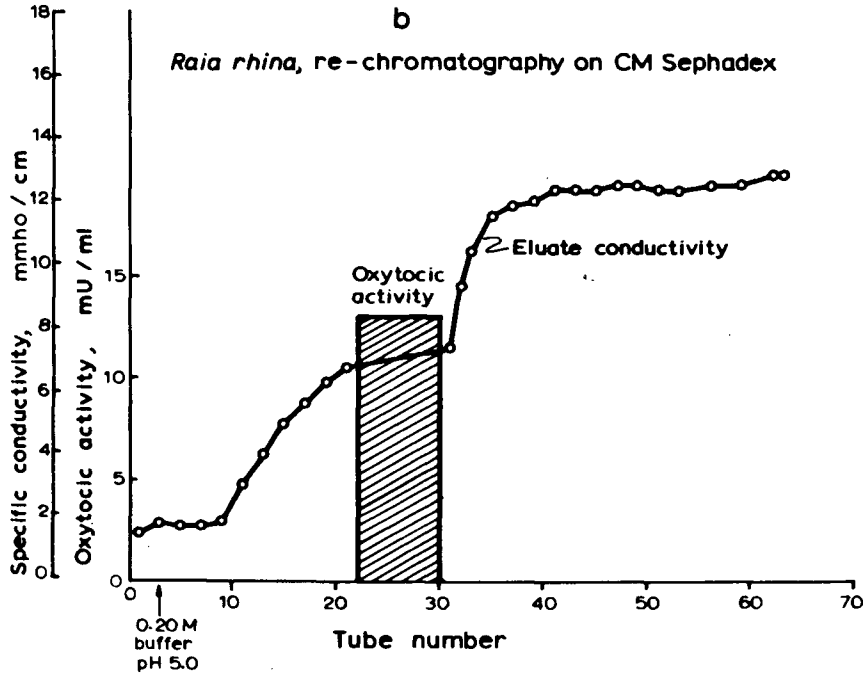
The eluate from the DEAE Sephadex was adjusted to pH 5.0 and specific conductivity 0.16 mmho/cm by dropwise addition of acetic acid and dilution with water to a final volume of 7300 ml. It was applied to a 35 cm x 0.9 cm column of CM Sephadex, Lot 8852, which had been equilibrated with 0.002 M NH_4acetate, pH 5.0. The neurohypophysial principles were adsorbed onto the exchanger. They were eluted with NH_4acetate buffer which increased stepwise in concentration from 0.002 M (pH 5.0) to 0.15 M (pH 5.0) and then to 0.20 M (pH 7.0).

Three fractions of biological activity were eluted from the column. The peaks of activity were not assayed throughout their length. This was in order to conserve the small amount of neurohypophysial principle in the eluates, and to avoid introducing contamination which might have been significant in relation to the amount of active peptide. The beginning and end of each fraction was located, and its total activity and position in the eluate is indicated in Figure 4a.

Figure 4a. Chromatogram of the partially purified neurointermediate lobe extract of Raia rhina on a 35 cm x 0.9 cm column of CM Sephadex. The activity (1,827 mU rat uterus activity, no Mg^{++} present) was applied in 7,300 ml of solution at pH 5.0 and specific conductivity 0.16 mmho/cm, and was eluted with NH_4acetate buffer of increasing concentration, from 0.002 M (pH 5.0): 0.15 M NH_4acetate (pH 5.0) was introduced stepwise at tube 5, and 0.20 M NH_4acetate (pH 7.0) was introduced stepwise at tube 47. Empty circles = specific conductivity of the eluate. Hatched columns = rat uterus activity, without Mg^{++}: fraction 1 = 356 mU; fraction 2 = 652 mU. Cross-hatched column = antidiuretic activity: fraction 3 = 209 mU. Volume of tubes 1-47 = 4.6 ml, of tubes 48-90 = 3.5 ml.

Figure 4b. Chromatogram of combined fraction 1 and fraction 2 of Raia rhina (purified on Sephadex G-15 and on CM Sephadex - see Fig. 4a, above) on a second 35 cm x 0.9 cm column of CM Sephadex. The activity was applied in 150 ml of solution at pH 5.0 and specific conductivity 1.6 mmho/cm, and was eluted with NH_4acetate buffer of increasing concentration, from 0.022 M (pH 5.0): 0.20 M NH_4acetate (pH 5.0) was introduced into the buffer reservoir at tube 3. Empty circles = specific conductivity of the eluate. Hatched column = oxytocic activity (rat uterus activity, without Mg^{++}); 439 mU. Tube volumes = 4.6 ml.


a
Raia rhina, chromatography on CM Sephadex
Specific conductivity, mmho / cm
Oxytocic activity, mU / ml
Antidiuretic activity, mU/ml
Eluate conductivity
Fraction 1
Fraction 2
Fraction 3
0·15 M buffer pH 5.0
0·20 M buffer pH 7·0
Tube number


b
Raia rhina, re-chromatography on CM Sephadex
Specific conductivity, mmho / cm
Oxytocic activity, mU / ml
Eluate conductivity
Oxytocic activity
0·20 M buffer pH 5.0
Tube number

Two of the fractions were eluted separately during the course of the wash with 0.15 M buffer (pH 5.0); this followed the behavior which had been found for the neutral peptide, oxytocin (Syntocinon), during preliminary experiments. The third fraction was eluted at pH 7.0 by 0.20 M NH_4acetate. Fraction 1, tubes 9-17 inclusive, contained 356 mU of rat uterus activity in a combined volume of 41.4 ml. Fraction 2, tubes 24-35 inclusive, contained 652 mU rat uterus activity in 55.2 ml. The third fraction, tubes 73-84, had an antidiuretic activity of 209 mU in 38 ml (Table XIV, page 134).

b. The basic fraction

The third fraction of activity was detected by its antidiuretic activity. It was eluted from the gel in the same position as the basic peptide from Squalus acanthias (Fig. 9, page 133; also see Sawyer, 1967). The Squalus principle was identified as arginine vasotocin. Arginine vasotocin occurs in other elasmobranch species, including skate (Acher et al., 1965; 1967; Chauvet et al., 1965; Sawyer, 1967), and has been found to be the basic peptide in many non-mammalian vertebrates (Table II, page 13). On this preliminary basis the third fraction from Raia rhina was tentatively regarded as arginine vasotocin.

c. Rechromatography of the combined neutral fraction on CM Sephadex

During earlier purifications of the neurohypophysial principles produced by the dogfish Squalus acanthias, it had been found that two neutral fractions were eluted from CM Sephadex, in the same positions in the eluate as fractions 1 and 2 found here for Raia rhina (Fig. 9, page 133). Careful comparison of the two Squalus fractions had indicated that their neurohypophysial peptide contents were identical, and that it was probable that the splitting of the neutral principle on the resin had resulted from the high volumes in which the samples had been applied to the columns (Section IIC7, page 141). The Raia rhina principles had also been loaded onto the resin in a high volume (7300 ml). Since pharmacological studies of the Raia extract had pointed to a single neutral peptide, glumitocin (Section B3, page 82), it seemed reasonable to suppose that fraction 1 and fraction 2 of this species also contained identical principles. Therefore, in order to conserve material for later amino acid analyses, they were combined.

Further, it was anticipated, on the basis of earlier similar experiments with elasmobranch material (Section IIC7g, page 151), that if the combined Raia rhina activity were applied to the CM Sephadex column in a reduced volume, then the "fraction 1" portion would be eluted in the position of "fraction 2", and contaminants which might be present in

"fraction 1" would be separated away. In order to obtain this reduced volume, and because all the activity was expected to be eluted at the higher conductivity of fraction 2, the solution to be applied to the column was not diluted back to a specific conductivity of 0.16 mmho/cm, as in the initial CM Sephadex procedure, but only to the specific conductivity of 1.6 mmho/cm (pH 5.0).

Because of these considerations, the neutral fractions were treated as follows: The two neutral fractions from the initial CM Sephadex purification were combined into a total volume of 96.6 ml, which contained a total of 1008 mU oxytocic activity (rat uterus, without magnesium). This solution was lyophilized for the removal of ammonium buffer salts, and the powder was taken up in 30 ml of weak buffer solution (0.002 M NH_4acetate, pH 5.0). This was then diluted to a specific conductivity of 1.6 mmho/cm, at pH 5.0, in a final volume of 150 ml, which represented only 2% of the volume applied in the earlier CM Sephadex run. The solution was applied to a 35 cm x 0.9 cm column of CM Sephadex, Lot 8852, which had been equilibrated with 0.022 M NH_4acetate (pH 5.0). The activity was adsorbed onto the gel, and was eluted on an NH_4acetate gradient from 0.022 M (pH 5.0) to 0.20 M (pH 5.0). The oxytocic activity appeared in a single fraction between specific conductivities of 6.8 mmho/cm and 7.4 mmho/cm (tubes 22-30 inclusive, Fig. 4b); this justified the decision

to combine the two oxytocic fractions, and again suggested that they contained the same principle. All the tubes which contained activity were combined. The total activity was 439 mU, in a volume of 33.5 ml.

4. Amino Acid Analysis of the Oxytocic Principle

The oxytocic principle of another skate, Raia ocellata, had been purified by a single elution from CM Sephadex (Perks and Sawyer, 1965), and it was anticipated that the neutral principle from R. rhina, which had been submitted twice to chromatography on this same exchanger, would also be pure at this stage. Therefore the solution containing the oxytocic principle was lyophilized for removal of buffer salts, and the purified peptide was hydrolyzed in 6 N HCl at 107°C for 18 hours. The amino acid content of the hydrolysate was determined on a Biocal BC-200 Amino Acid Analyzer. Three analyses were made. The results are given in Table IX.

It can be seen that the purification of the neutral principle was not complete. Nevertheless, a cautious interpretation of the data was possible. The basic amino acids - histamine, lysine, and arginine - were present in the hydrolysate in considerable proportion. However, the behavior of the peptide on CM Sephadex (Fig. 4, a and b), and the low ratio of antidiuretic to oxytocic activity of the crude extract (R_{ADH} = 0.02; Section B1, page 70) had indicated

TABLE IX

Amino Acid Analyses of the Purified Neutral Principle from Raia rhina

Amino Acid	Molar ratio to glutamic acid				
	Raia rhina principle				Glumitocin
	Sample 1[a]	Sample 2	Sample 3	Average values	
Neutral and acidic amino acids					
Cysteic acid	0.47	0.43	0.67	0.52	2
Aspartic acid	1.23	1.19	1.09	1.17	1
Serine	0.76	0.62	1.76	0.71	1
Glutamic acid	1.00	1.00	1.00	1.00	1
Proline	1.03	1.02	1.16	1.07	1
Glycine	1.84	1.76	1.90	1.83	1
Isoleucine	0.58	0.54	0.62	0.58	1
Tyrosine	0.76	0.85	1.38	1.00	1
Threonine	0.32	0.24	0.32	0.29	0
Alanine	0.25	0.35	0.33	0.31	0
Valine	0.39	0.38	0.41	0.39	0
Methionine	0.88	0.85	0.95	0.89	0
Leucine	0.34	0.29	0.38	0.34	0
Phenylalanine	0.13	0.34	0.53	0.33	0
Basic amino acids					
Histamine	1.65	0.85	2.19	1.56	0
Lysine	+ve[b]	+ve	+ve	+ve	0
Arginine	0.43	0.39	4.17	1.66	0
Ammonia	+ve	+ve	+ve	+ve	4
Total amino acids	Micromoles per unit oxytocic activity				
Neutral and acidic amino acids	2.356	2.566	2.260	2.394	–

[a] All samples were from one hydrolysate of CM Sephadex-purified material. Basic, neutral and acidic amino acids were determined from each sample. Sample 1=88 mU oxytocic activity (no Mg^{++} present). Sample 2=88 mU. Sample 3=264 mU.

[b] +ve: present in hydrolysate; molar ratio not calculated.

that basic residues did not form part of its molecule; a similar situation has been reported from the closely parallel study of the neutral principle of the skate, Raia ocellata, and the basic residues were shown to be extrinsic to the active molecule (Perks and Sawyer, 1965). It had been found during previous purifications of the Squalus neutral fraction that some basic amino acids were still present in the preparation after its elution from CM Sephadex, but these could be successfully removed by chromatography on SE Sephadex (Section IIC5, 6; pages 130, 137: Table XV; page 136). Unfortunately, there was insufficient Raia rhina material for this further step to be attempted, because the probable loss of material would have eliminated any possible amino acid analysis.

Of the neutral and acidic amino acids present in the hydrolysate, the amino acids which constitute the glumitocin molecule predominated over the others, with the exception of methionine. Cysteic acid, the oxidation product of the cysteine residue which occurs in glumitocin in twice the proportion of the other amino acid constituents, was in low proportion. However, cysteine is known to break down during hydrolysis (Chauvet et al., 1963); analogy with all other neurohypophysial principles would suggest the presence of two cysteine residues, and such an assumption is justified by the pharmacological properties of the skate material. The amount of methionine in the hydrolysate was sufficiently high

(molar ratio to glutamic acid = 0.89) for it to be considered part of the peptide. However, methionine had also been present as a contaminant in the Squalus preparation after its elution from CM Sephadex (Table XV); despite its presence, it could be shown that it could be removed from the principle by the use of SE Sephadex. Therefore, it is considered probable that the methionine found here was not part of the peptide, and that chromatography on SE Sephadex might have separated it from the R. rhina principle, if sufficient material had been available to include this extra step. The exclusion of methionine from the active peptide is further supported by the pharmacological evidence presented earlier.

The specific activity of the peptide was calculated from the rat uterus activity (no Mg^{++} present) of the hydrolyzed sample and from the number of micromoles of glutamic acid it contained; the value was found to be 4.6 IU/mg. This provides additional support for the suggestion that the peptide might be glumitocin. Glumitocin has a much lower specific activity than the other known neutral principles - 8 IU/mg (Table VIII, page 88; also Acher et al., 1965), as compared to 450 IU/mg for oxytocin, 290 IU/mg for mesotocin (Sawyer, 1965) and 125-150 IU/mg for isotocin (Sawyer, 1965; Wilson and Smith, 1969). The activity of the R. rhina principle was in the same low range as the activity of glumitocin.

D. Summary of the Studies on the Neurohypophysial Principles of Raia rhina

1. The potencies of neurointermediate lobe extracts of Raia rhina were found to vary upon repeated rat uterus assays, both in the presence and the absence of magnesium ions, and on the milk-ejection assay. Exact values appeared to depend on the particular assay preparation used, as well as on the concentration and nature of the peptide. This indicated the importance of assaying an unknown against a standard of the same molecular structure whenever it is possible. Nevertheless, despite the caution necessary in interpreting assay values, no alternative exists when dealing with a totally unknown peptide. The existence of a variability in assay values obtained for an unknown does not detract from the validity of this evidence as an indication that there is a difference between the unknown and the standard; further, repetitive agreement in assay values between two peptides increases the possibility that the two are the same, although it does not prove that this is the case.

2. Two principles, one neutral and one basic, were separated from the Raia rhina extract:

a. The basic principle was not precisely characterized; however, analogy with other elasmobranchs, including skate, and the similarity of the chromatographic behavior of this antidiuretic material with that of the arginine vasotocin identified in Squalus, suggested that it

is also arginine vasotocin.

b. The following evidence suggested that the neutral principle was glumitocin (4-ser, 8-glu (NH_2) oxytocin);

i. The results of the pharmacological comparison between the crude extract and synthetic glumitocin (Table VI, page 83) were consistent with the presence of a mixture of glumitocin with arginine vasotocin, or with any of the known naturally occurring antidiuretic principles; with the provision that the proportions in which the principles occurred in the extract were the same as those found when the neutral (oxytocic) and basic (antidiuretic) fractions were separated on a CM Sephadex column.

ii. The amino acid analyses of the neutral principle (Table IX) revealed the amino acids of glumitocin, plus methionine. The basic amino acids - histamine, lysine, and arginine - were also present but were considered to be extrinsic to the active molecule. Experiments with the principles of Squalus acanthias suggested that the methionine found here was a contaminant, and not an intrinsic part of the peptide; this was supported by the pharmacological data, since a substitution or addition of methionine would be expected to disturb the agreement between the biological activities of the extract and those of synthetic glumitocin.

iii. The specific activity of the neutral principle was low, and comparable to that of glumitocin.

II. STUDIES OF THE NEUROHYPOPHYSIAL PRINCIPLES OF THE DOGFISH, SQUALUS ACANTHIAS, (PACIFIC VARIETY)

A. Introduction

This species had already been studied by other workers, but with conflicting results. Heller and Pickering (1961) had resolved a mixture of the two oxytocic principles, E_1 and E_2, by paper chromatography of pituitary extracts of the Atlantic variety of this species. In contrast, Perks (1966) had been unable to distinguish more than one active fraction, EOP I, when using the same method, and Sawyer (1965) had resolved only one oxytocin-like fraction by ion exchange chromatography of neurohypophysial material from the Pacific variety of this species.

An attempt was made to explain this conflict and to elaborate on the nature of the principle or principles produced by this species. This was done by pharmacological and chromatographic studies of crude extracts, and by the preparation of purified principles which could then be subjected to more rigorous pharmacological, chromatographic and chemical examination. The synthesis of glumitocin, a known elasmobranch principle, during the course of this study (Manning et al., 1968) permitted the direct pharmacological comparison of the Squalus principle against a peptide of possibly identical structure.

The dogfish was of additional interest because it provided a contrast with the skate, Raia rhina, the other species studied here. Squalus acanthias is a shark, and it

has a discrete neurohypophysial structure (Meurling, 1962) as opposed to the diffuse type found among the species of Raia (Meurling, 1967a). An attempt was made to determine whether these differences between the two species would be paralleled by differences in their neurohypophysial peptides.

B. Pharmacological and Chromatographic Studies of the Neurointermediate Lobe Extracts

1. Biological Activities and Activity Ratios of the Crude Extracts

Several crude extracts were assayed against synthetic oxytocin (Syntocinon, Sandoz Pharmaceuticals) for rat uterus activity, both in the presence and absence of magnesium ions, and for milk-ejection activity. They were assayed against vasopressin (Pitressin, Parke Davis) for antidiuretic activity. The ratios of their activities were compared to those of other elasmobranchs. The results, shown in Table X, were comparable to those found for this species by other workers (Sawyer et al., 1961; Perks and Dodd, 1963a; Sawyer, 1965; 1967; Swiatkiewicz, 1968). The levels of oxytocic activity ranged from 11.6 to 32.2 mU/mg dried tissue. These were higher than the levels found for Raia rhina, the other species studied here, but were comparable to the levels of activity reported for several other elasmobranch species (Perks and Dodd, 1963a).

The activity ratios were also similar to those of other elasmobranchs (Perks and Dodd, 1963a). The ratio of antidiuretic to rat uterus activity was typically low (R_{ADH} = 0.03 or 0.04) and suggested that an antidiuretic-

Table X

The Activities and Activity Ratios of Crude Neurointermediate Lobe Extracts of *Squalus acanthias*

Sample number[a]	Activities[b] (mU/mg dried tissue)				Activity ratios		
	RU[c]	ME	ADH	RU+Mg	R_{ME}	R_{ADH}	R_{Mg}
	Values found in this study						
27-39 (incl) (998 NIL)	25.0 ± 2.6	55.1 ± 13.0	0.70 ± 0.29	51.4 ± 10.1	2.2 ± 0.5	0.03 ± 0.01	2.1 ± 0.5
25 (21 NIL)	12.3 ± 0.7	-	0.48 ± 0.15	55.2 ± 16.8	-	0.04 ± 0.01	4.5 ± 1.4
26 (26 NIL)	11.6 ± 1.3	-	0.45 ± 0.22	34.2 ± 13.4	-	0.04 ± 0.02	2.9 ± 1.1
36 (5 NIL)	32.2 ± 5.0	-	-	63.8 ± 12.0	-	-	2.0 ± 0.5[d]

Table X continued

Values found by other workers					
Location	Rat uterus activity (mU/mg dried tissue)	Activity ratios			Reference
		R_{ME}	R_{ADH}	R_{Mg}	
British Columbia	-			1.2-3.2	Swiatkiewicz, 1968
Washington State	-	1.6[e]	0.02[e]	1.6±0.1	Sawyer, 1965
Maine	30 ±4	1.9[e]	0.03[e]	1.3[e]	Sawyer et al., 1961
Virginia	-	-	0.06	2.6±0.1	Sawyer, 1967
Scotland	57.0±3.8	-	0.09	-	Perks and Dodd,1963a
Scotland	17	2.6	0.07	-	Perks and Dodd,1963a
Scotland	35 ±3.5	1.8[e]	0.02[e]	2.0[e]	Sawyer et al., 1961

[a]As in Appendix I.

[b]Assays calculated by the 4-point method of Holton (1948); whenever three or more groups were obtained the fiducial limits are expressed, P = 0.05.

[c]Abbreviations used: RU = activity on rat uterus, in absence of magnesium. ME = activity on rabbit milk-ejection assay. ADH = activity on rat antidiuretic assay. RU+Mg = activity on rat uterus, in presence of 0.5 mM Mg^{++}. Activity ratios, R_{ME}, R_{ADH}, and R_{Mg}, are the ratios of the activity found on each assay (ME, ADH, and RU+Mg) to the activity found on the rat uterus assay, without magnesium (RU).

[d]Also listed in Table XII.

[e]Calculated from the published data.

pressor principle was either absent or present in very small amounts. The ratio of milk-ejection to rat uterus activity (R_{ME} = 2.2 ± 0.5) characterized the oxytocic fraction as EOP I, according to the definition of Sawyer (1964). This principle has been reported in seven other species (Perks and Dodd, 1963a; Perks, 1966) and is distinguished from EOP II by possessing a lower value for R_{ME}. Values have not been reported for the magnesium potentiation of the EOP I produced by these other species, but the values found here for Squalus acanthias (R_{Mg} = 2.0 to 4.5) are comparable to those found previously for the same species. This potentiation does not distinguish the Squalus principle from either E_2 (R_{Mg} = 2) or from EOP II (R_{Mg} = 2.6) but it does differentiate it from the E_1 principle found in certain species (R_{Mg} = 6) and from glumitocin (R_{Mg} = 10). However, in view of the observations made on the variability of magnesium potentiations (Section IB2, page 71) this characteristic must be viewed with considerable scepticism.

2. Studies of Acetone-Extractable Biological Activities

A possible reason why the two neutral principles, E_1 and E_2, which were found in this species by Heller and Pickering (1961), could not be distinguished by either Sawyer (1965) or Perks (1966) might have been the loss of one of the peptides into the acetone in which the glands were collected. The preferential extraction of one peptide (oxytocin) over

another (vasopressin) had been observed by Heller and Lederis (1959) when the posterior pituitary glands from infant rats were collected in acetone.

Therefore the acetone into which the glands of samples 35 and 36 (Appendix I) were dissected, and the two changes of acetone in which they stood (at approximately 12°C) for the first hour after dissection were combined for each sample. A total of 250 ml of acetone extract was obtained from each set of glands. The acetone in which the glands were stored (at approximately 12°C) between 72 and 144 hours after dissection was also kept. The four samples were flash evaporated to the point of dryness. The residues were taken up in 0.25% acetic acid solution and assayed on the rat uterus for oxytocic activity. The results, listed in Table XI, show that the acetone in which the glands stood for the first hour after dissection contained activity, 488 ± 31 mU rat uterus activity (without Mg^{++}) from sample 35 and 706 ± 102 mU rat uterus activity (without Mg^{++}) from sample 36, but that no activity was lost from the glands between 72 and 144 hours after dissection.

The acetone-extractable activity was inactivated by incubation with sodium thioglycollate. This indicated that it was in fact due to the presence of neurohypophysial hormone rather than to some other oxytocic factor which might have been leached from the glands. At least 80% and 85% of the

Table XI

The Oxytocic Activity Extracted from Two Samples of Squalus acanthias Neurointermediate Lobes by the Acetone Used for Collection

Sample number[a]	Number of glands	Storage in acetone		Rat uterus activity in the acetone		Inactivation by sodium thioglycollate
		Hours after dissection	Volume of acetone (ml)	Total actvity (mU)	% of total activity in glands	
35	50	0-1	250	488 ± 31	-	>80%
		72-144	50	none	-	-
36	79	0-1	250	706 ± 102	3.7	>85%
		72-144	36	none	-	-

[a] As in Appendix I.

activity lost from samples 35 and 36, respectively, was destroyed upon 4 1/2 and 1 1/2 hours exposure to sodium thioglycollate (Table XI).

In sample 36, it was possible to show that the activity recovered from the acetone accounted for only a small proportion of that present in the glands - only about 4% of the total activity in the sample. Five glands from this sample were extracted in 0.25% acetic acid and found to have a rat uterus activity of 30.1 ± 2.9 mU/mg dried tissue. This was extrapolated to an activity of 18,476 mU for the total sample of 613.2 mg dried tissue (Appendix I). The 706 mU recovered from the acetone accounted for 3.7% of the total (Table XI).

The acetic acid extract of sample 36 and the activity recovered from its "first hour" acetone were compared for magnesium potentiation and for chromatographic behavior. These two criteria distinguish principle E_1 from E_2. The two unknowns were assayed side by side on the same rat uteri, both with and without magnesium ions present. Their resulting magnesium potentiations were similar, 2.0 ± 0.5 for the standard extract of the glands, and 2.1 ± 0.5 for the acetone soluble residue (Table XII); neither approximated to the high values reported for principle E_1 in some species (Heller and Pickering, 1961; Heller and Roy, 1965a; 1965b; Roy, 1969). The extract and the acetone soluble residue were applied side

Table XII

The Magnesium Potentiation and Chromatographic Behavior of a Crude Extract of Squalus acanthias Neurointermediate Lobes, and of the Activity Recovered from the Acetone in which the Glands were Collected

Source of activity	Magnesium potentiation (R_{Mg})	Chromatographic behavior[a] (R_F)
Acetic acid extract[b]	2.0 ± 0.5[c]	0.4 - 0.7
Acetone soluble residue[d]	2.1 ± 0.5	0.4 - 0.8

[a]Solvent = n-butanol:acetic acid:water, 4:1:5.

[b]Crude extract of five glands from Sample 36 (Appendix I).

[c]Also listed in Table X.

[d]Residue recovered from the 250 ml of acetone into which the glands of Sample 36 were dissected and stored for one hour.

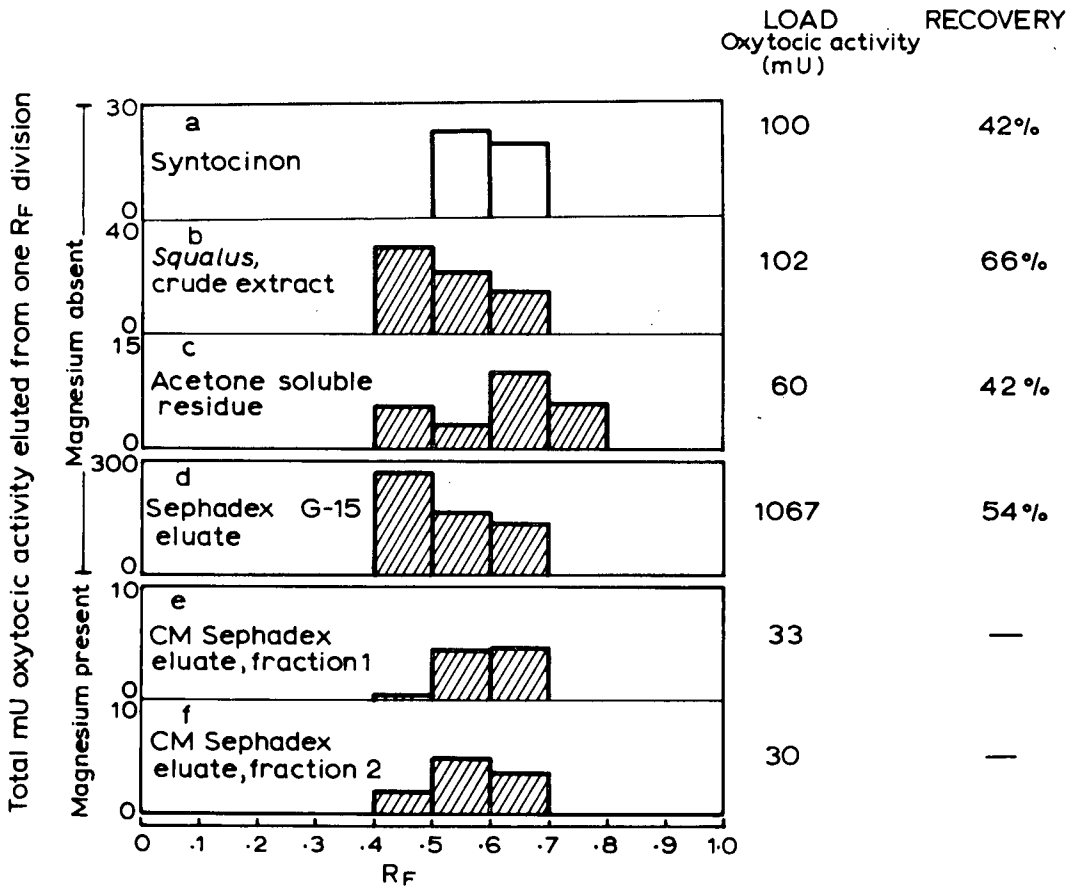
by side to a paper chromatogram which was then developed in n-butanol:acetic acid:water = 4:1:5 - the system used by Heller and Pickering (1961) to separate E_1 from E_2. Oxytocic activity was found in the eluates of R_F 0.4-0.7 in the case of the extract, and R_F 0.4-0.8 for the acetone soluble residue (Table XII; Fig. 5, b and c). No activity was found for either the extract or the residue in the eluates of R_F 0-0.4, where E_1 would be expected to run. Nor was there evidence for the incomplete separation of E_1 from E_2. The potentiation of the tailing edge of the oxytocic peak, R_F 0.4-0.5, was 2.3 for the extract and 1.8 for the acetone soluble residue. These values were similar in each case to the overall potentiation of 2.0 for the total active eluate of the extract and 2.4 for the total active eluate of the acetone soluble residue.

Therefore, although approximately 4% of the activity of the glands was lost into the collecting acetone, this activity appeared similar to the principle extracted later by acetic acid; and there was no evidence for the presence of the slow running, and sometimes highly potentiated, principle E_1 in either fraction.

C. The Purification of the Extract

An attempt was made to purify the neurohypophysial principles produced by this species so that they could be subjected to more rigorous pharmacological and chromatographic examination, and to chemical analysis. The general

Figure 5. Descending paper chromatograms of Squalus acanthias neurointermediate lobe extract, at various stages of purification, on Whatman 3MM paper, in n-butanol:acetic acid: water = 4:1:5, at room temperature. Samples used; a: synthetic oxytocin (Syntocinon, Sandoz), b: crude extract of five glands from Sample 36 (see Appendix I), c: residue recovered from the acetone into which the glands of Sample 36 were dissected and stored for one hour, d: extract after partial purification on Sephadex G-15 (Preparation 2), e: oxytocic fraction 1 (Preparation 2), eluted from CM Sephadex, f: oxytocic fraction 2 (Preparation 2), eluted from CM Sephadex. Loads are stated as mU oxytocic activity = mU rat uterus activity in the absence of Mg^{++}.


LOAD
Oxytocic activity (mU)
RECOVERY
Total mU oxytocic activity eluted from one RF division
Magnesium absent
Magnesium present
a
Syntocinon
100
42%
b
Squalus, crude extract
102
66%
c
Acetone soluble residue
60
42%
d
Sephadex G-15 eluate
1067
54%
e
CM Sephadex eluate, fraction 1
33
—
f
CM Sephadex eluate, fraction 2
30
—
30
0
40
0
15
0
300
0
10
0
10
0
0 .1 .2 .3 .4 .5 .6 .7 .8 .9 1.0
RF

purification scheme, which is outlined in Figure 6a, included gel filtration of the extract, followed by three ion exchange chromatography procedures, and a final purification by paper chromatography. The procedures were complicated by the need to combine fractions from different purifications in order to conserve enough material to allow for amino acid analysis. This is shown in the more detailed outline of the purification scheme presented in Figure 6b. At various stages in their purification, as indicated in the two figures, the principles were subjected to pharmacological or chromatographic studies, or to hydrolysis and amino acid analysis.

1. Extraction of the Glands

With the exception of the five glands which were used in the previous experiment (Section B2, page 108) the glands of samples 27-39 (Appendix I) were taken for purification. The 998 acetone-dried glands, weighing 6500 mg, were extracted in 0.25% acetic acid at 40 mg dried tissue/ml. The rat uterus activity (without Mg^{++}) of the crude extract was 998 ± 105 mU/ml, or 25.0 ± 2.6 mU/mg dried tissue (Table X). It's Lowry peptide content was 7.95 mg/ml and the ratio of oxytocic activity to Lowry peptide was 125.5 mU/mg. The extract was stored frozen until required. No loss of activity was incurred, even after storage of up to 15 months.

2. Preliminary Purification on Sephadex G-15

A. The extent of purification

The active peptides were separated away from the larger

Figure 6a. General outline of the procedures followed during the purification of the active principles from the neurointermediate lobe (NIL) of Squalus acanthias (Pacific variety).

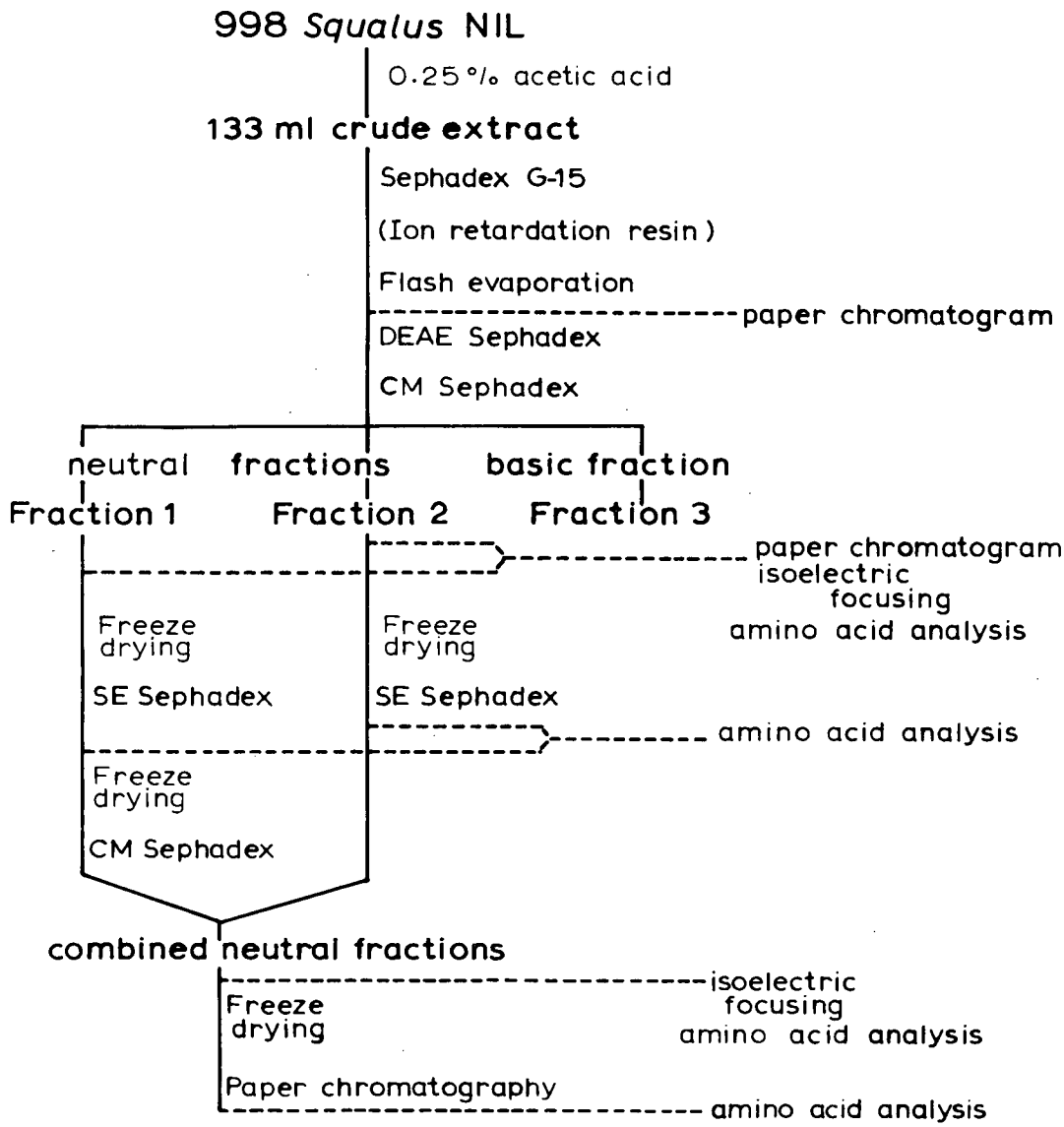


Figure 6a. General outline of the procedures followed during the purification of the active principles from the neurointermediate lobe (NIL) of *Squalus acanthias* (Pacific variety).

Figure 6b. Detailed outline of the procedures followed during the purification of the active principles from the neurointermediate lobe (NIL) of Squalus acanthias (Pacific variety).

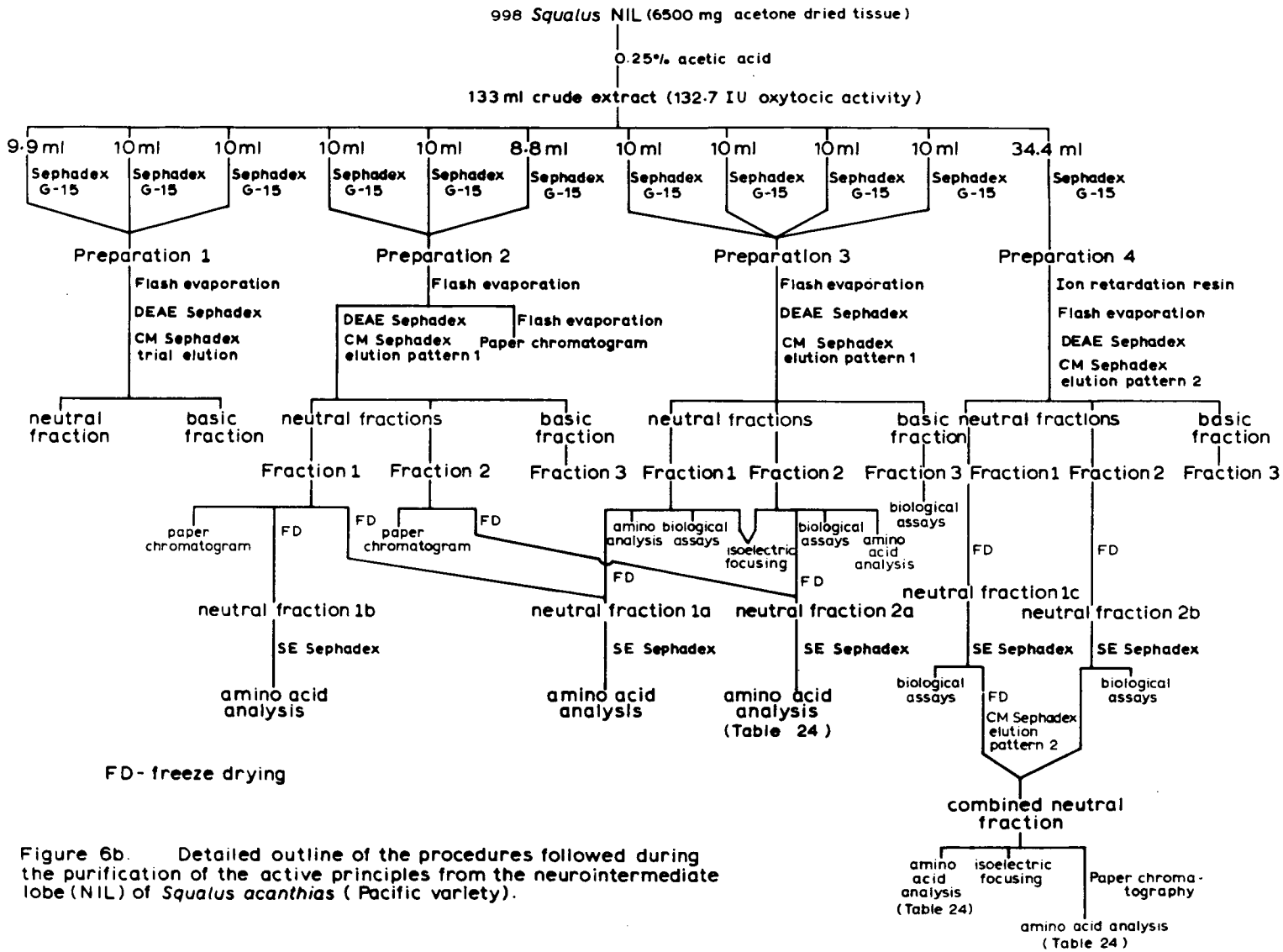


Figure 6b. Detailed outline of the procedures followed during the purification of the active principles from the neurointermediate lobe (NIL) of *Squalus acanthias* (Pacific variety).

protein molecules in the extract by passage of the extract through a 90 cm x 2.4 cm column of Sephadex G-15. This was the first time a Sephadex gel with such a low exclusion limit (molecular weight of approximately 1500) had been used in the purification of elasmobranch principles. The higher degree of purification afforded by this gel was especially advantageous to elasmobranch extracts, which have such a low level of oxytocic activity (Perks and Dodd, 1963a; Perks, 1966).

The extract was passed through the column in ten portions of 10 ml each. Figure 7 shows the separation of the active principles from the larger proteins in the eluate. The results of each run are summarized in Table XIII. An average of 92% of the Lowry peptide was separated away from the oxytocic principles, and the ratio of oxytocic activity to Lowry peptide was increased approximately 13-fold. The extract was diluted 9-fold by this procedure. In an attempt to decrease the dilution, the remaining 34.4 ml of extract was applied to the column in one lot. The separation of the active peptides from the larger protein molecules was not as complete as when the samples were applied in 10 ml amounts, but 86% of the Lowry peptide was removed and a 6.2-fold purification was achieved. The reduction in purification was offset by a 50% reduction in the dilution of the principles.

Figure 7. Chromatogram of 10 ml of crude Squalus acanthias neurointermediate lobe extract on a 90 cm x 2.4 cm column of Sephadex G-15. Empty triangles = Lowry peptide content of the eluate. Empty circles = oxytocic activity (rat uterus activity, without Mg^{++}) of the eluate.

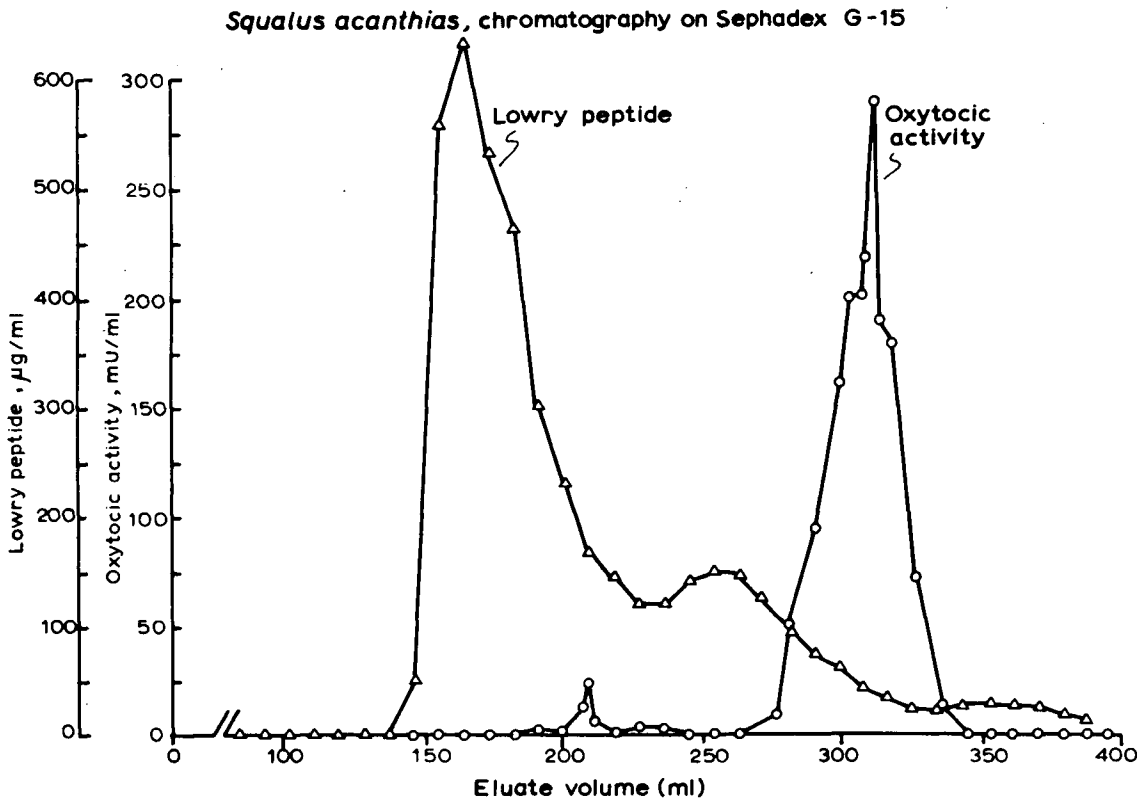

Squalus acanthias, chromatography on Sephadex G-15
Lowry peptide
Oxytocic activity
Lowry peptide, µg/ml
Oxytocic activity, mU/ml
Eluate volume (ml)
0
100
150
200
250
300
350
400
50
600
500
400
300
200
100
0
300
250
200
150
100
50
0

Table XIII

The Filtration of Squalus acanthias and Raia rhina Neurointermediate Lobe Extract Through Sephadex G-15

Experiment number	Sample volume (ml)	Position of minor oxytocic peak in the eluate (cumulative volume, ml)	Position of major oxytocic peak in the eluate (cumulative volume, ml)	Volume of the major peak (ml)	Dilution of activity in major peak	"Preparation" number assigned to the combined major peaks	Specific activity - ratio of oxytocic acitivty to Lowry peptide (mU/mg)	% Lowry peptide removed from crude extract	% Recovery of oxytocic activity	Degree of purification
Crude extract of Squalus acanthias										
1	9.9	195-210	249-339	90	9.1x					
2	10	192-215	252-344	92	9.2	1	1,954	94	100	15.6x
3	10	a	261-351	90	9.0					
4	10	a	255-345	90	9.0					
5	10	a	253-337	84	8.4	2	1,218	92	—	—
6	8.8	a	261-354	93	10.6					
7	10	190-212	271-354	83	8.3					
8	10	162-173	225-308	83	8.3	3	1,397	91	100	11.1x
9	10	174-210	244-332	88	8.8					
10	10	173-201	253-345	92	9.2					
11	34.4	a	198-343	145	4.2	4	783	86	91	6.2x
Crude extract of Raia rhina										
1	30	a	201-340	139	4.6	—	27	77	82	3.8x
2	35	a	195-340	145	4.1					

[a]There was no attempt to locate the minor peak.

This was an advantage in view of the already low level of oxytocic activity in the extract.

The major oxytocic fractions (see Table XIII) eluted from runs 1-3 were combined as "Preparation 1", and from runs 4-6 and 7-10 as "Preparation 2" and "Preparation 3", respectively. The active eluate from the eleventh run became "Preparation 4". Each was processed separately.

b. The minor oxytocic peak

A small amount of oxytocic activity (1.4% of the total) was eluted from the column along with the Lowry peptide and in advance of the main oxytocic peak (Fig. 7, Table XIII). This might have represented som oxytocic agent other than a neurohypophysial principle. However when two samples of this preliminary activity were incubated for several hours with sodium thioglycollate, they were completely inactivated. Therefore this minor peak probably represented neurohypophysial peptide which may still have been in association with the carrier protein. Such a phenomenon has been described for the passage of pituitary extracts of the cobra, Naja naja, through Sephadex G-25 (Pickering, 1967).

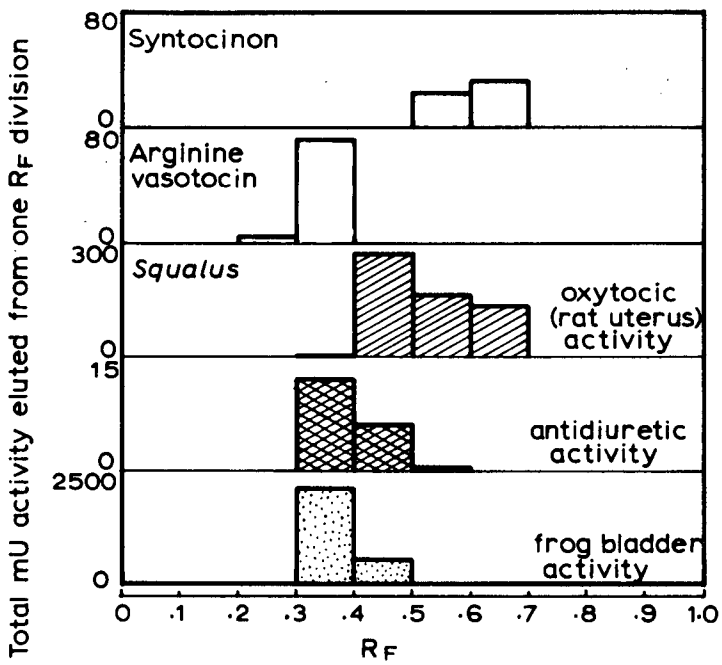
c. Paper chromatography of the Sephadex G-15 eluate

Trace amounts of a basic principle, which was pharmacologically similar to arginine vasotocin, had been separated away from the main neutral fraction of Squalus acanthias by ion exchange chromatography (Sawyer, 1965). However it had

not been detected in paper chromatograms of crude extracts of the same species (Perks, 1966), possibly because the danger of overloading the paper had limited the amount of extract which was applied.

An attempt was made here to demonstrate the basic peptide by paper chromatography, this time using partially purified extract so that higher loads of activity could be applied. A 3.4 ml sample of the Sephadex G-15 eluate (Preparation 2) containing 1067 mU oxytocic activity was reduced by flash evaporation to a volume of 0.3 ml, with no loss of activity. It was applied to a paper chromatogram beside an arginine vasotocin standard. After the chromatogram had been developed in n-butanol:acetic acid:water = 4:1:5, the synthetic arginine vasotocin was located by rat uterus assay in the eluate of R_F 0.3-0.4. A total of 577 mU of oxytocic activity was recovered from the chromatogram of the Squalus extract, mainly in the eluates of R_F 0.4-0.7 (Fig. 8). However a trace amount of oxytocic activity (4.5 mU) occurred in the eluate of R_F 0.3-0.4. This eluate also contained most of the antidiuretic and frog bladder activity recovered from the paper (12 mU and 2136 mU, respectively) and its activity ratios, R_{ADH} = 2.7 and R_{FB} = 475, were comparable to the values of R_{ADH} = 2.0 and R_{FB} = 271-910 reported for arginine vasotocin (Sawyer et al., 1961; Sawyer, 1965). Such a high ratio of frog bladder to rat uterus activity is a peculiar characteristic of arginine vasotocin; the only other oxytocin analogue known to have an R_{FB} at all

Figure 8. Descending paper chromatogram of Squalus acanthias neurointermediate lobe extract (Preparation 2, after partial purification on Sephadex G-15) on Whatman 3MM paper, in n-butanol:acetic acid:water = 4:1:5, at room temperature. The three diagrams for the Squalus extract show the distribution of the three activities: rat uterus activity, without Mg^{++} (also shown in Figure 5d); rat antidiuretic activity; and frog bladder activity. Loads are stated as mU oxytocic activity = mU rat uterus activity in the absence of Mg^{++}.



	LOAD Oxytocic activity (mU)	RECOVERY
Syntocinon	200	29%
Arginine vasotocin	230	32%
Squalus oxytocic (rat uterus) activity	1067	54%

comparable to that of arginine vasotocin is 8-lysine oxytocin (R_{FB} = 190; Sawyer, 1965). However the 8-lysine peptide differs from arginine vasotocin in having a low R_{ADH} of 0.31, rather than a value of R_{ADH} = 2.

3. Passage of the Extract through Ion Retardation Resin

The salt content of the extracts necessitated a high dilution before the principles could be adsorbed onto the subsequent cation exchangers. Therefore in the purification of Preparation 4 a procedure was introduced for the removal of salt from the solution. The active eluate from the Sephadex G-15 column was passed through a 40 cm x 0.9 cm column of Biorad AG11A8 Ion Retardation Resin. The specific conductivity of the solution, which also contained the 0.2 M acetic acid eluant of the Sephadex G-15 (specific conductivity 0.71 mmho/cm) was reduced from 1.23 mmho/cm to 0.94 mmho/cm by this procedure. No loss of oxytocic activity was detected.

4. Passage of the Extract through DEAE Sephadex

Although the neurohypophysial peptides are not themselves adsorbed onto DEAE Sephadex, other proteins or peptides in the sample do become attached to the gel, and so are separated away from the active principles. This step had been found necessary for the subsequent adsorption of the oxytocic principle from the skate, Raia ocellata, onto the cation exchanger, CM Sephadex (Perks and Sawyer, 1965), and so it was adopted here.

Each preparation was adjusted to pH 7.0 and specific conductivity 0.21 mmho/cm by dropwise addition of NH_4OH and dilution with water. Each was passed through a separate 35 cm x 0.9 cm column of DEAE Sephadex which had been equilibrated with 0.002 M NH_4acetate (pH 7.0).

There was no loss of oxytocic activity from the samples during their passage through this exchanger. The Lowry peptide content of the eluate could be detected only in the case of Preparation 2, where it was found that 19% of the Lowry peptide in the sample had been removed by this procedure. The ratio of oxytocic activity to Lowry peptide content had increased from 1,218 mU/mg to 1,758 mU/mg, a 1.4 fold purification of the active principles.

5. Chromatography on CM Sephadex

a. The passage through the column

The eluate from each DEAE Sephadex column was adjusted to pH 5.0 and specific conductivity 0.16 mmho/cm by dropwise addition of acetic acid and dilution with water. Each preparation was applied to a separate 35 cm x 0.9 cm column of CM Sephadex, which had been equilibrated with 0.002 M NH_4-acetate (pH 5.0).

The neurohypophysial peptides were adsorbed onto this exchanger, but could be eluted off on an NH_4 acetate gradient of increasing concentration. Preparation 1 was used to establish the concentrations of buffer which were necessary to elute

the principles. The eluate containing the neutral fraction was not used further in the present experiments. It was lyophilized and sent to Dr. W. Sawyer, Columbia University, for further study. His findings are considered in the Discussion, Chapter 4.

The elution patterns from the other three columns (Preparations 2, 3, and 4) were all similar (for example, Fig. 9). Two neutral peaks appeared on the gradient between 0.002 M NH_4 acetate (pH 5.0) and either 0.05 M (pH 5.0), in the case of Preparations 2 and 3 (CM Sephadex Lot T06071), or 0.10 M (pH 5.0) in the case of Preparation 4 (CM Sephadex Lot 8852). A third, basic, peak was eluted by 0.20 M NH_4-acetate (pH 7.0). Table XIV shows the amounts of activity in each peak.

b. The chemical analysis of the two neutral peaks

The appearance of two neutral fractions at different positions on the eluting gradient raised the possibility of the presence of two neutral principles. The properties of the two fractions were compared carefully, and the results will be considered in Section C7, page 141.

A pure preparation of oxytocic principle had been obtained from the skate Raia ocellata by the above procedures (Perks and Sawyer, 1965) and it was anticipated that the neutral principle(s) from Squalus might also be pure at this stage. Therefore, a sample of each of the two fractions was

Figure 9. Chromatogram of partially purified neurointermediate lobe extract of Squalus acanthias (Preparation 3; Sephadex G-15 purified) on a 35 cm x 0.9 cm column of CM Sephadex. The activity (22, 701 mU rat uterus activity, without Mg^{++}) was applied in 4,364 ml of solution at pH 5.0 and specific conductivity 0.16 mmho/cm, and was eluted with NH_4acetate buffer of increasing concentration, from 0.002 M (pH 5.0): 0.05 M NH_4acetate (pH 5.0) was introduced into the buffer reservoir at tube 2, and 0.20 M NH_4acetate (pH 7.0) was introduced at tube 153. Solid circles = specific conductivity of the eluate. Empty circles = rat uterus activity, without Mg^{++}: fraction 1 = 9,392 mU; fraction 2 = 7,036 mU; fraction 3 = 446 mU. Tube volumes = 3.4 ml.

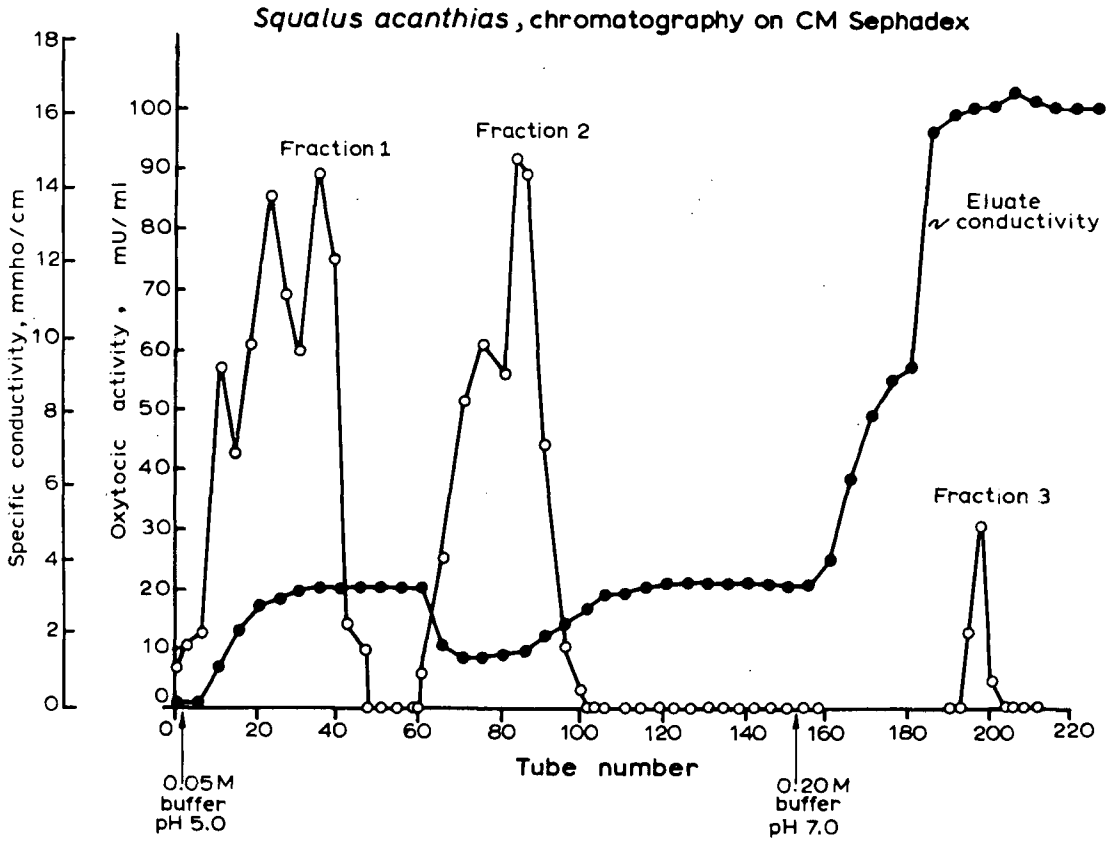

Squalus acanthias, chromatography on CM Sephadex
Specific conductivity, mmho/cm
Oxytocic activity, mU/ml
Fraction 1
Fraction 2
Fraction 3
Eluate
conductivity
Tube number
0.05 M buffer pH 5.0
0.20 M buffer pH 7.0

Table XIV.

The Chromatography of *Squalus acanthias* and *Raia rhina* Neurohypophysial Principles on CM Sephadex, Following Purification on Sephadex G-15 and DEAE Sephadex

Activity applied to column		Eluate				
Preparation	Load, mU(RU)[a]	Neutral principles		Basic principle		Total recovery of rat uterus activity
		Peak 1	Peak 2	Peak 3		
		mU(RU)	mU(RU)	mU(RU)	mU(ADH)[b]	
Squalus 2	25,016	>10,339	5,061	-	1,261	-
Squalus 3	22,701	9,392	7,036	446	763	74%
Squalus 4	31,086	15,168	7,127	453	-	73%
Raia	1,827	356	652	-	209	-

[a](RU) = rat uterus activity, in absence of magnesium.

[b](ADH) = rat antidiuretic activity.

hydrolyzed and subjected to amino acid analysis so that its purity could be estimated. The results are given in Table XV, column A. Each hydrolysate contained more than the nine amino acid residues that could be expected for a purified neurohypophysial principle. Therefore it was necessary to subject each fraction to a further purification procedure, which will be described in Section C6, page 137.

c. The pharmacological analysis of the basic peak

The third, basic, fraction eluted from the CM Sephadex contained approximately 2% of the total rat uterus activity in the eluate (see Table XIV). The small amount present prevented any attempt to obtain a chemical analysis of the amino acid composition of this peak, and therefore studies were confined to a pharmacological analysis.

A principle which was pharmacologically similar to arginine vasotocin had been demonstrated earlier by paper chromatography (Section C2c, page 125; also see Table XVI). An attempt was made here to confirm this identification. The third peak from the CM Sephadex was assayed against synthetic oxytocin (Syntocinon) for rat uterus and frog bladder activities, and against vasopressin (Pitressin) for antidiuretic activity. These activities are listed in Table XVI, part A. The resulting activity ratios, R_{ADH} = 2.0 and R_{FB} = 177, were again comparable to those of arginine vasotocin.

For more conclusive evidence the Squalus principle was

Table XV

Amino Acid Analyses of Squalus acanthias Neutral Fraction 1 and Neutral Fraction 2 at Two Stages of Purification

Amino acid	Molar ratio to glutamic acid			
	A Purified on CM Sephadex		B Purified on both CM Sephadex and SE Sephadex	
	Fraction 1[a]	Fraction 2	Fraction 1	Fraction 2
Neutral and acidic amino acids				
Cysteic acid	0.07	0.13	0.26	0.52
Ethanolamine	+ve[b]	--ve	–ve	–ve
Aspartic acid	1.93	1.97	3.30	2.33
Threonine	0.26	0.21	0.35	0.32
Serine	0.79	0.94	1.95	1.14
Glutamic acid	1.00	1.00	1.00	1.00
Proline	0.74	0.35	2.13	1.38
Half cystine	–ve	–ve	–ve	0.83
Glycine	7.93	1.56	6.21	2.62
Alanine	0.17	–ve	0.89	0.75
Valine	0.62	0.50	0.73	0.95
Methionine	0.44	–ve	0.08	–ve
Isoleucine	0.75	0.55	1.21	1.13
Leucine	0.90	0.56	1.07	0.80
Tyrosine	0.49	0.59	0.97	0.36
Phenylalanine	0.18	0.49	–ve	–ve
Basic amino acids				
Lysine	2.13	0.90	1.59	0.30
Histidine	–ve	0.55	0.04	–ve
Arginine	7.28	14.86	–ve	–ve
Ammonia	+ve	+ve	+ve	+ve
Total amino acids	Micromoles per unit oxytocic activity			
Neutral and acidic	0.0940	0.0822	0.0847	0.0699
Basic	0.0443	0.1515	0.0045	0.0015

[a]Samples used for analyses (oxytocic activity, without Mg^{++}):

A. Purified on CM Sephadex:

Fraction 1: from Preparation 3. Neutral and acidic amino acid values are the average values from two analyses - - of 836 mU and of 1255 mU. Basic amino acid values are from one analysis of 418 mU.

Fraction 2: from Preparation 3. Neutral and acidic amino acid values were determined from 1327 mU; basic amino acid values, from 1327 mU.

B. Purified on CM and SE Sephadex:

Fraction 1: neutral fractions 1a and 1b (fraction 1 from Preparations 2 and 3). Neutral and acidic amino acid values were determined by averaging the mean values from two analyses - - of 1235 mU and of 722 mU (neutral fraction la)– with the values from one analysis of 1180 mU (neutral fraction 1b). Basic amino acid values are the average values from two analyses -- of 542 mU (neutral fraction 1a) and of 1062 mU (neutral fraction 1b).

Fraction 2: neutral fraction 2a (fraction 2 from Preparations 2 and 3, combined). Neutral and acidic amino acid values were determined from 1441 mU; basic amino acid values, from 1441 mU.

[b]+ve: present; molar ratio not calculated.
–ve: not detected during analysis.

then assayed directly against synthetic arginine vasotocin for rat uterus activity, with and without magnesium ions, for frog bladder activity, and for antidiuretic activity. Table XVI, part B shows that the activity of the Squalus principle relative to arginine vasotocin was constant on all four assays, with the resulting activity ratios - D_{Mg} = 0.94 ± 0.16, D_{FB} = 0.96, and D_{ADH} = 0.89 ± 0.21. This provided strong evidence for the similarity of the Squalus principle and arginine vasotocin: since arginine vasotocin and 8-lysine oxytocin are alone in their high frog bladder activity but are distinguished from each other by the higher antidiuretic activity of arginine vasotocin, it is almost certain that the value of D_{FB} would have been less than 1.0 if the Squalus principle were neither arginine vasotocin nor 8-lysine oxytocin, and that the value of D_{ADH} would have been less than 1.0 if it were 8-lysine oxytocin rather than arginine vasotocin. Neither the value for D_{FB} nor D_{ADH} of the Squalus principle differed from 1.0, and so its identity with arginine vasotocin is highly probable.

6. Purification of the Two Neutral Fractions on SE Sephadex

The two neutral peaks from each CM-Sephadex column were lyophilized separately for the removal of ammonium buffer salts. The dried residues of peaks 1 and 2 from Preparations 2 and 3 were stored at -18°C for periods of up to 5 months. Between 40% and 80% of the oxytocic activity was lost from these samples, and so it was necessary to combine the

Table XVI

The Activities and Activity Ratios of the Basic Neurohypophysial Principle from *Squalus acanthias*

Basic principle	A Assayed against normal standards[a]				
	Activities[b] (mU/ml)			Activity ratios	
	RU[c]	ADH	FB	R_{ADH}	R_{FB}
CM Sephadex-purified (Preparation 3, Peak 3)	30.4	59.2	5375	2.0	177
Eluted from paper, R_F 0.3-0.4 (Preparation 2)	4.5	12	2136	2.7	475
Arginine vasotocin					
Sawyer (1965)				1.6	910
Munsick *et al.* (1960)				2.0	271

	B Assayed against synthetic arginine vasotocin						
	Activities[b] (ng/ml)				Activity ratios		
	RU[c]	RU+Mg	ADH	FB	D_{Mg}	D_{ADH}	D_{FB}
CM Sephadex-purified (Preparations 1, 2, 3 mixed; Peak 3)	196 ± 21	184 ± 27	175 ± 38	189	0.94 ± 0.16	0.89 ± 0.21	0.96

Table XVI continued

[a]Normal standards = synthetic oxytocin (Syntocinon, Sandoz; 10 IU/ml) for rat uterus and frog bladder assays, or arginine/lysine vasopressin (Pitressin, Parke Davis; 20 IU/ml) for the rat antidiuretic assay.

[b]Assays calculated by the 4-point method of Holton (1948); whenever three or more groups were obtained the fiducial limits are expressed, P = 0.05.

[c]Abbreviations: RU, RU+Mg, ADH; as in Table X. FB = activity on frog bladder assay. Activity ratios, R_{ADH}, R_{FB}, D_{Mg}, D_{ADH}, and D_{FB}, are the ratios of the activity found on each assay (RU+Mg, ADH, FB) to the activity found on the rat uterus assay (RU); where R indicates that the assays were performed against the normal standards (footnote a), and D indicates that the assays were performed against a different peptide (arginine vasotocin).

corresponding peaks from the two columns.

Peak 1 from Preparation 3 and part of peak 1 from Preparation 2 were combined as "neutral fraction 1a" (3,055 mU rat uterus activity, no Mg^{++}). The remainder of peak 1 from Preparation 2 (approximately 2,410 mU) became "neutral fraction 1b". Peak 2 from Preparations 2 and 3 were combined as "neutral fraction 2a" (3,513 mU). The two peaks from Preparation 4 had not been stored after lyophilization, and only 22% and 5% of the oxytocic activity had been lost from peak 1 and peak 1, respectively, by the freeze drying process itself. Peak 1 became "neutral fraction 1c" (11,814 mU) and peak 2 became "neutral fraction 2b" (6,782 mU).

The amino acid analyses of the CM Sephadex eluates (Table XV, column A) had shown that at this stage of purification neither fraction 1 nor fraction 2 contained a pure neurohypophysial peptide. Wilson (1968) had found that elution from two cation exchangers, at two different pH's, was necessary for the purification of isotocin from the salmon Onchorhynchus tschawytscha. Therefore the Squalus principles were subjected to further purification on SE Sephadex, at a pH of 2.45. The three samples of neutral fraction 1 and the two samples of fraction 2 were separately adjusted to pH 2.45 and specific conductivity 1.85 mmho/cm by dropwise addition of formic acid and dilution with water to volumes ranging from 63 ml to 219 ml. Each was applied to a separate 35 cm x 0.9 cm column of SE Sephadex which had been equilibrated

with 0.14 M NH_4 formate (pH 2.45). The activity was adsorbed onto the gel and eluted off on an NH_4 formate gradient between 0.14 M (pH 2.45) and 1.5 M (pH 2.45), as shown in Figure 10. The recovery of oxytocic activity from the gel was almost complete, between 92% and 97%.

After their elution from SE Sephadex, samples of each of the two neutral fractions were lyophilized separately for the removal of NH_4 formate, hydrolyzed, and analyzed for amino acid content. The analyses are listed in Table XV, column B. They showed that the main effect of the SE Sephadex was to separate basic amino acid residues away from the neurohypophysial principles. The data show that 90-99% of the basic amino acid residues, still present in each fraction after elution from CM Sephadex, were removed by the SE Sephadex. The SE exchanger also removed 10%-15% of the neutral and acidic amino acids present after purification on CM Sephadex. Methionine and ethanolamine were removed from fraction 1 and phenylalanine from both fractions. The analyses of the two fractions were qualitatively similar and both indicated more than the anticipated nine residues of a neurohypophysial peptide. This will be considered again in Sections C7f and C8b, pages 151 and 158.

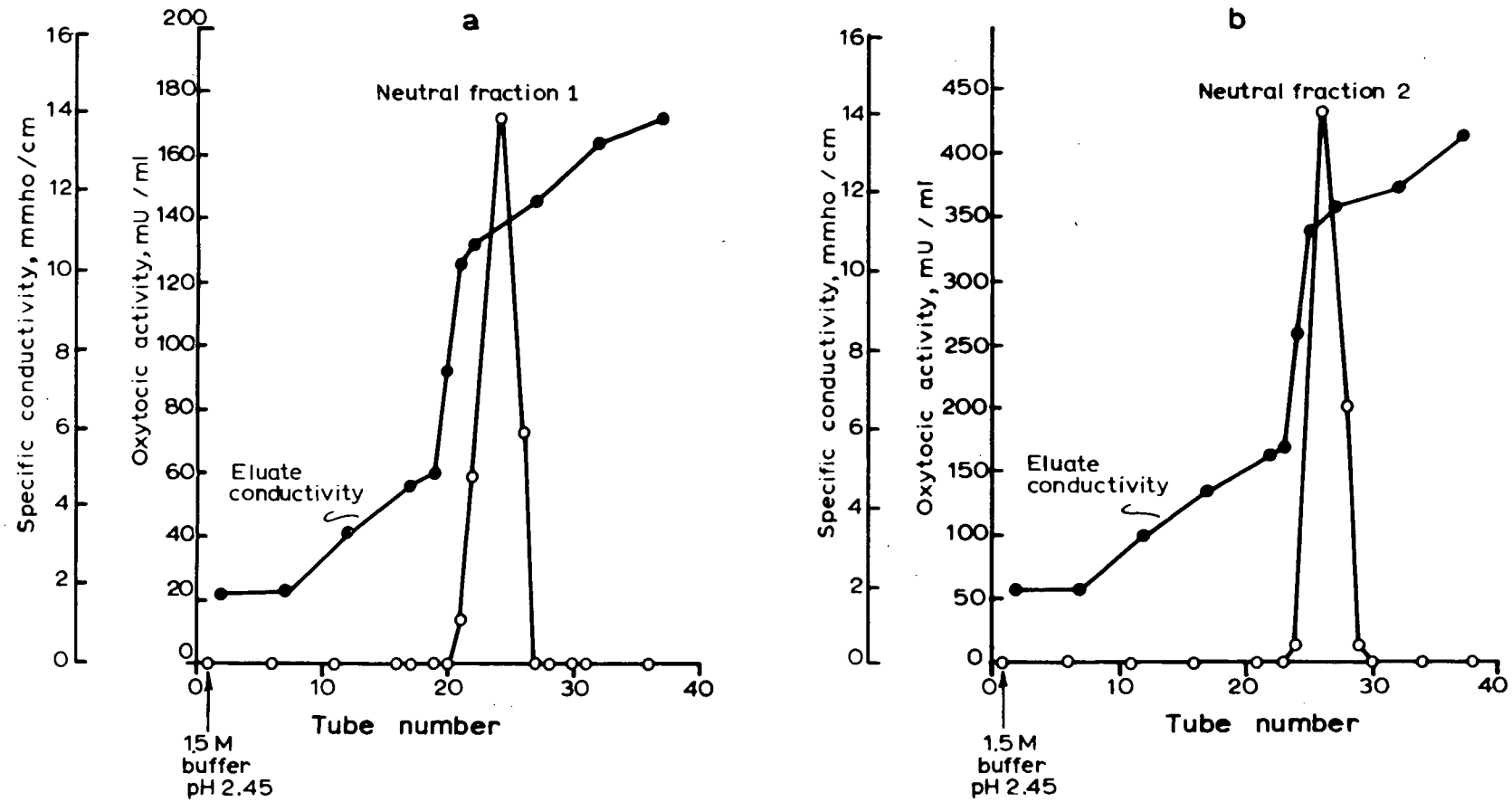
7. Comparative Studies of the Two Neutral Fractions

The repeated elution of two neutral peptide fractions from the CM Sephadex raised the possibility that two distinct neutral principles were elaborated by this species. A

Figure 10a. Chromatogram of Squalus acanthias neutral fraction 1a (fraction 1 from Preparations 2 and 3, combined; CM Sephadex-purified) on a 35 cm x 0.9 cm column of SE Sephadex. The sample (3,055 mU rat uterus activity, without Mg^{++}) was applied in 170 ml of solution at pH 2.45 and specific conductivity 1.85 mmho/cm, and was eluted with NH_4formate buffer of increasing concentration, from 0.14 M (pH 2.45): 1.5 M NH_4formate (pH 2.45) was introduced into the buffer reservoir at tube 1. Solid circles = specific conductivity of the eluate. Empty circles = rat uterus activity, without Mg^{++}. Recovery of activity = 95%. Tube volumes = 3.4 ml.

Figure 10b. Chromatogram of Squalus acanthias neutral fraction 2a (fraction 2 from Preparations 2 and 3, combined; CM Sephadex-purified) on a 35 cm x 0.9 cm column of SE Sephadex. The sample (3,513 mU rat uterus activity, without Mg^{++}, in a volume of 110 ml) was applied to the column and eluted under the same conditions as fraction 1a (see above). Solid circles = specific conductivity of the eluate. Empty circles = rat uterus activity, without Mg^{++}. Recovery of activity = 92%. Tube volumes = 3.4 ml.

Squalus acanthias, chromatography on SE Sephadex



demonstration of further differences between the two fractions would verify previous reports of two neutral principles in this species (Heller and Pickering, 1961). This was important, since the occurrence of more than one neutral peptide within any species is rare. Therefore a careful comparison of the properties of the two fractions was made. The results will be discussed in turn.

a. The comparison of the pharmacological activities of the neutral peaks, by assay against synthetic oxytocin (Syntocinon)

Each of the two fractions was assayed against synthetic oxytocin for rat uterus activity, both with and without magnesium ions, for milk-ejection activity, and for frog bladder activity. Both fractions were assayed together on the same preparations. Table XVII shows that their relative activities were similar. There was no difference between the magnesium potentiations (R_{Mg}) of the two fractions, or between their ratios of milk-ejection to rat uterus activity (R_{ME}). This was significant since these two ratios have been used to distinguish between other elasmobranch principles (see Table III, page 33). The apparent difference between the two ratios of frog bladder activity to rat uterus activity (R_{FB}) cannot be regarded as significant, since each estimate of frog bladder activity was based on only one response to the unknown.

b. The comparison of the pharmacological activities of the neutral peaks, by assay of one fraction against the other

The two fractions were assayed directly against each

Table XVII

The Activities and Activity Ratios of the Two Neutral Fractions[a] from *Squalus acanthias*

Fraction	Activities[b] (mU/ml)				Activities ratios		
	RU[c]	RU+Mg	ME	FB	R_{Mg}	R_{ME}	R_{FB}
1	58.7 ± 8.0	120.3 ± 34.0	107.1 ± 58.4	405	2.1 ± 0.6	1.8 ± 1.0	6.9
2	49.9 ± 6.2	115.8 ± 31.4	86.1 ± 67.1	860	2.3 ± 0.7	1.7 ± 1.3	17.2

[a] CM Sephadex eluates (Preparation 3).

[b] Assays calculated by the 4-point method of Holton (1948); whenever three or more groups were obtained, the fiducial limits are expressed, P = 0.05.

[c] Abbreviations as in Table X and XVI.

other, again for rat uterus activity, both with and without magnesium ions, for milk-ejection activity, and also for antidiuretic activity. Table XVIII shows that the activity of fraction 2 relative to the activity of fraction 1 was constant on all four assays, as would be expected if the two principles were identical. Thus no difference was demonstrated between the two fractions by pharmacological comparison, either by assay side by side against synthetic oxytocin, or by the direct assay of one fraction against the other, a method which eliminates errors due to variations in the sensitivity of assay preparations to standards or unknowns.

c. Paper chromatography of the two fractions

The two fractions were applied to adjacent origins on Whatman 3MM paper and the chromatogram was developed in n-butanol:acetic acid:water = 4:1:5. The eluates were assayed on the rat uterus in the presence of magnesium, and the results are shown in Figure 5, e and f, page 115. Both fractions exhibited the same behavior and were located in R_F 0.4-0.7. Since neither occupied the position of the principle E_1, which is slow-running in this solvent system, it is apparent that the two Squalus fractions found here did not correspond to the principles E_1 and E_2 which had been reported earlier in this species (Heller and Pickering, 1961). No difference between the two Squalus fractions was demonstrated by this procedure.

Table XVIII

The Activities and Activity Ratios of Squalus acanthias Neutral Fraction 2[a], Assayed against Neutral Fraction 1[a]

Volume of fraction 2	Acitvities[b]--equivalent volume (ml) of fraction 1				Activity ratios		
	RU[c]	RU+Mg	ME	ADH	D_{Mg}	D_{ME}	D_{ADH}
1 ml	0.70 ± 0.10	0.65 ± 0.09	0.75 ± 0.18	0.68 ± 0.34	0.94 ± 0.18	1.08 ± 0.26	0.97 ± 0.49

[a]SE Sephadex eluates (Preparation 4).

[b]Assays calculated by the 4-point method of Holton (1948); whenever three or more groups were obtained the fiducial limits are expressed, P = 0.05.

[c]Abbreviations as in Table X; except the activity ratios, D_{Mg}, D_{ME}, and D_{ADH}, where D indicates that the assays were not performed against the normal standards.

d. Isoelectric focusing of the two fractions

A mixture of the two fractions was subjected to isoelectric focusing, a sensitive technique which can separate compounds differing in isolectric point by as little as 0.02 pH units (Vesterberg and Svensson, 1966). This method had not previously been applied in the study of neurohypophysial principles. It is an important technique, in view of the similarity of these peptides and of the difficulty in distinguishing them by other means.

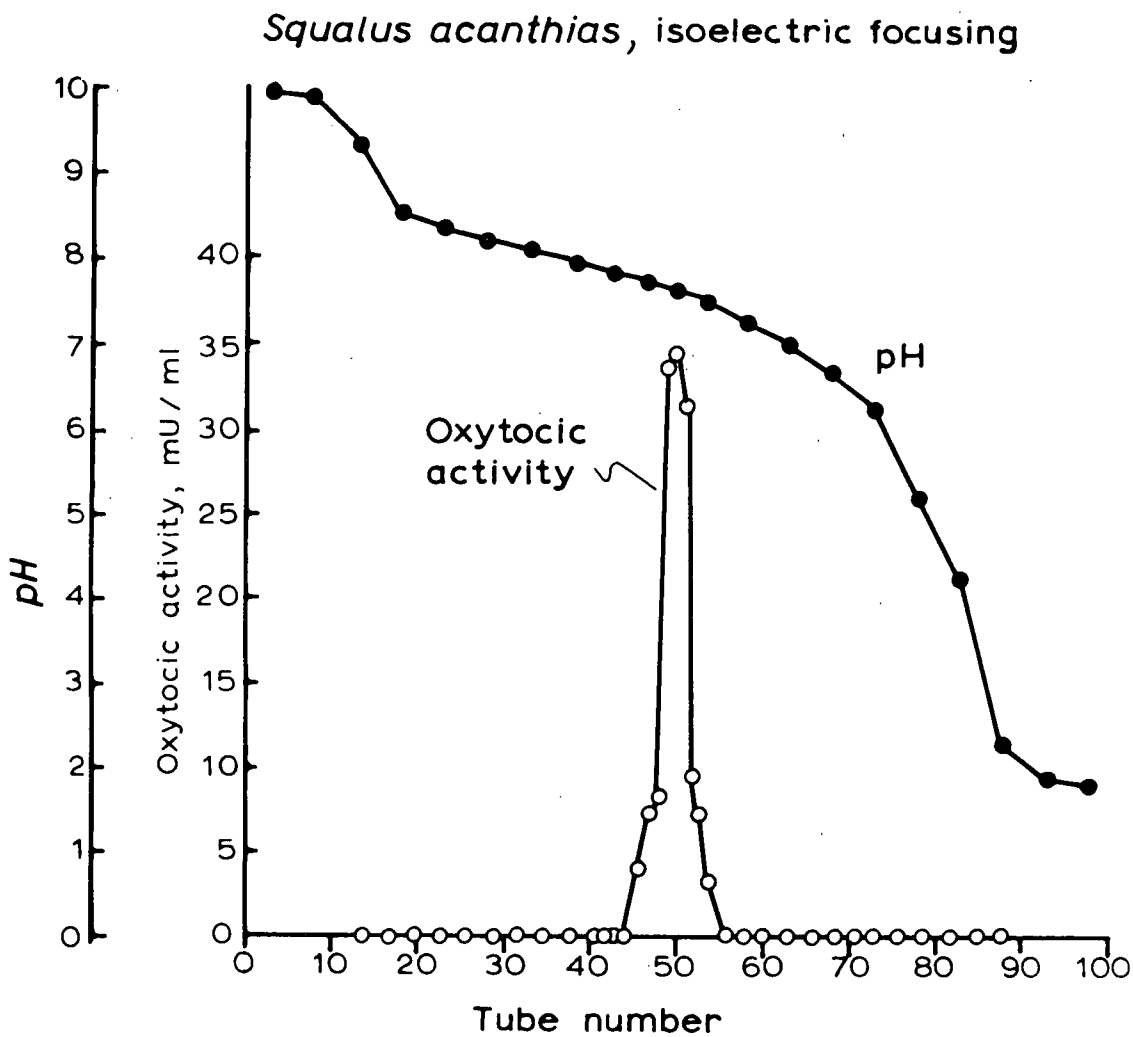
A sample of 400 mU oxytocic activity (200 mU from each fraction) of SE Sephadex-purified material was subjected to 300 volts for 48 hours in a pH range 3-10; and a second sample of 391 mU (261 mU from fraction 1, 130 mU from fraction 2) of CM Sephadex-purified material was subjected to 800 volts for 25 hours in a narrower pH range of 7-9. In each case 40% of the activity was recovered from the column.

Once again, there was no difference in the behavior of the two fractions. They were not resolved in either run; they migrated in the pH gradient as one peak, and stabilized at pH 7.62 (Fig. 11).

e. The comparison of the behavior of the two fractions on SE Sephadex

Since the two neutral fractions had been eluted from the CM Sephadex at different buffer concentrations, their behavior on SE Sephadex, also a cation exchanger, was of interest. However they appeared similar in their behavior

Figure 11. Isoelectric focusing of a mixture of Squalus acanthias neutral fraction 1 and neutral fraction 2 (Preparation 3, Sephadex G-15- and CM Sephadex-purified) in a pH range of 7-9 at 800 volts for 25 hours. Load = 391 mU rat uterus activity, without Mg^{++} (261 mU of fraction 1 and 130 mU of fraction 2). Solid circles = pH of eluate. Empty circles = oxytocic activity (rat uterus activity, without Mg^{++}). Recovery of activity = 40%. Tube volumes = 1.1 ml.


Squalus acanthias, isoelectric focusing
pH
Oxytocic activity, mU / ml
Oxytocic activity
pH
Tube number
0
1
2
3
4
5
6
7
8
9
10
0
5
10
15
20
25
30
35
40
0
10
20
30
40
50
60
70
80
90
100

on this exchanger (see Section C6 above, page 137). Both were eluted in the same position on the NH_4 formate gradient, as is illustrated in Figure 10. The three samples of fraction 1 appeared within the specific conductivities of 5.8 and 11.6 mmho/cm and the two samples of fraction 2 were eluted between 6.9 and 11.8 mmho/cm.

f. The comparison of the amino acid composition of the two fractions

After purification on SE Sephadex a sample of each fraction was hydrolyzed and analyzed for amino acid composition (Section C6 above). The results were listed in Table XV, column B (page 136). The analyses of the two fractions were qualitatively similar. Fraction 1 contained relatively more glycine than fraction 2, and there were differences between their tyrosine and cysteic acid contents. However the extra glycine in fraction 1 was later removed by rechromatography on CM Sephadex (see Section g below), and so it did not form a part of the active peptide. The recovery of tyrosine and cysteic acid from the hydrolysis procedure is known to vary (Chauvet et al., 1963) and so quantitative differences in these amino acid residues may not indicate any real difference between the peptides in the two fractions.

g. Rechromatography of fraction 1 on CM Sephadex

The two oxytocic fractions had been distinguished by the buffer concentrations at which they were eluted from CM Sephadex. However, a further difference between them could

not be demonstrated, even by such diverse means as pharmacological comparisons, paper chromatography, ion exchange chromatography, isoelectric focusing, or comparisons of their amino acid compositions (Section a-f, above).

Therefore an attempt was made to demonstrate the identity of the two principles, by rechromatography of fraction 1 on CM Sephadex. After its elution from SE Sephadex a sample of fraction 1 (7,900 mU rat uterus activity, no Mg^{++} present, from Preparation 4) was lyophilized for the removal of NH_4formate. The residue (containing 6,400 mU rat uterus activity in the absence of Mg^{++}, a recovery of 81%, and also a little remaining electrolyte) was taken up in 100 ml water. The specific conductivity of the resulting solution was 0.16 mmho/cm and the pH was 5.0. It was applied, without further adjustment, to a 35 cm x 0.9 cm column of CM Sephadex, Lot 8852, which had been equilibrated with 0.002 M HN_4acetate (pH 5.0), the same conditions as prevailed during the first chromatography of Preparation 4 on CM Sephadex. However Preparation 4 had been applied to the first CM Sephadex column in a larger volume, 2,485 ml.

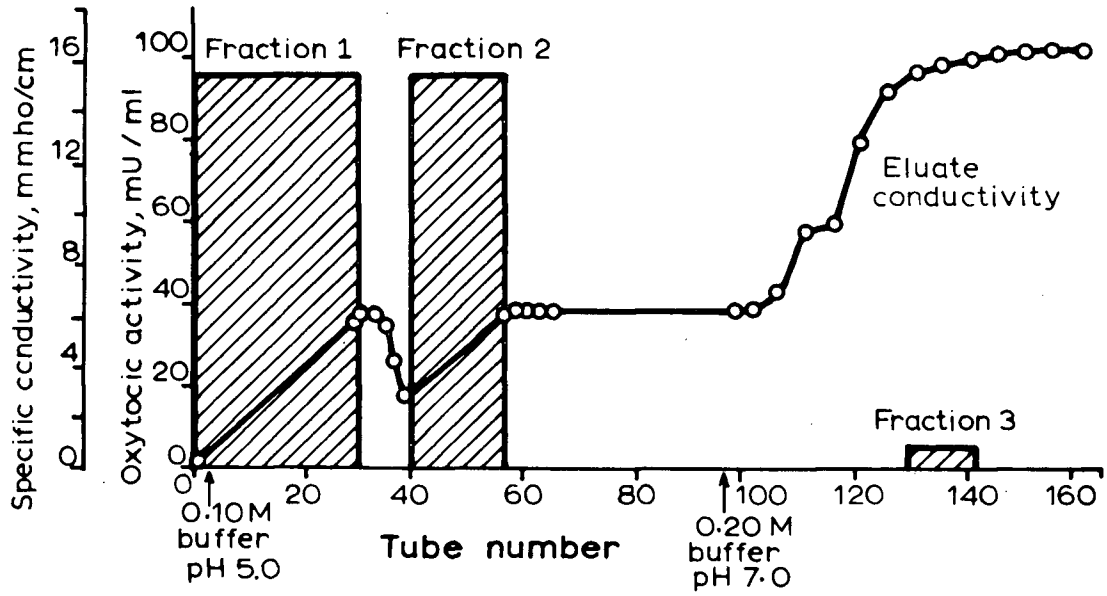
Peak 1 of Preparation 4 had come off the first CM Sephadex column between the specific conductivities of 0.16 mmho/cm and 6.2 mmho/cm, while peak 2 had appeared only after the specific conductivity had reached 6.2 mmho/cm (Fig. 12a). This time, when fraction 1 was reloaded onto the resin in the much smaller volume of 100 ml, it appeared in the position of

Figure 12a. Chromatogram of partially purified neurointermediate lobe extract of Squalus acanthias (Preparation 4; Sephadex G-15-purified) on a 35 cm x 0.9 cm column of CM Sephadex. The activity (31, 086 mU rat uterus activity, without Mg^{++}) was applied in 2,485 ml of solution at pH 5.0 and specific conductivity 0.16 mmho/cm, and was eluted with NH_4acetate buffer of increasing concentration, from 0.002 M (pH 5.0): 0.10 M NH_4acetate (pH 5.0) was introduced into the buffer reservoir at tube 3, and 0.20 M NH_4acetate (pH 7.0) was introduced at tube 97. Empty circles = specific conductivity of the eluate. Hatched columns = rat uterus activity, without Mg^{++}: fraction 1 = 15,168 mU; fraction 2 = 7,127 mU; fraction 3 = 453 mU. Tube volumes = 4.8 ml.

Figure 12b. Chromatogram of Squalus acanthias fraction 1 (Preparation 4; purified on Sephadex G-15 and on CM Sephadex - see Fig. 12a, above - and also on SE Sephadex) on a second 35 cm x 0.9 cm column of CM Sephadex. The activity (6,400 mU rat uterus activity, without Mg^{++}) was applied in 100 ml of solution at pH 5.0 and specific conductivity 0.16 mmho/cm, and was eluted with NH_4acetate buffer of increasing concentration, from 0.002 M (pH 5.0): 0.10 M NH_4acetate (pH 5.0) was introduced into the buffer reservoir at tube 4. Empty circles = specific conductivity of the eluate. Hatched column = oxytocic activity (rat uterus activity, without Mg^{++}); 6,138 mU. Tube volumes = 4.8 ml.

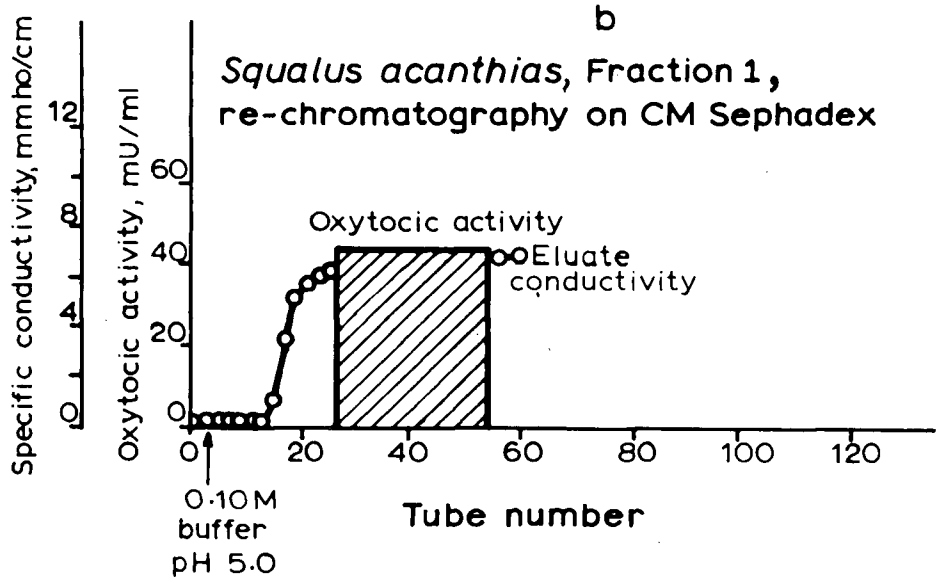
a

Squalus acanthias, chromatography on CM Sephadex



b

Squalus acanthias, Fraction 1, re-chromatography on CM Sephadex



fraction 2, at a specific conductivity of 6.2 mmho/cm (Fig. 12b).

On the basis of this experiment and the preceeding comparisons of the two fractions, it was concluded that the neurohypophysial peptide contents of the two were identical.

The appearance of the neutral principle at two different positions on the elution gradient might be attributed here to the large volumes in which the samples were applied to the exchanger (2,007 ml, 4,364 ml, 2,485 ml for Preparations 2, 3, and 4, respectively). It is worth noting that the neutral peptide of the Raia rhina extract, tentatively identified as glumitocin alone (Section I B3 and C4, pages 82 and 98) was also eluted from CM Sephadex in two fractions when the exchanger was loaded at a high volume, but that it emerged as a single peak when the two fractions were recombined and applied to a second column in a small volume.

8. The Nature of the Single Neutral Fraction

The preceeding experiments suggested that the Squalus extract contained only one neutral fraction. An attempt was made to determine its nature by pharmacological study and by the analysis of its amino acid content.

a. Pharmacological studies of the neutral fraction

The activity ratios for crude neurohypophysial extracts of Squalus acanthias (Table X, page 106) had indicated that the oxytocin-like principle was of the EOP I type. This was confirmed by the values for R_{ME} of 1.8 ± 1.0 and 1.7 ± 1.3

found for "neutral fraction 1" and "neutral fraction 2" after their separation on CM Sephadex (Table XVII, page 145). However since glumitocin had been identified in four species of skate (Acher et al., 1965; 1967; Chauvet et al., 1965) and had been tentatively identified here in the longnose skate, Raia rhina, (Section I B3 and I C4, pages 82, 98) it was of interest to know whether it might also be the principle produced by the shark, Squalus acanthias. This was not unlikely, since neurohypophysial extracts of three of the skates found to produce glumitocin - Raia clavata, Raia batis, and Raia rhina - had appeared to contain principles of the EOP I type (Perks and Dodd, 1963a; data presented here in Section I B1, page 70).

Therefore a purified preparation of the Squalus neutral principle was assayed against synthetic glumitocin for rat uterus activity, both in the presence and absence of magnesium ions, and for milk-ejection activity. The results are listed in Table XIX. The potency of the Squalus principle relative to glumitocin was different on each of the three assays. The ratio of its activity on the rat uterus in the presence of Mg^{++} to its activity in the absence of Mg^{++}, $D_{Mg} = 0.49 \pm 0.06$, demonstrated the lack of identity between the two peptides. This was verified by the ratio of milk-ejection activity to rat uterus activity, $D_{ME} = 0.20$. These results confirmed the findings of Sawyer et al. (1969), who found that the principle produced by Squalus acanthias, Atlantic variety, was different from glumitocin.

Table XIX

The Activities and Activity Ratios of the Oxytocin-like Fraction[a] from Squalus acanthias, Assayed against Synthetic Glumitocin

Activities[b] (μg/ml)			Activity ratios	
RU[c]	RU + Mg	ME	D_{Mg}	D_{ME}
23.4 ± 1.9	11.5 ± 1.3	4.7	0.49 ± 0.06	0.20

[a]SE Sephadex eluate of neutral fraction 1a (= Preparations 2 and 3, mixed; Peak 1).

[b]Assays calculated by the 4-point method of Holton (1948); whenever three or more groups were obtained, the fiducial limits are expressed, P = 0.05.

[c]Abbreviations as in Table X; except the activity ratios, D_{Mg} and D_{ME}, where D indicates that the assays were performed against a principle (glumitocin) other than the normal standard.

This species, then, appeared to produce a principle other than the glumitocin of the skates. It might be that it produces a mixture of glumitocin and another peptide, so that the mixture appears pharmacologically different from glumitocin. However, chromatographic evidence (Sawyer, personal communication) and theoretical considerations (presented in the Discussion, Chapter 4) suggest that glumitocin would not be present in any possible Squalus peptide mixture. A third possibility is that the Squalus oxytocin-like fraction consists of a mixture of principles, none of which is glumitocin.

b. Amino acid analyses of the neutral fraction

A further attempt was made to determine the nature of the Squalus neutral principle by an analysis of its amino acid composition. Several analyses were made; these will be outlined in turn:

i. The analysis of "fraction 2" after purification on SE Sephadex

The amino acid analyses of the SE Sephadex-purified fraction 1 and fraction 2 (from combined Preparations 2 and 3; see Table XV, column B, page 136) suggested that "fraction 2" was the purer preparation of the Squalus oxytocin-like principle. The SE Sephadex had not removed the glycine which had been eluted from the CM Sephadex into "fraction 1"; and after elution from SE Sephadex, "fraction 1" contained 0.085 μ mole of neutral and acidic amino acid residues per unit of oxytocic

activity, while "fraction 2" contained only 0.070 μ moles. Nevertheless "fraction 2" contained more than the nine amino acid residues typical of a single neurohypophysial principle.

ii. The analysis of the combined fractions

An attempt was made to verify the above result by an analysis of material which had been purified independently from the previous sample. Fraction 1 and fraction 2 from Preparation 4 (6.2 IU and 4.7 IU rat uterus activity in the absence of Mg^{++}, respectively) were combined after fraction 1 had been rechromatogrammed on CM Sephadex (see Section 7g above, page 151). A sample of the combined material was hydrolyzed and analyzed. The results are listed in Table XX, column 2. The extra glycine of the "fraction 1" portion (see Table XV, Column B) appeared to have been removed during its rechromatography on CM Sephadex. Table XX shows that this analysis of material from Preparation 4 was similar to the previous analysis of material from combined Preparations 2 and 3 (Table XV, column B; repeated in Table XX, column 1).

It was possible that some free amino acids had not been separated from the neurohypophysial peptide by any of the purification procedures which had been used. Therefore an unhydrolyzed sample of the combined fractions 1 and 2 from Preparation 4 (642 mU rat uterus activity, no Mg^{++} present) was analyzed for free amino acids. Only two could be detected, serine and glycine (Table XXI), and the analysis of the hydrolyzed sample of Preparation 4 (Table XX, column 2) has been

Table XX

Amino Acid Analyses of the Purified Neutral Fraction from Squalus acanthias

Amino acid	Molar ratio to glutamic acid			
	Fraction 2 (CM and SE Sephadex-purified)[a]	Fractions 1 and 2, combined (CM, SE, and CM Sephadex-purified)		Average values
			(plus paper chromatography)	
	1	2	3	4
Neutral and acidic amino acids				
Cysteic acid	0.97[b]	0.84	1.56	1.12
Aspartic acid	2.33	1.94	2.21	2.16
Threonine	0.32	–ve[c]	–ve	0.11
Serine	1.14	0.87[d]	1.00	1.00
Glutamic acid	1.00	1.00	1.00	1.00
Proline	1.38	1.63	2.87	1.96
Half cystine	0.83	–ve	–ve	–
Glycine	2.62	2.09[d]	2.28	2.33
Alanine	0.75	0.64	0.62	0.67
Valine	0.95	0.88	1.05	0.96
Methionine	–ve	–ve	–ve	–ve
Isoleucine	1.13	1.01	1.08	1.07
Leucine	0.80	0.89	1.13	0.94
Tyrosine	0.36	0.29	–ve	0.22
Phenylalanine	–ve	0.17	–ve	0.06
Basic amino acids				
Lysine	0.30	–	–ve	–
Histidine	–ve	–	–ve	–
Arginine	–ve	–	–ve	–
Ammonia	+ve	–	+ve	–
Total amino acids	Micromoles per unit oxytocic activity			
Neutral and acidic	0.0699	0.0523	0.0685	
Basic	0.0015	–	–ve	

[a]Samples used for analyses:

Column 1: see Table XV, footnote a: Fraction 2, purified on both CM and SE Sephadex.

Column 2: combined neutral fractions 1c and 2b (fractions 1 and 2 from Preparation 4); both fractions purified on CM and SE Sephadex, and fraction 1c then rechromatogrammed on CM Sephadex. Neutral and acidic amino acid values were determined from 1280 mU oxytocic activity. Basic amino acid values were not determined.

Column 3: combined neutral fractions 1c and 2b (as in column 2), further purified by paper chromatography. All amino acids were determined from one sample, estimated to contain 603 mU oxytocic activity.

[b]Corrected to include the half cystine.

[c]+ve: present; molar ratio not calculated. –ve: not detected during analysis.

[d]Corrected for the presence of serine and glycine in the unhydrolyzed sample (see Table XXI).

Table XXI

Amino Acid Analysis of the Unhydrolyzed, Purified Neutral Fraction[a] from Squalus acanthias

Amino acid	Micromoles in unhydrolyzed sample (642 mU)	Molar ratio to the glutamic acid in the hydrolyzed sample (Table XX, column 2)[b]
Cysteic acid	-ve[c]	
Aspartic acid	-ve	
Threonine	-ve	
Serine	0.0011	0.40
Glutamic acid	-ve	
Proline	-ve	
Glycine	0.0011	0.39
Alanine	-ve	
Valine	-ve	
Methionine	-ve	
Isoleucine	-ve	
Leucine	-ve	
Tyrosine	-ve	
Phenylalanine	-ve	

[a]Combined neutral fractions 1 and 2 from Preparation 4; as in Sample 2, Table XX. Neutral and acidic amino acids = analysis of 642 mU of unhydrolyzed sample. Basic amino acids not determined.

[b]Corrected for the ratio of the unhydrolyzed sample to the hydrolyzed sample (642 mU: 1280 mU).

[c]-ve: not detected during analysis.

corrected for these two residues. It was also possible that some contamination might have been introduced in the HCl which was used for the hydrolyses. Therefore a sample of the HCl was subjected to a blank hydrolysis procedure and then to amino acid analysis. It was found to contain several acids, but they were in small proportion to the amounts of peptide that were hydrolyzed. Table XXII shows the low molar ratio of these contaminants to the glutamic acid found in hydrolysates 1 and 2 of Table XX (column 1 and 2). Hydrolysate 3 (Table XX, column 3) was prepared with a different lot of HCl.

iii. The analysis of the combined fractions after paper chromatography

It was possible that the procedures which were followed had not succeeded in separating a contaminating peptide away from the oxytocic principle. Therefore the remaining unhydrolyzed portion of the combined fractions 1 and 2 from Preparation 4 (3.64 IU rat uterus activity in the absence of Mg^{++}) was subjected to additional purification by a further method, based on different principles - by partition chromatography, on paper, using n-butanol:acetic acid:water = 4:1:5. Oxytocic activity was located at R_F 0.25-0.60 and was eluted into 0.25% acetic acid. The eluate was centrifuged for removal of cellulose, and lyophilized. The residue was hydrolyzed and analyzed for amino acid content. The results of the analysis are listed in Table XX, column 3. A comparison of the two analyses of the sample, before and after its paper chroma-

Table XXII

Amino Acid Analysis of the Hydrochloric Acid Used for Hydrolyses[a]

Amino acid	Micromoles in 4 ml HCl	Molar ratio[b] to the glutamic acid in	
		Hydrolysate 1 (Table XX, column 1)	Hydrolysate 2 (Table XX, column 2)
Cysteic acid	-ve[c]	-	-
Aspartic acid	0.00063	0.08	0.02
Threonine	-ve	-	-
Serine	0.00015	0.02	0.01
Glutamic acid	0.00013	0.02	0.01
Proline	-ve	-	-
Glycine	0.00109	0.14	0.04
Alanine	0.00015	0.02	0.01
Valine	-ve	-	-
Methionine	-ve	-	-
Isoleucine	-ve	-	-
Leucine	-ve	-	-
Tyrosine	-ve	-	-
Phenylalanine	-ve	-	-
Lysine	0.00070	0.09	0.03
Histidine	-ve	-	-
Arginine	-ve	-	-

[a] Used for all hydrolyses, except the hydrolysis of the sample purified by paper chromatography (Table XX, column 3).

[b] Corrected for the proportion of the total hydrolysate which was used in analysis.

[c] -ve: not detected during analysis.

tography (Table XX, columns 2 and 3) shows that no further purification was achieved by this step.

iv. An interpretation of the analyses of the neutral fraction

If an allowance is made for the breakdown of cystine and tyrosine during hydrolysis, these three similar analyses of the purified material (Table XX) suggest the presence of the amino acid residues of a mixture of two neurohypophysial peptides, present in equimolar amounts and having an average potency of 109 IU/mg. Destruction of cystine and tyrosine during hydrolysis is known to occur (Chauvet et al., 1963) and it is particularly marked when minimal amounts of peptide are present in the hydrolysis mixture, as was the case here. All the naturally occurring neurohypophysial peptides contain single residues of asparagine, proline, and glycine, in addition to cystine and tyrosine (see Table I, page 6) and these three residues (asparagine hydrolyzed to aspartic acid) were found in double proportion in the Squalus hydrolysate. The six residues which were present in single proportion in the hydrolysate- serine, glutamic acid, alanine, valine, isoleucine, and leucine- could occupy the remaining three positions in each of two peptide molecules; it is the amino acids of these positions, position 3, 4, and 8, which have been found to vary in nature.

D. Summary of the Studies on the Neurohypophysial Principles of *Squalus acanthias*

1. Early pharmacological and chromatographic studies suggested that *Squalus acanthias* produces only an oxytocin-like principle (Perks *et al.*, 1960; Sawyer *et al.*, 1961; Perks and Dodd, 1963a; Perks, 1966). However it was found here, in agreement with Sawyer (1965; and later, 1967) that *Squalus* elaborated a small amount of basic peptide, which accounted for approximately 2% of the total rat uterus activity of the extract, in addition to the oxytocic fraction. The basic principle was identified as arginine vasotocin on the basis of:

a. Its mobility on paper chromatography (Fig. 8, page 128).

b. Its pharmacological agreement with arginine vasotocin, both (i) when assayed by the classical methods against synthetic oxytocin (Syntocinon) and vasopressin (Petressin), (Table XVIA, page 138): and, more conclusively, (ii) when assayed directly against synthetic arginine vasotocin (Table XVIB).

2. a. The neutral fraction was eluted from the CM Sephadex in two peaks (Fig. 9, page 133). This raised the possibility of the presence of two neutral principles within a single species - a rare occurrence. However, a careful comparison of the two peaks by pharmacological studies (Tables XVII, XVIII, pages 145, 147), by paper chromatography

(Fig. 5e, f, page 115), by ion exchange chromatography on SE Sephadex (Fig. 10, page 143), by isoelectric focusing (Fig. 11, page 150), and by amino acid analysis (Table XV, column B, page 136) failed to show any difference between them; and rechromatography of peak 1 on CM Sephadex showed that it moved identically to peak 2, when it was applied to the column in low volume (Fig. 12a, b, page 154). This evidence strongly suggested that the two neutral fractions were identical in their neurohypophysial peptide contents, and they were combined for further analysis. The splitting of the oxytocic principle on this ion exchanger may have resulted from the large volumes in which the samples were applied during the preparative purification procedures.

2. b. The neutral fraction appeared pharmacologically different from glumitocin, the principle found in the skates (Table XIX, page 157). This was important because both the Squalus principle and the principle found in most skates had appeared to be of the EOP I type; the pharmacological disagreement between the Squalus principle and glumitocin suggested that glumitocin was not the neutral principle found in all elasmobranchs.

2. c. The amino acid analyses of the purified neutral fraction (Table XX, page 160) were the first analyses to be made of the oxytocic principle of a shark. They revealed more amino acid residues than would be expected for a single

neurohypophysial peptide, but which could represent a mixture of two such principles, present in equimolar amounts and having an average activity of 109 IU/mg.

Chapter 4

DISCUSSION

I. STUDIES OF THE NEUROHYPOPHYSIAL PRINCIPLES OF THE SKATE, *RAIA RHINA*

1. The Variability in the Apparent Biological Potency of Crude Extracts and of Glumitocin

The unexpected variation which was found in the magnesium potentiation values for neurointermediate lobe extracts of *Raia rhina* (ex. R_{Mg} = 2.5 to 16.8; see Table V, page 79) reflected changes in the apparent potency of the extracts as judged by either of the two assays used-- rat uterus with magnesium ions, or rat uterus in the absence of magnesium (Chapter 3, Section IB2, page 71).

The causes of the variation in potency are unknown, since the mechanism of the hormones' action on the uterus and the role played by magnesium in this process are uncertain (Munsick and Jeronimus, 1965; Bentley, 1965; Walter *et al.*, 1968; Krejčí and Poláček, 1968). The potency of a neurohypophysial peptide other than oxytocin is determined by the absolute amount of the agent, and by the difference between the sensitivity of the assay preparation to the unknown peptide and to the oxytocin standard. On each type of assay- rat uterus, milk-ejection, etc.- the difference in the

responses of individual preparations to oxytocin and to its analogues has been relatively constant and characteristic for each analogue, as evidenced by the apparent stability of the biological properties which have appeared to characterize each of the neurohypophysial principles (Perks and Dodd, 1963a; Follett and Heller, 1964a; 1964b; Sawyer, 1968). However, it is possible that some preparations may make an anomalous distinction between oxytocin and the unknown, with a resulting variability in the potency of the analogue upon repeated assays. Further, the particular difference in structure between oxytocin and certain of its analogues, such as the R. rhina principle, might promote such anomalous behavior in the case of a particular principle. This might be expected when the unknown principle is derived from a class of animals which is as widely removed from the mammals as the elasmobranch fish.

One factor which might influence the behavior of the rat uterus is its stage in the estrous cycle at the time of assay. Munsick and Jeronimus (1965) found that the potencies of some analogues, as measured both with and without Mg^{++}, were significantly affected by the estrous condition of the uteri on which they were assayed. However the widest variation they found in potentiation was only from 1.23 to 1.98, values which were obtained from assays of arginine vasotocin on uteri which were taken from rats in metestrous-diestrous and induced estrous, respectively. In the studies presented

here, the wide range of potentiations was obtained on uteri all of which were judged to be in the estrous state. However, in this regard Staples and Geils (1965) have reported that approximately twelve hours before proestrous the rat vaginal smear consists of large cornified cells, and these authors caution that the evaluation of the smear is not as straight-forward as has been supposed. Nevertheless even if some of the smears taken in the present study were thus misidentified, variation of the magnitude observed by Munsick and Jeronimus could not account for the total variation which was observed here. Also, in this regard, Sawyer et al. (1969) observed a range of magnesium potentiations of from 1.46 to 4.90 for synthetic glumitocin even when the uteri used for assay were taken from rats pretreated with diethylstilbestrol. Therefore, while differences in the estrous condition of the uteri may contribute to the variability in magnesium potentiation, they are unlikely to be the sole cause.

In addition to the variability found in magnesium potentiations, some variability was also observed in the milk-ejection activities of the unknowns when they were assayed against oxytocin. Two different potencies, the second being 100% greater than the first, were found for both crude R. rhina extract and for a solution of synthetic glumitocin when they were assayed together against synthetic oxytocin on two different occasions (see Table VIII, page 88); naturally, the corresponding ratios of milk-ejection to rat uterus activity

(R_{ME}) reflected these differences.

Pharmacological activity ratios have been used extensively to characterize neurohypophysial peptides. In particular, values for magnesium potentiation (R_{Mg}) and relative milk-ejection activity (R_{ME}) have been used to distinguish between the numerous postulated elasmobranch principles (see Table III, page 33). In view of the observations presented here, in which the variability of these parameters has been demonstrated, it is clear that they may be used to distinguish neurohypophysial peptides from oxytocin itself, if oxytocin is used as the standard preparation, but that the values obtained by independent, "classical" methods of assay are an unreliable criterion for comparing two unknowns - at least in the case of elasmobranch extracts. Consequently, some of the distinctions between the many elasmobranch principles may prove to be invalid, and it is possible that the confusing array of these agents - EOP I, EOP II, glumitocin, the E_1 principles, and the E_2 principles - might be considerably simplified, in the following manner.

The E_1 and E_2 principles (the chromatographically slow-moving and chromatographically fast-moving components, respectively, of the oxytocic activity found in certain species) have been found to vary in their magnesium potentiations; three groups of values have been observed for the R_{Mg} of the slow-moving component (R_{Mg} values of 6, 2, and 1), and two groups of values have been found for the fast-moving

component (R_{Mg} values of 2 and 1; see Table III, page 33). Therefore it has been suggested (Heller and Roy, 1965b; Roy, 1969) that the nature of each of the two chromatographic components may vary between species. However, the total range of magnesium potentiation values shown by each fraction (slow-moving fraction: R_{Mg} = 0.95-6.3; fast-moving fraction: R_{Mg} = 0.85-2.6; see Table III) is considerably smaller than the range of values shown either by synthetic glumitocin (R_{Mg} = 1.46-9.5; see below) or by "natural" glumitocin as postulated in the Raia rhina extract (R_{Mg} = 1.8-17.5, Table IV, page 73). The capriciousness of this ratio as it applies to elasmobranch principles strongly suggests that the three groups of values found for E_1 may all characterize a single slow-moving component and that the two groups of values found for E_2 may also characterize a single fast-moving agent. Other pharmacological evidence is compatable with this suggestion. The slightly potentiated E_1 agent (R_{Mg}=2) and the more highly potentiated E_1 agent (R_{Mg}=6) appeared similar in their relative guinea pig milk-ejection activities and in their relative rat antidiuretic activities (Roy, 1969). The non-potentiated E_1 principle was not assessed for these activities. The non-potentiated E_2 principle did not differ from the potentiated E_2 agent in its relative chicken blood pressure activity. However, the two principles, E_1 and E_2, are still distinguished by their different mobilities on paper chromatography (see Table III) and by their different natriferic

activities, where this latter characteristic has been investigated (Roy, 1969).

Glumitocin, chemically identified in several species of skate, has been distinguished from the other elasmobranch neutral principles by its high, ten-fold, magnesium potentiation (Acher et al., 1965; see Table III); a sample of synthetic glumitocin was found here to have a similarly high potentiation (R_{Mg} = 9.5; Table VIII, page 88). However, during the course of this work, and in addition to the observations made here, Sawyer et al. (1969) found a lower range of values (R_{Mg} = 1.46-4.90) for the same sample of synthetic glumitocin; the different potentiations observed for synthetic glumitocin in the two laboratories (R_{Mg} = 1.46-9.5) included values which had been reported previously for EOP I (R_{Mg} = 1.2-3.2), EOP II (R_{Mg} = 2.6), E_1 (R_{Mg} = 0.95-6.3), and E_2 (R_{Mg} = 0.85-2.6; see Table III). Therefore glumitocin cannot now be shown to be different from any of the E_1, E_2, EOP I, or EOP II principles on the basis of its magnesium potentiation; further, in view of the range of R_{Mg} values that might be attributed to each of the single E_1 and E_2 principles (see above), no distinction can reliably be made between the magnesium potentiations of any two of the postulated elasmobranch neutral principles. However, despite doubts concerning the validity of the criteria which have been used to distinguish the various elasmobranch principles, it is fair to point out that even in the case of agents which show no reliable differentiation by the

methods used, the chemical nature may still prove to vary.

No difference has yet been demonstrated between E_2 and EOP I. Their R_{Mg}'s have appeared similar and they have the same chromatographic mobility in n-butanol:acetic acid: water = 4:1:5 (see Table III). Other comparative data is lacking. E_2, EOP I, and glumitocin have all been reported in the same species, Raia clavata (Heller and Roy, 1965a; Perks and Dodd, 1963a; Acher et al., 1965). This strongly suggests that the three are identical, although it does not prove that this is the case. On the other hand, the amino acid content of glumitocin is identical to that of EOP II, and although the amino acid sequence could be different in the two peptides, it is also possible that it could be the same. The paper chromatographic behavior of either glumitocin or EOP II has not been determined and so it cannot be compared to that of E_1, E_2, or EOP I.

The distinction between EOP I and EOP II has been based on the difference between their relative potencies on the milk-ejection assay (R_{ME}). In the study presented here, this value has been found to vary in the case of both synthetic glumitocin (R_{ME} = 8.2 and 15.6) and of the R. rhina extract (R_{ME} = 4.4, 8.3, and 17.5). Another value for the R_{ME} of synthetic glumitocin has been reported by Sawyer et al. (1969) as 5.3. The values of R_{ME} which characterize the EOP I principle range from 1.5 to 4.5 in different species

(Perks and Dodd, 1963a; Sawyer, 1965; see Table III, page 33). The values for R_{ME} of the EOP II principle are in a higher range, given as 8.5 and 10.3 (Perks and Sawyer, 1965; see Table III). However, the R_{ME} value for synthetic glumitocin ranges from 5.3 to 15.6 (Sawyer et al., 1969; data presented here), and this range stretches almost down to the values for EOP I, and up beyond those for EOP II. More important, "natural" glumitocin, as postulated in the R. rhina extract (see Section 2, below), gave a range of R_{ME} from 4.4 to 17.5, values which include ratios ascribed to both EOP I and EOP II. These data suggest that the R_{ME} ratio is an unreliable criterion for distinguishing between EOP I and EOP II, and further, they suggest that no distinction can be made between glumitocin and either EOP I or EOP II on this basis.

It may be concluded that a critical assessment of the data available at this time fails to show a clear distinction between the principles EOP I, EOP II, glumitocin, and E_2, and suggests that the wide variety of elasmobranch neutral principles may possibly be reduced to only two - E_1, which is clearly differentiated from E_2 and EOP I by its chromatographic behavior (Heller and Pickering, 1961; Heller and Roy, 1965a; 1965b; Roy, 1969) but which is not always present (Perks, 1966; Sawyer, 1967; Swiatkiewicz, 1968; data presented here), and glumitocin. However, the limited criteria available at present may fail to distinguish all peptides which might exist within the pituitaries of the different species.

2. The Identity of the Neurohypophysial Principles of Raia rhina

Although the classical methods of biological assay have yielded much important information on neurohypophysial principles, this study has shown that inconsistencies may occur in the values obtained, since some assay preparations may make anomalous distinctions between the different chemical structures of the unknown and of the standard. However this can be turned to advantage in establishing the identity of an unknown by comparing it directly with synthetic peptides other than oxytocin (Syntocinon). Two identical peptides, assayed one against the other, will have the same relative activities on every assay preparation, and on each type of assay. If the peptides are not identical, however, the activity of the one relative to the other may well vary between assays.

The advent of synthetic glumitocin made it possible for the skate principle to be assayed against a molecule of possibly identical structure, and the results of these direct assays suggested that glumitocin is in fact the neutral principle in this species (see Chapter 3, Section IB3, page 82). The amino acid analyses of the neutral principle (Table IX, page 99) strongly supported the pharmacological evidence that the peptide is glumitocin (see Chapter 3, Section IC4, page 98).

The identification of the R. rhina principle as glumitocin, when it had first appeared to correspond to the EOP I principle, is consistent with the suggestion that the variety of elasmobranch principles might possibly be reduced to two - E_1 and glumitocin. However it should be noted that the Squalus principle, which had also appeared to be EOP I, was found here to be neither glumitocin nor E_1. It appeared to consist of a mixture of two peptides, neither of which has been previously demonstrated. This will be discussed in later sections.

A small amount of basic principle was separated away from the neutral peptide of Raia rhina. It was not subjected to further study; however, it was tentatively suggested to be arginine vasotocin on the basis of the similarity of its chromatographic behavior to that of the arginine vasotocin found in Squalus extracts and on the ubiquity of arginine vasotocin as the basic peptide in non-mammalian vertebrates (Chapter 3; Section I C3b). The presence of arginine vasotocin in the R. rhina extract would account for 9.5% of the total oxytocic activity (arginine vasotocin: $R_{ADH} = 2.0$; Sawyer, 1961) but it would account for only 0.7% of the total peptide by weight in a corresponding mixture of arginine vasotocin and glumitocin.

II. STUDIES OF THE NEUROHYPOPHYSIAL PRINCIPLES OF THE DOGFISH, *SQUALUS ACANTHIAS* (PACIFIC VARIETY)

1. The Identity of the Basic Principle of Squalus acanthias

The amount of basic peptide present in the extract was relatively very small, approximately 2% of the total oxytocic activity which was eluted from the CM Sephadex. It resembled arginine vasotocin both chromatographically (Fig. 8, page 128) and pharmacologically (Table XVI A, page 138). Arginine vasotocin is notable for the remarkably high and characteristic value of its relative frog bladder activity. The *Squalus* principle showed a similarly high activity on the frog bladder when the assays were performed against the usual oxytocin standard (Syntocinon).

This evidence confirmed Sawyer's observations on the same species (Sawyer, 1967). The identity of the *Squalus* basic peptide with arginine vasotocin was further substantiated by the more sophisticated technique of direct pharmacological comparison with the synthetic peptide (Table XVI B), a method which had not been carried out previously. The activity of the *Squalus* principle relative to synthetic arginine vasotocin was constant on the rat uterus, both in the presence and absence of magnesium, on the antidiuretic assay, and on the frog-bladder assay. These results indicate that the *Squalus* basic neurohypophysial peptide is almost certainly identical to arginine vasotocin. However, chemical confirmation is still required for complete certainty.

Arginine vasotocin is the basic peptide elaborated by other non-mammalian vertebrates, usually in higher proportions than are found in elasmobranchs. In Squalus the presence of arginine vasotocin would account for only 2.3% of the total neurohypophysial activity (rat uterus activity), and only 2.4% of the total weight of neurohypophysial peptide present.

2. Considerations of the Identity of the Neutral Principle of Squalus acanthias

a. Behavior of the neutral fraction on CM Sephadex

The nature of the Squalus neutral principle is more elusive. It was eluted from CM Sephadex in two fractions (Fig. 9, 12a; page 133, 154). However these two fractions were indistinguishable from one another by any method that was attempted (see Chapter 3, Section IIC7, page 141).

The apparent similarity of the principle in each of the two fractions was substantiated when fraction 1 (Preparation 4) was reapplied to a CM Sephadex column in a much smaller volume than had been used in the first purification (100 ml instead of 2485 ml); subsequently, it eluted in the same position as its sister peak, fraction 2 (Fig. 12b; page 154). Further, it could be shown that chromatography of a single peptide, applied to the column in large volume, resulted in the production of two artifact peaks. This was demonstrated when the neutral fraction of Raia rhina, postu-

lated to be the single principle glumitocin, was also split into two active peaks by the original procedure. Because of these considerations, the two neutral fractions separated from Squalus extracts (Preparation 4) by the use of CM Sephadex were combined for subsequent study; this allowed sufficient material for the possibility of chemical analysis.

b. Chromatographic and pharmacological characteristics of the neutral principle

The pharmacological activity ratios of the crude extracts (Table X, page 106) and of the purified neutral fraction (Table XVII, page 145) suggested the neutral principle is an EOP I type of elasmobranch peptide which, as discussed in the previous section (Section I, above) is not clearly distinguished from glumitocin and might possibly be identical to it. However the direct pharmacological comparison of the purified agent against synthetic glumitocin (Table XIX, page 157) showed that the two principles were different. This confirmed the results of Sqwyer et al. (1969) on the same species, Atlantic variety, and it is important because it suggests that glumitocin is not a neutral principle common to all elasmobranchs.

A first alternative to the identification of the Squalus neutral principle with glumitocin, is that the unknown may be a mixture of the two principles, E_1 and E_2, as found by Heller and his co-workers in many elasmobranch species. E_1 and E_2 have been distinguished by their different mobilities

on paper chromatograms (developed in n-butanol:acetic acid: water = 4:1:5); in some species they have also differed in their magnesium potentiations, so far as this criterion is reliable (see Table III, page 33). However, there was no evidence for the presence of the chromatographically slow-moving component, E_1, in the Pacific variety of Squalus acanthias, as the following evidence will show:

1. Paper chromatograms of:

a) Squalus crude extract (Fig. 5b, page 115),

b) Squalus neurohypophysial material partially purified by filtration through Sephadex G-15 gel (Fig. 5d), and

c) the Squalus neutral fraction after elution from CM Sephadex - both the fraction 1 component (Fig. 5e) and the fraction 2 component (Fig. 5f) - were developed in n-butanol:acetic acid:water = 4:1:5. All showed only one area of oxytocic activity, from R_F 0.4 to 0.7, the position characteristic of EOP I and of E_2. The chromatograms will be discussed in turn:

a. The glands had been extracted at a high concentration (40 mg dried tissue/ml) and it was possible that the protein content of the crude extract prevented a clear resolution of E_1 from E_2 on the chromatogram. However, there was no evidence for the presence of E_1 in the tailing edge of the active peak, as judged by the magnesium potentia-

tions of the eluates (see Chapter 3, Section IIB2). Although magnesium potentiation values have been shown to be an unreliable criterion for comparing elasmobranch agents, it is still necessary to use these results in discussion in order to relate the present findings to work previously published.

b. The amount of Sephadex G-15-purified material loaded onto the chromatogram was high (1067 mU), and in this case only, a small amount of oxytocic activity (1% of the total) was found in the eluate of R_F 0.3-0.4. This activity had the pharmacological characteristics of arginine vasotocin (Table XVIA, page 138). E_1 had been reported in a similar position, R_F 0.35-0.45, on a paper chromatogram of Squalus extract (Heller and Pickering, 1961). In the present case, it is unlikely that the small amount of arginine vasotocin postulated in R_F 0.3-0.4 could have masked the presence of E_1 in the same eluate, for the following reasons:

i. The activity in R_F 0.3-0.4 accounted for only 1% of the total rat uterus activity (no Mg^{++} present) recovered from the chromatogram, and E_1 is reported to occur in much larger amounts - from one third of the total activity (Pickering, personal communication to Perks, 1960) to one half of the total activity (Roy, 1969).

ii. The presence of E_1 as a high proportion of the total activity (one third to one half) would

have been expected to lower the ratios R_{FB} and R_{ADH} of the eluate of the slow-running component (R_F 0.3-0.4) to values below those of arginine vasotocin. This was not the case (Table XVIA, page 138).

iii. The slow-moving E_1 agent has been reported in positions as low as R_F 0.04 upon paper chromatography of neurohypophysial material from other elasmobranch species (ex. Raia clavata; Heller and Roy, 1965a). No activity was detected in R_F 0-0.3 of the Squalus chromatogram. While the E_1 principle might have been confined to one R_F unit, R_F 0.3-0.4, this would be somewhat surprising in view of the size of the load which was applied to the chromatogram and the proportion in which E_1 is reported to occur.

iv. Paper chromatograms of the neutral principle, after it had been separated from the arginine vasotocin on CM Sephadex, showed no activity in R_F 0.3-0.4.

2. It should be noted that the two neutral fractions which were eluted from CM Sephadex did not correspond to the E_1 and E_2 principles. In fact, they were indistinguishable, and neither had the high magnesium potentiation sometimes characteristic of E_1 (Table XVII, page 145), nor exhibited its slow-running chromatographic behavior (Fig. 5, e and f; page 115).

3. There is the possibility that the E_1

principle of Squalus might have been lost in the processing of the glands or of the extract. This is unlikely, for the following reasons:

a. Only about 4% of the total oxytocic activity (rat uterus activity, without Mg^{++}) of the glands was lost into the acetone in which they were collected (Table XI, page 110). Further, this activity did not differ, by the criteria which distinguishes E_1 from E_2, from the material which was later extracted from the glands by acetic acid. It resembled the crude acetic acid extract in its magnesium potentiation value of approximately 2 and in its fast-running behavior upon paper chromatography in n-butanol:acetic acid: water = 4:1:5 (Table XII, page 112; Fig. 5, b and c, page 115). In contrast, E_1 is chromatographically slow-running and in some species it may be distinguished from the E_2 principle by its higher R_{Mg} values (Table III, page 33). There was no evidence for an incomplete separation of E_1 from E_2 on the chromatogram of the acetone soluble fraction, as judged by the magnesium potentiation of the eluates, so far as this is admissable evidence (see Chapter 3, Section II B2).

b. During the passage of the extract through Sephadex G-15, approximately 1.4% of the oxytocic activity was eluted with the Lowry peptide peak in advance of the main oxytocic peak (Fig. 7, page 123). It is unlikely that this material could account for the "missing" E_1 principle since

E_1 is reported to occur in amounts approximately equal to those of the E_2 principle (Pickering, personal communication to Perks, 1960; Roy, 1969). Also, it is unlikely that a mixture of two neutral neurohypophysial principles such as E_1 and E_2 would have been resolved on Sephadex G-15 when, as demonstrated by subsequent paper chromatography and ion exchange chromatography of the main eluate peak, the basic peptide, arginine vasotocin, had remained in association with the neutral fraction throughout the gel filtration procedure (Figs. 8 and 9, pages 128 and 133).

c. Amino acid analyses of the neutral fraction.

The pharmacological and chromatographic characteristics of the Squalus neutral principle demonstrated that it is neither oxytocin, glumitocin, nor a mixture of the E_1 and E_2 principles. However, the amino acid analyses of the purified material (Table XX) do suggest that it may be a mixture of two neurohypophysial peptides, present in equal amounts and having the high average potency of 109 IU/mg.

It is not likely that all the amino acids in the hydrolysate would have occurred together in one very long peptide. Such a structure has not been found among naturally occurring neurohypophysial hormones. Furthermore, studies of synthetic peptides with slightly elongated molecules show that additional amino acid residues in the chain lower the potency (Berde and Boissonnas, 1966), and the activity of

the Squalus neutral fraction is high.

It is possible that the amino acid analyses reflected only one neurohypophysial peptide, together with another contaminating peptide or with free amino acids which had not been separated away during the purification. However, the following evidence is strongly in favour of a "pure" preparation of a peptide mixture:

1. The analysis of an unhydrolyzed sample of purified material revealed only small amounts of two free amino acids - serine and glycine (Table XXI, page 161) - and the analysis of the corresponding hydrolyzed sample (Table XX, column 2, page 160) has been corrected for these two residues.

2. The HCl used in the hydrolyses was also analyzed for free amino acids, and only minimal amounts were detected (Table XXII, page 163).

3. The amino acids in the hydrolysates were present in a molar ratio to glutamic acid of 2:1 or 1:1. Although not impossible, it would be a notable coincidence if the free amino acids, or acids due to another non-active peptide, happened to be present in such precise amounts.

4. In this and other laboratories, purification procedures similar to those followed here have been used successfully to produce effectively pure neurohypophysial

preparations. In fact, the present procedure included one more step - the final paper partition chromatography - than was required to prepare pure isotocin from salmon (Wilson and Smith, 1969), and two more steps - chromatography on SE Sephadex and on paper - than were needed to isolate glumitocin from another elasmobranch, Raia ocellata (Perks and Sawyer, 1965; also see Sawyer et al., 1969).

5. The analysis of the neutral fraction from Perparation 4 after its elution from the final paper chromatogram (Table XX, column 3, page 160) was similar to the analysis made after the preceeding chromatography on SE Sephadex (Table XX, column 2). It would be most surprising if a non-neurohypophysial contaminating peptide showed both the same electrostatic behavior as the neurohypophysial principle upon ion exchange chromatography at two different pH's - 5.0 and 2.45 -, and also the same relative solubility in the solvent system used for the partition chromatography on paper.

6. The average specific activity of the peptide mixture is 109 IU/mg. This is in the range of activity of two other naturally occurring neutral analogues of oxytocin - mesotocin, 290 IU/mg; and isotocin, 150 IU/mg (Sawyer, 1965) - and is much higher than the activity of glumitocin, 8 IU/mg (Table VIII, page 88). It is also within the range of, or higher than, the activity of the many

synthetic analogues which have been studied, with the exception of desamino-oxytocin (Sawyer, 1965; Chan et al., 1963). This high average activity speaks for a "pure" preparation; it also indicates that if glumitocin were itself present, it would be mixed with a further peptide of remarkably high activity.

7. Recently Sawyer (personal communication) has resolved two different samples of the purified Squalus neutral fraction into two components using partition chromatography on Sephadex columns. One of the samples was prepared in Sawyer's laboratory with neurointermediate lobes taken from Squalus acanthias (Atlantic variety) caught off the coast of Virginia, and the other, Squalus acanthias (Pacific variety), was purified in this laboratory, a part of Preparation 1. The separations were achieved in three different solvent systems:

1) 1-butanol:1.5% pyridine in 3.5% acetic acid = 1.1
2) 1-butanol:ethanol:1.5% pyridine in 3.5% acetic acid = 7:2:9
3) 1-butanol:ethanol:1.5% pyridine in 3.5% acetic acid = 17:1:18

Whether or not these two components correspond to the two peptides in the mixture reported here has not been established. However the independent resolution of the neutral fraction of Preparation 1 into two components by column partition

chromatography provides powerful support for the existence of a peptide mixture, as suggested here.

It is surprising that the powerful electrofocusing technique did not achieve a resolution of the two possible peptides. It is possible that the two have identical isoelectric points. Another explanation is that one peptide is less active than the other. This would give rise to two possibilities. Firstly, while there was no evidence of "notching" in the oxytocic peak (Fig. 11, page 150), it is still possible that there was a partial resolution of two principles of unequal activity. Secondly, the recovery of oxytocic activity from the experiment suggested that if the ratio of the specific activities of the two peptides (which amino acid analysis suggested were equimolar) were in the order of 10:1, the less active principle, which may have been well separated from the more active one, would have escaped detection on the rat uterus assay.

There have been other reports of a mixture of neutral peptides occurring within a single species. For example, analysis of the neutral fraction from the cobra, Naja naja, has revealed the amino acids of an equimolar mixture of oxytocin and mesotocin, and its biological activity ratios support this (Pickering, 1967). Pharmacological activity ratios suggest that other reptiles, the grass snake, green turtle, and Loggerhead turtle, may also elaborate an equimolar mixture of oxytocin and mesotocin (Follett, 1967).

Within certain elasmobranch species, including Squalus acanthias, two neutral principles E_1 and E_2 have been reported (Heller and Pickering, 1961; Heller and Roy, 1965a; 1965b; 1967; Roy, 1969), although others (Perks, 1966; Sawyer, 1967; Swiatkiewicz, 1968; data presented here) have been unable to repeat this chromatographic resolution, either in the case of Squalus or of other elasmobranch species. It was demonstrated above that the peptide mixture suggested here does not correspond to a mixture of E_1 and E_2. Also, it has already been noted that the two peptides suggested by the amino acid analyses do not correspond individually to the two neutral peaks which were eluted off the CM Sephadex.

d. Theoretical considerations and speculations of possible structure

At this time the amino acid content and molecular structure of each of the two possible peptides can only be inferred, and the following must be regarded as speculation. Taking the amino acid sequence of the known naturally occurring peptides as a guide, the duplicate amino acid residues found in the Squalus neutral fraction can be arranged into two peptides with the following structure:

1 2 3 4 5 6 7 8 9
cys-tyr---------asp(NH_2)-cys-pro-----gly(NH_2)

Since the oxytocic activity of Squalus neurointermediate lobe extract is entirely destroyed by incubation

with 0.01 M sodium thioglycollate (Perks and Dodd, 1963b; Swiatkiewicz, 1968), the normal cystine ring is assumed to be present in both peptides. Positions 3, 4, and 8 would then remain unfilled, and it is in these positions that amino acid substitutions have taken place in the naturally occurring analogues of oxytocin. The six remaining residues, serine, glutamine, alanine, valine, leucine, and isoleucine, would be distributed among these six unoccupied positions.

Glumitocin, already known to be an elasmobranch peptide, might be considered as a first possibility for one of the Squalus principles. The necessary amino acids for the structure 3-isoleucine, 4-serine, and 8-glutamine were present in the analysis. However there is the following pharmacological and chromatographic evidence against the presence of glumitocin. Glumitocin has a low activity of approximately 10 IU/mg and an $R_{FB} = 0.8 \pm 0.1$ (Sawyer, personal communication, 1968). Therefore its companion peptide (if present in equimolar amount, as indicated by the amino acid analyses) must have an activity of approximately 208 IU/mg and an R_{FB} of at least 7 for the average Squalus activity of 109 IU/mg and even the minimum R_{FB} value observed for the neutral fraction ($R_{FB} = 6.9$; Table XVII, page 145) to be realized. Similar high R_{FB} values for the neutral fraction of Squalus have been reported previously ($R_{FB} = 3.0$ or 4.2; Sawyer, 1965; 1967). With the exception of arginine vasopressin, all the oxytocin analogues with an R_{FB} value greater than one have an intact

oxytocin ring, and those with an R_{FB} of less than one do not do so (Sawyer et al., 1961; 1970). Therefore an intact oxytocin ring would be expected in the second peptide. However the presence of both glumitocin (3-isoleucine, 4-serine, 8-glutamine) and a second peptide with the oxytocin ring structure (3-isoleucine, 4-glutamine, 8-alanine/valine/leucine) is incompatable with the Squalus amino acid content.

More direct evidence against the identity of one of the Squalus components with glumitocin comes from partition chromatography on Sephadex columns. Sawyer (personal communication) has shown that glumitocin runs with a different R_F from the two Squalus components in the three solvent systems listed above (page 188).

Three other naturally occurring neutral peptides, oxytocin, mesotocin, and isotocin, remain as possibilities. Mesotocin and isotocin can be eliminated immediately. Each possesses a duplicate isoleucine residue and the Squalus analysis yielded only one. Oxytocin is also an ulikely candidate. Its activity of 450 IU/mg would mean an average Squalus activity of at least 225 IU/mg, even if the second peptide were almost inert; the observed value was only 109 IU/mg. Also, since the Squalus neutral fraction showed high relative frog bladder activity (R_{FB}=6.9, and 17.2; see Table XVII, page 145), the value of R_{FB}=1 for oxytocin would require a second almost inert peptide to have a very high R_{FB} value and therefore an intact oxytocin ring. The amino acids of

the Squalus neutral fraction cannot satisfy the requirements of two such rings. Further, Sawyer (1967) has demonstrated a difference between these three peptides and the Squalus neutral fraction during partition column chromatography in 1-butanol:benzene:pyridine:0.1% acetic acid = 6:2:1:9, despite the fact that the Squalus neutral fraction moved as a single peak in this solvent system.

Therefore it appears that neither of the possible Squalus peptides has been demonstrated previously among the known neurohypophysial hormones. In view of the high Squalus R_{FB} value, one of the peptides might be assumed to have an intact oxytocin ring (Sawyer et al., 1961; 1970; Sawyer, 1965; 1967). The amino acids found in Squalus allow for one of a possible four such analogues, 8-serine, 8-valine, 8-alanine, or 8-leucine oxytocin. The 8-leucine peptide is oxytocin itself, and as discussed above, it is probably not present. Of the three remaining analogues, all have an activity of at least 140 IU/mg, an R_{FB} value greater than one, and a low R_{ADH} value (Sawyer et al., 1970).

8-alanine oxytocin, with an activity of approximately 154 IU/mg and $R_{FB} = 2$ is the least satisfactory possibility since its accompanying peptide would be expected to have an activity of 64 IU/mg and an R_{FB} of at least 19, and again the amino acids found in Squalus do not permit two intact oxytocic rings. Furthermore, Sawyer (personal communication)

has shown that 8-alanine oxytocin runs with a different R_F from both of the Squalus components during partition column chromatography in the three different solvent systems listed above (page 188).

Possible variation in the specific activities and in the relative frog bladder activities of both the oxytocin analogues and the Squalus neutral fraction upon repeated assays, makes uncertain any speculation on the identity of the Squalus peptides. Therefore it is important that there is chromatographic evidence as well as a possible pharmacological basis for the theoretical elimination of these peptides - oxytocin, glumitocin, isotocin, mesotocin, and 8-alanine oxytocin - from the Squalus peptide mixture.

The remaining possibilities for a peptide with an intact oxytocin ring are 8-valine oxytocin and 8-serine oxytocin. These two molecules have not been shown to differ chromatographically from the Squalus neutral fraction and so the possibility of their presence in the Squalus peptide mixture can only be considered on pharmacological grounds. The 8-serine analogue has an activity of 145 IU/mg and an R_{FB} of 9 (Sawyer et al., 1970). The companion peptide might then be expected to have an R_{FB} of approximately 3 to account for all the frog bladder activity of the Squalus neutral fraction (R_{FB} = 6.9; Table XVII, page 145). However the relative frog bladder activity of the Squalus fraction has varied on repeated assays,

although its value for R_{FB} has always appeared greater than one. R_{FB} values of 6.9 and 17.2 were reported here (Table XVII) and lower values of 3.0 and 4.2 have been reported elsewhere (Sawyer, 1965; 1967). While the frog bladder activity of 8-serine oxytocin might be insufficient to account for all the frog bladder activity in an equimolar peptide mixture whose overall R_{FB} was 6.9, it could account for the total frog bladder activity of a mixture whose value for R_{FB} was 3. In this latter case the second peptide would be required to contribute very little frog bladder activity, and need not be presumed to possess an intact oxytocin ring.

The last peptide to be considered is 8-valine oxytocin. Two values have been reported for the specific activity of 8-valine oxytocin - 200 IU/mg and 265 IU/mg (Sawyer et al., 1970). The value of R_{FB} for this peptide is reported to be 6 (Sawyer et al., 1970). If the activity of the peptide were taken as the lower value of 200 IU/mg, then 8-valine oxytocin could account for all the frog bladder activity in an equimolar mixture of peptides characterized by an R_{FB} of 3.0 or 4.2, the lower values reported for the Squalus neutral fraction (Sawyer, 1965; 1967); it could not account for all the frog bladder activity if the R_{FB} of the peptide mixture were 6.9, the higher value for Squalus found here. The second peptide in the mixture would require a high value for R_{FB} of 17 (and an intact oxytocin ring) for an overall R_{FB} value of 6.9 to be

realized. If, on the other hand, the activity of 8-valine oxytocin were taken to be the higher value of 265 IU/mg, the peptide could account for all the frog bladder activity in a peptide mixture whose R_{FB} was 6.9; it could not account for all the frog bladder activity in a mixture characterized by higher values for this ratio, and a value for R_{FB} of 17.2 was also found here for the Squalus neutral fraction. (The values for R_{FB} of 6.9 and 17.2 were obtained for peak 1 and peak 2 of the neutral fraction, respectively; the neurohypophysial peptide content of the two peaks was considered identical and so the two values of R_{FB} are each considered to represent the entire neutral fraction). On the basis of the data available at the present time, it must be concluded that there is no clear pharmacological evidence for the elimination of either 8-valine oxytocin or 8-serine oxytocin from the postulated Squalus peptide mixture.

The limited chromatographic evidence is also inconclusive. On partition column chromatography in 1-butanol: ethanol:1.5% pyridine in 3.5% acetic acid = 17:1:18, 8-valine oxytocin runs with the same R_F as one of the peaks of the Squalus neutral fraction (Sawyer, personal communication). However not too much emphasis should be placed on this. In the same solvent system oxytocin runs with the same R_F as the other Squalus peak, and oxytocin is not considered to be a component of the Squalus mixture.

Both 8-valine and 8-serine oxytocin are feasible

elasmobranch peptides in terms of the genetic code. The most widespread of the neurohypophysial hormones is arginine vasotocin which occurs in all vertebrate classes and is the only one so far known to be elaborated by the cyclostomes. Because of this it is considered the most primitive of the peptides, the one which gave rise through mutation to the others, which are of more limited occurrence. According to the genetic code as presented by Vliegenthart and Versteig (1967), 8-serine oxytocin could have developed from arginine vasotocin as a result of one mutation. The formation of 8-valine oxytocin would have required an intermediate compound such as 8-glycine oxytocin or oxytocin itself (8-leucine oxytocin).

On the other hand oxytocin is also fairly widespread. It has been identified in mammals and birds and there are reports of its possible occurrence in reptiles, amphibians, lungfish, and in the holocephalian Hydrolagus collei. If it is assumed that the presence of the same peptide in two species indicates its presence in a common ancestor then it follows that oxytocin must have appeared before the dichotomy between the line leading to the cartilaginous fish on one hand and that leading to all other jawed vertebrates on the other (Sawyer, 1968). If oxytocin is indeed the primitive molecule, both 8-valine and 8-serine oxytocin could have arisen from it by a single mutation.

If one of the peptides should be 8-valine oxytocin, then the serine residue would be found in the second peptide.

Apart from glutamine the only amino acid in the 4 position of the naturally occurring neurohypophysial peptides is in fact serine, which is found in isotocin of the teleosts and in glumitocin of some elasmobranchs. The second peptide might then be expected to be a 4-serine analogue.

Should the first peptide prove to be 8-valine oxytocin and the second to have a 4-serine ring, then there are two possibilities for the structure of the second peptide. These are 3-leucine, 4-serine, 8-alanine oxytocin or else 3-alanine, 4-serine, 8-leucine oxytocin. A 3-leucine substitution greatly reduces activity, and an 8-alanine substitution also has this effect (Sawyer, 1965; Berde and Boissonnas, 1966); the unknown peptide accompanying 8-valine oxytocin would be expected to have low activity. The effect of a 3-alanine substitution on neurohypophysial peptide activity is not known; 8-leucine occurs in oxytocin itself and so this structure would not be expected to affect the activity of the peptide. 3-alanine, 4-serine, 8-leucine oxytocin is the more conservative molecule since both 4-serine and 8-leucine structures are known to occur naturally.

Alternatively, if the first peptide should be 8-serine oxytocin then the second peptide would have one of the six possible arrangements of leucine, valine, and alanine in positions 3, 4, and 8. Despite many interesting speculations, the true nature of the principles must remain unknown until

a final separation of the two possible components is achieved, probably by column partition chromatography.

Addendum: After the completion of this investigation, Acher et al. (1972) separated two oxytocic principles from Squalus acanthias by chromatoelectrophoresis. This study supported the presence of the mixture of principles found here. Amino acid analysis and partial Edman degradation suggested the following structures for these principles: 8-valine oxytocin ("valitocin") and 4-asparagine oxytocin ("aspartocin").

Ref: Ačher, R., J. Chauvet, and M.T. Chauvet. (1972). "Evolution moleculaire - identification de deux nouvelles hormones neurohypophysaires, la Valitocine (Val^{8}- ocytocine) et l'Aspartocin (Asn^{4}-ocytocine) chez un poisson selacien, l'Aiguillat (Squalus acanthias)," C.R.Acad.Sc. (Paris), 274:313-316

Chapter 5

SUMMARY

The vertebrate neurohypophysis generally contains at least two principles - a basic, antidiuretic peptide and a neutral, oxytocic agent (see Sawyer, 1968). The levels of activity in the elasmobranch neurointermediate lobe, as determined by the standard bioassay techniques, are so low (see Perks and Dodd, 1963a; Perks, 1966; this thesis, Tables IV and X) that early workers had difficulty in determining the presence of any neurohypophysial principles in these fish (Herring, 1908a; 1908b; 1913; 1915; Waring and Landgrebe, 1950). However, at the time this study was begun the existence of elasmobranch oxytocin-like agents had been firmly established, although their exact number and nature was uncertain; a little later, traces of a basic principle were observed in both skate and shark species (Maetz *et al.*, 1959; Sawyer *et al.*, 1961; Heller and Pickering, 1961; Perks and Dodd, 1963a; 1963b; Sawyer, 1965; Perks and Sawyer, 1965; Acher *et al.*, 1965; Chauvet *et al.*, 1965).

The neurohypophysial principles produced by two elasmobranch species - the skate, *Raia rhina*, and the dogfish shark, *Squalus acanthias* (Pacific variety) - were examined here.

The neurointermediate lobes of both species were found to contain low levels of activity, ranging from 12 to 32 mU/mg dried tissue (rat uterus activity, no Mg^{++}) for Squalus, and from 1 to 3 mU/mg dried tissue (rat uterus activity, no Mg^{++}) in the case of Raia.

In both species a small amount of basic peptide could be separated from the major neutral fraction by chromotography on CM Sephadex. The basic principle of Squalus appeared to be the widespread peptide, arginine vasotocin. This identification was based on the chromatographic and pharmacological similarity of the principle to synthetic arginine vasotocin; in particular, it was based on the new technique of direct pharmacological comparison of the principle to synthetic arginine vasotocin. The basic principle was isolated from Raia in only trace amounts, and it was not studied further; however it was tentatively identified as arginine vasotocin on the basis of the similarity of its behavior on ion exchange chromatography to the basic principle of Squalus.

A variety of neutral neurohypophysial principles - EOP I, EOP II, glumitocin, the E_1 principles, and the E_2 principles - had been reported among the elasmobranchs. These principles have been distinguished on the basis of their magnesium potentiation values (R_{Mg}) or their relative milk-ejection activities (R_{ME}): glumitocin and the E_1 principle (in some species) have been characterized by their high values

for R_{Mg}, and EOP II has been distinguished from EOP I on the basis of its high R_{ME} value.

However, the observations made here may invalidate these distinctions. When a series of crude neurointermediate lobe extracts of Raia rhina were assayed on the rat uterus, both in the presence of magnesium ions and in their absence, the resulting magnesium potentiation values (R_{Mg}) were found to cover an unexpectedly wide range. Further investigation showed that the potency of a single extract could vary significantly, whether assayed on the rat uterus in the presence or in the absence of magnesium ions; when pairs of extracts which had shown widely different potentiations were re-assayed jointly on the same assay preparations, the potentiations of the two extracts converged to closely similar values. These results suggested that the variability in potency was due to anomalies in the distinction which different assay preparations may make between the unknown peptide and the oxytocin standard. Some variability was also observed in the milk-ejection potencies of both Raia rhina extract and of synthetic glumitocin, and this was reflected in the corresponding ratios of milk-ejection activity to rat uterus activity (R_{ME}).

The variability found in these two ratios, when they are obtained by independent "classical" methods of assay, suggests that while they can be used to distinguish neurohypophysial peptides from oxytocin itself, they are an

unreliable criterion for comparing two unknowns- at least in the case of elasmobranch extracts. Nevertheless assay methods could be used in establishing the probable identity of an unknown by comparing the principle directly with synthetic peptides other than oxytocin; two identical peptides, assayed side by side, would have the same relative activity on every assay preparation and on each type of assay.

The variability found here for the values of R_{ME} and R_{Mg}, together with the other data available at this time, raised the interesting possibility that EOP I, EOP II, E_2, and glumitocin might prove to be the same, and that the variety of elasmobranch principles might be reduced to only two - E_1 and glumitocin. However, it must be noted that although the later studies on Raia were consistent with this possibility, the studies on Squalus indicated that its neutral fraction consisted of a mixture of two peptides, neither of which has been previously demonstrated.

There was no evidence for the presence of the chromatographically slow-moving agent, E_1, in either species studied here, although this principle had been reported to occur both in other species of skate, and in Squalus acanthias (Heller and Pickering, 1961; Heller and Roy, 1965a). Amino acid analyses of the Raia rhina principle indicated the presence of a single peptide, glumitocin. Paper chromatography of both Squalus crude extract and the purified Squalus neutral

fraction in n-butanol:acetic acid:water = 4:1:5 (the solvent system used by Heller to resolve E_1 from E_2) showed a single fast-running oxytocic peak, with no evidence for the presence of the E_1 principle in its tailing edge. In one case there was a trace of slower-running principle, but this agent showed the pharmacological characteristics (in particular, the high relative frog bladder activity) of arginine vasotocin. It was possible that an E_1 fraction could have been lost during the processing of the glands or of the extract. However, the small amount of activity which was lost during these procedures could not account for the high proportion of the total activity which has been attributed to the E_1 principle.

The results of the study of the neutral principle of Raia rhina were consistent with a single structure for EOP I and glumitocin. On first pharmacological examination the agent appeared to be of the EOP I type, which has been distinguished from the EOP II principle by its lower values for R_{ME}. Further pharmacological study, including direct comparison of the extract to the synthetic peptide, purification studies, and amino acid analyses, strongly indicated that this principle was glumitocin. Glumitocin had previously been identified in other species of skate.

The neutral fraction of the dogfish Squalus acanthias appeared to differ from glumitocin and from all other neurohypophysial principles which have been identified in nature.

Initially, pharmacological studies suggested that it resembled the neutral principle found in many species of sharks and in most skates, and designated as EOP I. However, while the EOP I principle of certain skates proved to be glumitocin, direct comparison between the purified Squalus fraction and synthetic glumitocin showed clearly that these two principles were not identical. Amino acid analyses of the purified neutral fraction suggested that it consisted of an equimolar mixture of two peptides. It should be noted that these two peptides would not correspond to a mixture of E_1 and E_2; there was no evidence for the presence of the E_1 principle in the extract. Also, the two peptides suggested by the amino acid analyses do not correspond individually to the two neutral peaks which were eluted off the CM Sephadex; no difference could be shown between the neurohypophysial activities of the two CM Sephadex peaks and so they were combined for further analyses. Chromatographic data and theoretical considerations suggested that neither of the two postulated Squalus principles was glumitocin or any other known neurohypophysial peptide. The high relative frog bladder activity of the neutral fraction suggested that one of the two principles might possess an intact oxytocin ring, and based on the limited data available at present, 8-valine oxytocin or 8-serine oxytocin were suggested as possible structures for this peptide. However, until the two postulated principles can be separated and individually analyzed, their true natures must remain unknown,

and any inferences that might be drawn concerning their structure must be regarded as speculation.

The presence of glumitocin in the skates and of a mixture of two new peptides in the dogfish shark raises several interesting possibilities. Firstly, there may be a wide-spread difference between the principles produced by the skates and the sharks. Secondly, it is possible that not only the skates, but also those other species which have a diffuse type of pars nervosa (see Meurling, 1967a) may synthesize glumitocin, while species such as Squalus which have a discrete pars nervosa structure (see Meurling, 1962) may elaborate a mixture of peptides. However, studies on the neurohypophysial principles produced by many more elasmobranch species is required before any such generalizations will be possible.

BIBLIOGRAPHY

Acher, R., and J. Chauvet. (1953). "La structure de la vasopressine de boeuf," Biochim. Biophys. Acta, 12: 487-488.

Acher, R., J. Chauvet, M. T. Lenci, F. Morel, and J. Maetz. (1960). "Présence d'une vasotocine dans la neurohypophyse de la grenouille (Rana esculenta L.)," Biochim. Biophys. Acta, 42:379-380.

Acher, R., J. Chauvet, M. T. Chauvet, and D. Crepy. (1961). "Les hormones neurohypophysaires des poissons: isolement d'une vasotocine du tacaud (Gadus luscus L.)," Biochim. Biophys. Acta, 51:419-420.

Acher, R., J. Chauvet, M. T. Chauvet, and D. Crepy. (1962). "Isolement d'une nouvelle hormone neurohypophysaire, l'isotocine, présente chez les poissons osseux," Biochim. Biophys. Acta, 58:624-625.

Acher, R., J. Chauvet, M. T. Chauvet, and D. Crepy. (1964). "Phylogénie des peptides neurohypophysaires: isolement de la mesotocine (ileu 8- ocytocine) de la grenouille, intermédiaire entre la ser 4- ileu 8- ocytocine des poissons osseux et l'ocytocine des mammifères," Biochim. Biophys. Acta, 90:613-615.

Acher, R., J. Chauvet, M. T. Chauvet, and D. Crepy. (1965). "Phylogénie des peptides neurohypophysaires: isolement d'une nouvelle hormone, la glumitocine (ser 4- gln 8- ocytocine) présente chez un poisson cartilagineux, la raie (Raia clavata)," Biochim. Biophys. Acta, 107: 393-396.

Acher, R., J. Chauvet, and M. T. Chauvet. (1967). "Phylogeny of the neurohypophysial hormones," Nature, 216:1037-1038.

Acher, R., J. Chauvet, and M. T. Chauvet. (1969). "The neurohypophysial hormones of reptiles: comparison of the viper, cobra, and elaphe active principles," Gen. Comp. Endocrinol., 13:357-360.

Adams, H. S. (1917). "The thermal decomposition of the oxytocic principle of pituitary solution," J. Biol. Chem., 30:235-242.

Bailey, J. L. (1967). Techniques in Protein Chemistry. 2nd ed. Amsterdam: Elsevier, p. 342.

Bargmann, W. (1955). "Weitere Untersuchungen am neurosekretorischen Zwischenhirn-Hypophysensystem," Z. Zellforsch., 42:247-272. Cited by P. Meurling. (1962). "The relations between neural and intermediate lobes in the pituitary of Squalus acanthias," Z. Zellforsch., 58:51-69.

Bargmann, W. (1960). "The neurosecretory system of the diencephalon," Endeavour, 19:125-133.

Bargmann, W., and E. Scharrer. (1951). "The site of origin of the hormones of the posterior pituitary," American Scientist, 39:255-259.

Baumgartner, E. A. (1915). "The development of the hypophysis in Squalus acanthias," J. Morphol., 26:391-446.

Beauvillain, A. (1943). "Titrage des préparations post-hypophysaires par leur seuil d'action sur l'utérus isolé de rat adulte," Compt. Rend. Soc. Biol., 137: 284-285.

Bentley, P. J. (1958). "The effects of neurohypophysial extracts on water transfer across the wall of the isolated urinary bladder of the toad Bufo marinus," J. Endocrinol., 17:201-209.

Bentley, P. J. (1965). "The potentiating action of magnesium and manganese on the oxytocic effect of some oxytocin analogues," J. Endocrinol., 32:215-222.

Berde, B., and R. A. Boissonnas. (1966). "Synthetic analogues and homologues of the posterior pituitary hormones," The Pituitary Gland (G. W. Harris and B. T. Donovan, eds.). London: Butterworth. Vol. 3, pp. 624-661.

Bergmann, M., and J. S. Fruton. (1941). "The specificity of proteinases," Advan. Enzymol., 1:63-98.

Braak, H. (1962). "Über die Gestalt des neurosekretorischen Zwischenhirn-Hypophysen-Systems von Spinax niger," Z. Zellforsch., 58:265-276.

Brownlee, K. A. (1965). Statistical Theory and Methodology in Science and Engineering. New York: Wiley, pp. 299-303.

Chan, W. Y., M. O'Connell, and S. R. Pomeroy. (1963). "Effects of the estrous cycle on the sensitivity of rat uterus to oxytocin and desamino-oxytocin," Endocrinol., 72:279-282.

Chauvet, J., M. T. Lenci, and R. Acher. (1960a). "L'ocytocine et la vasopressine du mouton: reconstitution d'un complexe hormonal actif," Biochim. Biophys. Acta, 38:266-272.

Chauvet, J., M. T. Lenci, and R. Acher. (1960b). "Présence de deux vasopressines dans la neurohypophyse de poulet," Biochim. Biophys. Acta, 38:571-573.

Chauvet, J., M. T. Chauvet, and R. Acher. (1963). "Les hormones neurohypophysaires des mammifères: isolement et characterisation de l'ocytocine et de la vasopressine de la baleine (Balaenoptera physalus L.)," Bull. Soc. Chim. Biol., 45:1369-1378.

Chauvet, J., M. T. Chauvet, D. Beaupain, and R. Acher. (1965). "Les hormones neurohypophysaires des raies: comparison des hormones du pocheteau blanc (Raia batis) et de la raie bouclée (Raia clavata)," Compt. Rend. Acad. Sc. Paris, 261:4234-4236.

Christ, J. F. (1966). "Nerve supply, blood supply, and cytology of the neurohypophysis," The Pituitary Gland (G. W. Harris and B. T. Donovan, eds.). London: Butterworth. Vol. 3, pp. 62-130.

Colbert, E. H. (1955). Evolution of the Vertebrates. Science editions. New York: Wiley, 1961, p. 49.

Dale, H. H. (1906). "On some physiological actions of ergot," J. Physiol. (London), 34:163-206.

Dale, H. H. (1909). "The action of extracts of the pituitary body," Biochem. J., 4:427-447.

Dicker, S. E. (1953). "A method for the assay of very small amounts of antidiuretic activity with a note on the antidiuretic titre of rat's blood," J. Physiol. (London), 122:149-157.

Dudley, H. W. (1920). "Some observations on the active principles of the pituitary gland," J. Pharmacol. Exptl. Therap., 14:295-312.

Du Vigneaud, V. (1956). "Trail of sulfur research: from insulin to oxytocin," Science, 123:967-974.

Du Vigneaud, V., C. Ressler, and S. Trippett. (1953). "The sequence of amino acids in oxytocin, with a proposal for the structure of oxytocin," J. Biol. Chem., 205: 949-957.

Du Vigneaud, V., C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis, and S. Gordon. (1953). "The synthesis of an octapeptide amide with the hormonal activity of oxytocin," J. Am. Chem. Soc., 75:4879-4880.

Du Vigneaud, V., H. C. Lawler, and E. A. Popenoe. (1953). "Enzymatic cleavage of glycinamide from vasopressin and a proposed structure for this pressor-antidiuretic hormone of the posterior pituitary," J. Am. Chem. Soc., 75:4880-4881.

Ferguson, D. R. (1969). "The genetic distribution of vasopressins in the peccary (Tayassu angulatus) and warthog (Phacochoerus aethiopicus)," Gen. Comp. Endocrinol., 12:609-613.

Ferguson, D. R., and B. T. Pickering. (1969). "Arginine and lysine vasopressins in the hippopotamus neurohypophysis," Gen. Comp. Endocrinol., 13:425-429.

Follett, B. K., and H. Heller. (1964a). "The neurohypophysial hormones of bony fishes and cyclostomes," J. Physiol. (London), 172:74-91.

Follett, B. K., and H. Heller. (1964b). "The neurohypophysial hormones of lungfishes and amphibians," J. Physiol. (London), 172:92-106.

Ginsburg, M., and M. Ireland. (1966). "The role of neurophysin in the transport and release of neurohypophysial hormones," J. Endocrinol., 35:289-298.

Gorbman, A., and H. A. Bern. (1962). A Textbook of Comparative Endocrinology. New York: Wiley, p. 18.

Green, J. D. (1966). "Microanatomical aspects of the formation of neurohypophysial hormones and neurosecretion," The Pituitary Gland (G. W. Harris and B. T. Donovan, eds.). London: Butterworth. Vol. 3, pp. 240-268.

Heinicke, E., and A. M. Perks. (1969). "The magnesium potentiation of the oxytocic activity of pituitary extracts from an elasmobranch (Raia rhina)," Gen. Comp. Endocrinol., 12:168-171.

Heller, H. (1941a). "The distribution of the pituitary antidiuretic hormone throughout the vertebrate series," J. Physiol. (London), 99:246-256.

Heller, H. (1941b). "Differentiation of an (amphibian) water balance principle from the antidiuretic principle of the posterior pituitary gland," J. Physiol. (London), 100:125-141.

Heller, H. (1963). "Pharmacology and distribution of neurohypophysial hormones," Symp. Zool. Soc. London, 9:93-106.

Heller, H., and K. Lederis. (1959). "Maturation of the hypothalamo-neurohypophysial system," J. Physiol. (London), 147:299-314.

Heller, H., and B. Pickering. (1961). "Neurohypophysial hormones of non-mammalian vertebrates," J. Physiol. (London), 155:98-114.

Heller, H., and B. P. Roy. (1965a). "Elasmobranch neurohypophysial hormones," J. Physiol. (London), 177: 50P-51P. (Abstract).

Heller, H., and B. P. Roy. (1965b). "The neurohypophysial hormones of some elasmobranch fishes," Gen. Comp. Endocrinol., 5:683. (Abstract).

Heller, H., and B. P. Roy. (1967). "Differences between the pharmacological properties of the neurohypophysial hormones of selachian and holocephalian cartilaginous fishes," Gen. Comp. Endocrinol., 9:458. (Abstract).

Herring, P. T. (1908a). "The physiological action of extracts of the pituitary body and saccus vasculosus of certain fishes," Quart. J. Exptl. Physiol., 1:187-188.

Herring, P. T. (1908b). "A contribution to the comparative physiology of the pituitary body," Quart. J. Exptl. Physiol., 1:261-280.

Herring, P. T. (1911). "The development of the elasmobranch pituitary," Quart. J. Exptl. Physiol., 4:183-191.

Herring, P. T. (1913). "Further observations upon the comparative anatomy and physiology of the pituitary body," Quart. J. Exptl. Physiol., 6:73-108.

Herring, P. T. (1915). "The origin of the active material of the posterior lobe of the pituitary body," Quart. J. Exptl. Physiol., 8:245-265.

Hoar, W. S., and C. P. Hickman. (1967). A Laboratory Companion for General and Comparative Physiology. Englewood Cliffs, N. J.: Prentice-Hall, p. 290.

Hogben, L. T. (1925). "Studies on the pituitary-V. The avine depressor response," Quart. J. Exptl. Physiol., 15:155-161.

Hogben, L. T., and G. R. de Beer. (1925). "Studies on the pituitary-VI. Localization and phyletic distribution of active materials," Quart. J. Exptl. Physiol., 15:163-176.

Holton, P. (1948). "A modification of the method of Dale and Laidlaw for standardization of posterior pituitary extract," Brit. J. Pharmacol., 3:328-334.

Jeffers, W. A., M. M. Livezey, and J. H. Austin. (1942). "A method for demonstrating an antidiuretic action of minute amounts of Pitressin: statistical analysis of results," Proc. Soc. Exptl. Biol. Med., 50:184-188.

Katsoyannis, P. G., and V. du Vigneaud. (1958). "Arginine-vasotocin, a synthetic analogue of the posterior pituitary hormones containing the ring of oxytocin and the side chain of vasopressin," J. Biol. Chem., 233:1352-1354.

Krejčí, I., and I. Poláček. (1968). "Effect of magnesium on the action of oxytocin and a group of analogues on the uterus in vitro," Europ. J. Pharmacol., 2:393-398.

Kunkel, H. G., S. P. Taylor, and V. du Vigneaud. (1953). "Electrophoretic properties of oxytocin," J. Biol. Chem., 200:559-564.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. (1951). "Protein measurement with the folin phenol reagent," J. Biol. Chem., 193:265-275.

Maetz, J., F. Morel, and B. Lahlouh. (1959). "Natriferin: a new hormonal principle in the neurohypophysis of certain vertebrates," Nature, 184:1236-1237.

Mellinger, J. (1960). "Contribution à l'étude de la vascularisation et du développement de la région hypophysaire d'un sélacien, Scyliorhinus caniculus (L)," Bull. Soc. Zool. Fr., 85:123-139.

Mellinger, J. (1962). "Existence de plusieurs systèmes neurosécrétoires hypothalamo-hypophysaires chex les poissons eleasmobranches Scyliorhinus caniculus et Sc. stellaris. Microscopie ordinaire et microscopie électronique," Compt. Rend. Acad. Sc. Paris, 255: 1789-1791.

Mellinger, J. (1966). "Variations de la structure hypophysaire chez les chondrichthyens: étude de l'ange de mer (Squatina) et de la pastenague (Trygon)," Ann. Endocrinol. (Paris), 27:439-450.

Mellinger, J., E. Follenius, and A. Porte. (1962). "Présence de terminaisons neurosécrétoires sur les capillaires primaires du système porte hypophysaire de la roussette (Scyliorhinus caniculus). Etude au microscope électronique," Compt. Rend. Acad. Sc. Paris, 254:1158-1159.

Meurling, P. (1960). "Presence of a pituitary portal system in elasmobranchs," Nature, 187:336-337.

Meurling, P. (1962). "The relations between neural and intermediate lobes in the pituitary of Squalus acanthias," Z. Zellforsch., 58:51-69.

Meurling, P. (1963). "Nerves of the neuro-intermediate lobe of Etmopterus spinax (Elasmobranchi)," Z. Zellforsch., 61:183-201.

Meurling, P. (1967a). "The vascularization of the pituitary in elasmobranchs," Sarsia, 28:1-104.

Meurling, P. (1967b). "The vascularization of the pituitary in Chimaera monstrosa (Holocephali)," Sarsia, 30: 83-106.

Meurling, P. (1967c). "Observations of nerve-types in the hypophysial stem of Raja radiata," Acta Univ. Lundensis, Sect. II, 19:1-20.

Meurling, P. (1967d). "Studies of innervation and vascularization of the pituitary in chondrichthyes" (Ph.D. thesis, University of Lund).

Meurling, P. (1970). "The arrangement of neurosecretory and catecholamine fibers in relation to the pituitary intermedia cells of the skate, Raja radiata," Z. Zellforsch., 108:81-92.

Munsick, R. A. (1960). "Effect of magnesium ion on the response of the rat uterus to neurohypophysial hormones and analogues," Endocrinology, 66:451-457.

Munsick, R. A. (1964). "Neurohypophysial hormones of chickens and turkeys," Endocrinology, 75:104-112.

Munsick, R. A. (1966). "Chromatographic and pharmacologic characterization of the neurohypophysial hormones of an amphibian and a reptile," Endocrinology, 78: 591-599.

Munsick, R. A., W. H. Sawyer, and H. B. van Dyke. (1960). "Avian neurohypophysial hormones: pharmacological properties and tentative identification," Endocrinology, 66:860-871.

Munsick, R. A., and S. C. Jeronimus. (1965). "Effects of diethylstilbestrol and magnesium on the rat oxytocic potencies of some neurohypophysial hormones and analogues," Endocrinology, 76:90-96.

Norris, H. W. (1941). The Plagiostome Hypophysis, General Morphology and Types of Structure. Lancaster, U.S.A.: Science Press. Cited by P. Meurling. (1967d). "Studies of innervation and vascularization of the pituitary in chondrichthyes" (Ph.D. thesis, University of Lund).

Oliver, G., and E. A. Schäfer. (1895). "On the physiological action of extracts of pituitary body and certain other glandular organs," J. Physiol. (London), 18:277-279.

Ott, I., and J. C. Scott. (1910). "The action of infundibulin upon the mammary secretion," Proc. Soc. Exptl. Biol. Med., 8:48-49.

Paton, D. N., and A. Watson. (1912). "The actions of pituitrin, adrenalin, and barium on the circulation of the bird," J. Physiol. (London), 44:413-424.

Perks, A. M. (1966). "Pharmacological and chromatographic studies of the neurohypopysial activities of the pituitary of further elasmobranch species," Gen. Comp. Endocrinol., 6:428-442.

Perks, A. M. (1969). "The neurohypophysis," Fish Physiology (W. S. Hoar and D. J. Randall, eds.). New York: Academic Press. Vol. 2, pp. 112-205.

Perks, A. M., M. H. I. Dodd, and J. M. Dodd. (1960). "A neurohypophysial principle in the elasmobranch pituitary," Nature, 185:850-851.

Perks, A. M., and M. H. I. Dodd. (1963a). "Evidence for a neurohypophysial principle in the pituitary gland of certain elasmobranch species," Gen. Comp. Endocrinol., 3:286-299.

Perks, A. M., and M. H. I. Dodd. (1963b). "The properties of the oxytocic, milk ejection and antidiuretic principle of the neurointermediate lobe of the elasmobranch pituitary," Gen. Comp. Endocrinol., 3:184-195.

Perks, A. M., and W. H. Sawyer. (1965). "A new neurohypophysial principle in an elasmobranch Raia ocellata," Nature, 205:154-156.

Pickering, B. T. (1967). "The neurohypophysial hormones of a reptile species, the cobra (Naja naja)," J. Endocrinol., 39:285-294.

Pickering, B. T., and S. McWatters. (1966). "Neurohypophysial hormones of the South American lungfish Lepidosiren paradoxa," J. Endocrinol., 36:217-218.

Pickering, B. T., and H. Heller. (1969). "Oxytocin as a neurohypophysial hormone in the holocephalian elasmobranch fish, Hydrolagus collei," J. Endocrinol., 45:597-606.

Pickford, M. (1966). "Neurohypophysis and kidney function," The Pituitary Gland (G. W. Harris and B. T. Donovan, eds.). London: Butterworth. Vol. 3, pp. 374-398.

Polenov, A. L., and M. A. Belenky. (1965). "Electron microscope observations of neurosecretory elements in the neurointermediate lobe in skates," Nature, 208: 94-95.

Popenoe, E. A., H. C. Lawler, and V. du Vigneaud. (1952). "Partial purification and amino acid content of vasopressin from hog posterior pituitary glands," J. Am. Chem. Soc., 74:3713.

Rasmussen, H., and L. Craig. (1961). "The isolation of arginine vasotocin from fish pituitary glands," Endocrinology, 68:1051-1055.

Roy, B. P. (1969). "Distribution of neurohypophysial hormones in some elasmobranch species," Gen. Comp. Endocrinol., 12:326-338.

Rurak, D., and A. M. Perks. (1971). Unpublished observations.

Russell, J. A. (1965). "The hypophysis," Physiology and Biophysics, nineteenth edition (T. C. Ruch and H. D. Patton, eds.). Philadelphia: Saunders, pp. 1089-1108.

Sawyer, W. H. (1960). "Increased water permeability of the bullfrog (Rana catesbiana) bladder in vitro in response to synthetic oxytocin and arginine vasotocin and to neurohypophysial extracts from non-mammalian vertebrates," Endocrinology, 66:112-120.

Sawyer, W. H. (1964). "Vertebrate neurohypophysial principles," Endocrinology, 75:981-990.

Sawyer, W. H. (1965). "Active neurohypophysial principles from a cyclostome (Petromyzon marinus) and two cartilaginous fishes (Squalus acanthias and Hydrolagus collei)," Gen. Comp. Endocrinol., 5:427-439.

Sawyer, W. H. (1966). "Biological assays for neurohypophysial principles in tissues and in blood," The Pituitary Gland (G. W. Harris and B. T. Donovan, eds.). London: Butterworth. Vol. 3, pp. 288-306.

Sawyer, W. H. (1967). "Chromatographic and pharmacological characteristics of the active neurohypophysial principles of the spiny dogfish Squalus acanthias," Gen. Comp. Endocrinol., 9:303-311.

Sawyer, W. H. (1968). "Phylogenetic aspects of the neurohypophysial hormones," Handbuch der experimentellen Pharmakologie (O. Eichler et al., eds.). Berlin: Springer-Verlag. New Series, Vol. 23, pp. 717-747.

Sawyer, W. H. (1969). "The active neurohypophysial principles of two primitive bony fishes, the bichir (Polypterus senegalis) and the African lungfish (Protopterus aethiopicus)," J. Endocrinol., 44:421-435.

Sawyer, W. H., R. A. Munsick, and H. B. van Dyke. (1959). "Pharmacological evidence for the presence of arginine vasotocin and oxytocin in neurohypophysial extracts from cold-blooded vertebrates," Nature, 184:1463-1465.

Sawyer, W. H., R. A. Munsick, and H. B. van Dyke. (1961). "Pharmacological characteristics of the active principles in neurohypophysial extracts from several species of fishes," Endocrinology, 68:215-225.

Sawyer, W. H., R. J. Freer, and T. C. Tseng. (1967). "Characterization of a principle resembling oxytocin in the pituitary of the holocephalian ratfish (Hydrolagus collei) by partition chromatography on Sephadex columns," Gen. Comp. Endocrinol., 9:31-37.

Sawyer, W. H., M. Manning, E. Heinicke, and A. M. Perks. (1969). "Elasmobranch oxytocin-like principles: comparisons with synthetic glumitocin," Gen. Comp. Endocrinol., 12:387-390.

Sawyer, W. H., J. W. M. Baxter, M. Manning, E. Heinicke, and A. M. Perks. (1970). "A fraction resembling oxytocin from Squalus acanthias: pharmacological comparisons with synthetic peptides," Gen. Comp. Endocrinol., 15:52-58.

Scharrer, E. (1952). "Das Hypophysen-Zwischenhernsystem von Scyllium stellare," Z. Zellforsch., 37:196-204.

Scharrer, E., and B. Scharrer. (1945). "Neurosecretion," Physiol. Rev., 25:171-181.

Schild, H. O. (1942). "A method of conducting a biological assay on a preparation giving repeated graded responses illustrated by the estimation of histamine," J. Physiol. (London), 101:115-130.

Sloper, J. C. (1966). "The experimental and cytopathological investigation of neurosecretion in the hypothalamus and pituitary," The Pituitary Gland (G. W. Harris and B. T. Donovan, eds.). London: Butterworth. Vol. 3, pp. 131-239.

Smith, M. I., and W. T. McClosky. (1924). "Studies on the bio-assay of pituitary extracts: concerning the use of a desiccated infundibular powder as a standard in the physiological evaluation of pituitary extracts," U.S. Hygienic Laboratory Bulletin, 138:1-37.

Staples, R. E., and H. D. Geils. (1965). "An observation on the vaginal smear of the rat," J. Endocrinol., 32:263-264.

Stendell, W. (1913). "Zur vergleichenden Anatomie und Histologie der Hypophysis cerebri," Arch. f. mikr. Anat., 82. Cited by E. A. Baumgartner. (1915). "The development of the hypophysis in Squalus acanthias," J. Morphol., 26:391-446.

Stendell, W. (1914). "Die Hypophysis cerebri," Lehrbuch der vergleichenden mikroskopischen Anatomie der Wirbeltiere (A. Oppel, ed.). Jena: Gustav Fischer. Vol. 8, pp. 19-26. Cited by P. Meurling. (1962). "The relations between neural and intermediate lobes in the pituitary of Squalus acanthias," Z. Zellforsch., 58:51-69.

Sterzi, G. (1909). Il Sistema Nervoso Centrale dei Vertebrati, II:I:1. Padova: Draghi. Cited by P. Meurling. (1962). "The relations between neural and intermediate lobes in the pituitary of Squalus acanthias," Z. Zellforsch., 58:51-69.

Stewart, A.D. (1968). "Genetic variation in the neurohypophysial hormones of the mouse (Mus musculus)," J. Endocrinol., 41:xix-xx.

Svensson, H. (1961). "Isoelectric fractionation, analysis, and characterization of ampholytes in natural pH gradients. 1. The differential equation of solute concentrations at a steady state and its solution for simple cases," Acta Chem. Scand., 15:325-341.

Svensson, H. (1962a). "Isoelectric fractionation, analysis, and characterization of ampholytes in natural pH gradients. 11. Buffering capacity and conductance of isoionic ampholytes," Acta Chem. Scand., 16:456-466.

Svensson, H. (1962b). "Isoelectric fractionation, analysis, and characterization of ampholytes in natural pH gradients. 111. Description of apparatus for electrolysis in columns stabilized by density gradients and direct determination of isoelectric points," Arch. Biochem. Biophys., Suppl. 1:132-138.

Swiatkiewicz, V. J. (1967). "Studies of the oxytocic activity of the neurointermediate lobe of Squalus acanthias (Pacific variety)" (M.Sc. thesis, University of British Columbia).

Van Dyke, H. B., and A. B. Hastings. (1928). "The response of smooth muscle in different ionic environments," Am. J. Physiol., 83:563-577.

Van Dyke, H. B., B. F. Chow, R. O. Greep, and A. Rothen. (1942). "The isolation of a protein from the pars neuralis of the ox pituitary with constant oxytocic, pressor and diuresis-inhibiting activities," J. Pharmacol. Exptl. Therap., 74:190-209.

Van Dyke, H.B., K. Adamsons Jr., and S. L. Engel. (1955). "Aspects of the biochemistry and physiology of the neurohypophysial hormones," Recent Progress in Hormone Research, 11:1-41.

Vesterberg, O., and H. Svensson. (1966). "Isoelectric fractionation, analysis, and characterization of ampholytes in natural pH gradients. IV. Further studies on the resolving power in connection with separation of myoglobins," Acta Chem. Scand., 20:820-834.

Vizsolyi, E., and A. M. Perks. (1969). "New neurohypophysial principle in foetal mammals," Nature, 223:1169-1171.

Vliegenthart, J. F. G., and D. H. G. Versteeg. (1967). "The evolution of the vertebrate neurohypophysial hormones in relation to the genetic code," J. Endocrinol., 38:3-12.

Vogt, M. (1953). "Vasopressor, antidiuretic, and oxytocic activities of extracts of the dog's hypothalamus," Brit. J. Pharmacol., 8:193-196.

Von den Velden, R. (1913). "Die Nierenwirkung von Hypophysenextrakten bein Menschen," Klin. Wschr., 50: 2083. Cited by H. Heller. (1963). "Pharmacology and distribution of neurohypophysial hormones," Symp. Zool. Soc. London, 9:93-106.

Walter, R., B. Dubois, and I. Schwartz. (1968). "Biological significance of the amino acid residue in position 3 of neurohypophysial hormones and the effect of magnesium on their uterotonic action," Endocrinology, 83:979-983.

Waring, H., and F. W. Landgrebe. (1950). "Hormones of the posterior pituitary," The Hormones (G. Pincus and K. V. Thimann, eds.). New York: Academic Press. Vol. 2, pp. 427-514.

Wilson, N. (1968). "Isolation and amino acid sequence of neurohypophysial hormones of Pacific chinook salmon (Oncorhynchus tschawytscha)" (Ph.D. thesis, University of British Columbia).

Wilson, N., and M. Smith. (1969). "Isolation and amino acid sequence of neurohypophysial hormones of Pacific chinook salmon (Oncorhynchus tschawytscha)," Gen. Comp. Endocrinol., 13:412-424.

APPENDIX VI

Source of the Neurointermediate Glands used in this Study

Sample number	Location of catch	Date of Catch	Storage in dry ice (DI) or acetone(A)	Number of glands	Total weight of glands (mg)	Average weight per gland (mg)
(the skate, Raia rhina)						
1	Kyugout Channel	Aug. '66	A	11	132.9	12.1
2	Oukounish Channel	Aug. '66	A	18	211.9	11.8
3	West coast of Vancouver Island	Aug. '66	A	4	49.3	12.3
4	Nanaimo, B.C.	July '66	A	3	39.0	13.0
5	La Perouse Banks	Aug. '66	A	33	436.1	13.2
6	La Perouse Banks	Aug. '66	A	15	193.4	12.9
7	South of Leonard Islands	Aug. '66	A	13	235.6	18.1
8	La Perouse Banks	Aug. '66	A	18	206.6	11.5
9	La Perouse Banks	Aug. '66	A	33	370.9	11.2
10	30 miles off Cape Scott	Aug. '66	A	4	51.6	12.9
11	30 miles off Cape Scott	Aug. '66	A	10	78.2	7.8
12	Kyugout Channel	Aug. '66	A	6	70.8	11.8
13	Stuart Channel	Jan. '67	A	18		
14	Pylades Channel	Jan. '67	A	30		
15	Point Grey, Vancouver	Feb. '67	A	1		
16	Nanaimo, B.C.	Feb. '67	A	3		
17	Nanaimo, B.C.	Mar. '67	A	2		
18	Dod's Narrows	Mar. '67	A	5	2166.6	8.6
19	La Perouse Banks	Mar. '67	A	55		
20	Dod's Narrows	June '67	A	10		
21	Friday Harbor, U.S.A.	Apr. '68	DI	13		
22	Friday Harbor, U.S.A.	May '68	DI	111		
23	Steveston, B.C.	Aug. '68	DI	3		
24	Steveston, B.C.	Oct. '68	DI	1		

APPENDIX I continued

Sample number	Location of catch	Date of Catch	Storage in dry ice (DI) or acetone(A)	Number of glands	Total weight of glands (mg)	Average weight per gland (mg)
(the dogfish, Squalus acanthias (Pacific variety)						
25	Nanaimo, B.C.	July '66	A	21	181.6	8.7
26	Nanaimo, B.C.	Aug. '66	A	26	232.5	8.9
27	West coast of Vancouver Island	Aug. '66	A	12	68.0	5.7
28	La Perouse Banks	Aug. '66	A	54	333.6	6.2
29	Roberts Banks	Nov. '66	A	50	245.0	4.9
30	Ladysmith, B.C.	Jan. '67	A	49	326.4	6.7
31	Nanaimo, B.C.	Mar. '67	A	32	291.2	9.1
32	Nanaimo, B.C.	Mar. '67	A	91	718.9	7.9
33	La Perouse Banks	Mar. '67	A	299	1722.8	5.8
34	La Perouse Banks	Mar. '67	A	219	1321.1	6.0
35	Everett, U.S.A.	Mar. '67	A	50	340.8	6.8
36	Everett, U.S.A.	Mar. '67	A	79	613.2	7.8
37	Everett, U.S.A.	Mar. '67	A	20	171.3	8.6
38	Everett, U.S.A.	Mar. '67	A	53	433.0	8.2

APPENDIX II

Standards used in Biological Assays

1. Synthetic oxytocin (Syntocinon, Sandoz Pharmaceuticals), 10 IU/ml. Diluted to 5 mU/ml and 10 mU/ml for the rat uterus assay (both in the presence and in the absence of Mg^{++}); diluted to 100 mU/ml for the rabbit milk-ejection assay; used undiluted in volumes of 25 and 50 µl for the frog bladder assay.

2. Synthetic glumitocin (supplied by Dr. M. Manning, McGill University through the kindness of Dr. W. Sawyer, Columbia University), dissolved at 1 mg/ml in an aqueous 0.25% solution of acetic acid containing 0.5% chlorobutanol. Diluted to 1 µg/ml for the rat uterus assay; diluted to 1.4 µg/ml for the rabbit milk-ejection assay; diluted to 0.01 mg/ml for the rat antidiuretic assay.

3. Synthetic arginine vasotocin (kindly supplied by Dr. B. Berde, Sandoz Laboratories), 0.1 mg/ml. Diluted to 0.1 µg/ml for the rat uterus assay and for the frog bladder assay; diluted to 0.0025 µg/ml for the rat antidiuretic assay.

4. Arginine/lysine vasopressin (Pitressin, Parke Davis), 20 IU/ml. Diluted to 0.5 mU/ml for the rat antidiuretic assay.

APPENDIX III

Solutions used in Biological Assays

---

1. Rat Uterus Assay: Bathing Solution

The bathing solution was formulated by van Dyke and Hastings (1928) and modified by Munsick (1960). The following aqueous stock solutions were prepared:

a. van Dyke-Hastings solution

NaCl	120.67 gm
$NaHCO_3$	46.62 gm
KCl	8.275gm
0.02% Na phenolsulfonephthalein	270 ml

made to 18 liters

b. phosphate buffer, pH 7.4

i) 22.714 gm Na_2HPO_4, made to 1 liter.

ii) 6.349 gm $NaH_2PO_4.H_2O$, made to 1 liter.

Solution (ii) was added to solution (i) until the pH of the combined solution reached 7.4 (Radiometer, pH meter type PHM 22).

c. 0.5 M $CaCl_2$ and 0.5 M $MgCl_2$

For each salt an approximately 1 M solution was prepared and its molarity was determined precisely by measurement of its chloride concentration. This was done with a chloridimeter (Aminco Cotlove Chloride Titrator, American Instrument) or by manual titration against an $AgNO_3$

APPENDIX III (continued)

solution (Fajans titration) as outlined by Hoar and Hickman (1967). The solution was then diluted appropriately to a 0.5 M concentration.

The above stock solutions were mixed to form the final bathing solution, with or without the presence of magnesium ions, according to the following formuli:-

	Mg^{++} present	Mg^{++} absent
van Dyke-Hastings soln.	1976 ml	1978 ml
phosphate buffer	20 ml	20 ml
0.5 M $CaCl_2$	2 ml	2 ml
0.5 M $MgCl_2$	2 ml	-
glucose	1 gm	1 gm

The solutions were gassed with a mixture of 5% CO_2 in air or oxygen until the pH reached 7.4. This was determined visually from the colour of the indicator present in the solution.

2. Milk-Ejection Assay: Dial Anaesthetic

The anaesthetic, prepared according to directions from the Ciba Co. (personal communication to Dr. A. M. Perks), consisted of monoethyl urea (10 gm), ethyl urethane (10 gm), and diallylbarbituric acid (2.5 gm) dissolved in water to a total volume of 25 ml over a warm (50°C) water bath. The solution was filtered through Whatman #1 paper and stored in

APPENDIX III (continued)

the cold (4°C).

3. Frog Bladder Assay: Ringer's Solution

The Ringer's solution was that used by Sawyer (1960). Two aqueous stock solutions were prepared:

Stock A

NaCl	94	gm
KCl	3.7	gm
$CaCl_2.2H_2O$	5.3	gm

made to 1 liter.

Stock B

$NaHCO_3$	40	gm
glucose	4	gm
$NaH_2PO_4.H_2O$	1.77	gm
phenol red	12	mg

made to 1 liter.

For each assay, 50 ml of stock A and 50 ml of stock B were mixed and made to 1 liter with water.

APPENDIX IV

Solutions Used In Non-Biological Measurements

1. Lowry Peptide Determination

a. Alkaline copper solution. 1 ml of 0.5% $CuSO_4.5H_2O$ in 1% potassium tartrate was mixed with 50 ml of 2% Na_2CO_3 in 0.1 N NaOH.

b. Phosphomolybdic-phosphotungstic acid reagent. 2 N Folin Ciocalteu Phenol reagent (Fisher Laboratory Chemical) was diluted 1:1 with water.

2. Isoelectric Focusing

a. Cathode solution. 0.4 ml of ethanolamine was mixed with 14 ml water and 12 gm sucrose was added.

b. Anode solution. 0.1 ml sulfuric acid was mixed with 10 ml water.

c. Ampholyte-sucrose fractions. "Ampholine" carrier ampholytes in 8% solution, and with pH ranges of 3-10 or 7-9 (LKB Produkter) were used. For each pH range two solutions were prepared:

i. Dense solution. 9.4 ml of the ampholyte mixture was made to 42 ml with water and 28 gm sucrose was added.

APPENDIX IV (continued)

ii. Less dense solution. 3.1 ml of the ampholyte mixture was made to 60 ml with water.

q The two solutions were combined in 24 different fractions, in which the volume of the dense solution per fraction increased steadily from zero to 4.6 ml. In each case the volume of less dense solution necessary to bring each fraction to a final volume of 4.6 ml was added. The peptide sample to be examined was incorporated into one of the middle fractions, and replaced its own volume of the less dense solution.