ISOLATION AND AMINO ACID SEQUENCE OF

NEUROHYPOPHYSIAL HORMONES OF

PACIFIC CHINOOK SALMON

(Oncorhynchus tschawytscha)

by

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ABSTRACT

The neurohypophysial hormones of Pacific chinook
salmon (Oncorhynchus tschawytscha) were purified and the
amino acid sequence of both hormones determined.

The extraction and purification procedure was de-
veloped in an effort to maximize the yields of pure hor-
mones. Pituitary glands were extracted at 4°C using 0.2 M
acetic acid. Purification consisted of gel filtration,
ultrafiltration, and ion exchange. Gel filtration on
Sephadex G-15 columns was used to separate neurohypophysial
peptides from high molecular weight material. Separation
of the two hormones was accomplished on one of three cation
exchangers: carboxymethylcellulose, phosphocellulose or
sulfoethyl-Sephadex. The hormones were eluted from cation
exchange columns using a sodium or ammonium ion concentra-
tion gradient at constant pH; pH 5 was used for chromato-
graphy on carboxymethylcellulose and on phosphocellulose;
pH 2.45 was used for chromatography on sulfoethyl-Sephadex.

The two hormones were purified further by rechroma-
tography of individual hormones on another cation exchange
medium. The material obtained by rechromatography was
pure as determined by amino acid analyses. The yields of

pure hormones at the end of purification procedure was 48% of the starting material. The specific activities of the two purified hormones were 145 and 229 oxytocic units per milligram for Hormone I and Hormone II respectively.

The amino acid sequence of Hormone I was determined by a combination of three methods: subtractive Edman degradation, partial acid hydrolysis, and Dansyl-Edman technique. The amino acid sequence of Hormone I was found to be that of 4-serine, 8-isoleucine oxytocin.

The amino acid sequence of Hormone II was determined by the Edman subtractive method, the Dansyl-Edman technique, and the mobility of the C-terminal residue on high voltage electrophoresis. The amino acid sequence of Hormone II was found to be that of 8-arginine oxytocin.

The two neurohypophysial hormones described from salmon have amino acid sequences identical with those described from four species of Gadiformes and one species of Cypriniformes by other workers. The position of Salmoniformes on the evolutionary tree of teleost fishes suggests that these structures are characteristic of a wide range of teleosts.

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FIGURE

A B B R E V I A T I O N S

U S E D

A.a.   -   amino acid

ala   -   alanine

arg   -   arginine

asn   -   asparagine

asp   -   aspartic acid

cys-cys   -   cystine

$CySO_3H$ -   cysteic acid

gln   -   glutamine

glu   -   glutamic acid

gly   -   glycine

$gly-NH_2$ -   glycinamide

his   -   histidine

ileu   -   isoleucine

leu   -   leucine

lys   -   lysine

met   -   methionine

phe   -   phenylalanine

pro   -   proline

ser   -   serine

trp   -   tryptophan

tyr   -   tyrosine

thr   -   threonine

val   -   valine

$A_{280\ nm}$ -   absorbance at 280 nm*

CM   -   carboxymethyl

C-terminal   -   carboxyterminal

DNS-dansyl   -   1-dimethylamino-naphthalene-5-sulfonyl-

DEAE   -   diethylaminoethyl

mho   -   1/ohm

μmole, μm   -   micromole

nm   -   nanometer

N-terminal   -   amino-terminal

p.s.i.   -   pounds per square inch

PTC-peptide   -   phenylthiocarbamyl peptide

SE   -   sulfoethyl

TCA   -   trichloroacetic acid

U   -   uridine

C   -   cytosine

A   -   adenosine

G   -   guanosine

* one absorbance unit is that amount of substance which when dissolved in 1 ml of solvent has an absorbance of 1.0 in a cell with a 10 mm light path (A.U.).

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To my children

Gregory, Katya, and Duncan

I. INTRODUCTION

(1) Background

Hormones having oxytocic, pressor and antidiuretic
activity have been found in the neurohypophyses of repre-
sentatives of all classes of vertebrates. The neurohypo-
physis is defined as the anatomical structure consisting of
hypothalamic nuclei of neurons, their axons passing down
the infundibular stalk, and the infundibular process or pars
nervosa of the pituitary gland (1). The biosynthesis of
the neurohypophysial hormones is thought to occur in the
cell bodies of neurons which comprise the hypothalamic
nuclei (2). In mammals nuclei supraopticus and paraven-
tricularis have been described and, in fishes, nucleus
praeopticus (3). The hormones are transported in associa-
tion with a protein or proteins by axoplasmic flow along
the infundibulum for storage in, and later release from,
the pars nervosa (4).

These peptide hormones contain a chain of nine amino
acids, which in general structure follow a uniform pattern
(Figure 1). They have a disulfide bridge between cysteine
residues at position 1 (the amino end of the peptide chain)
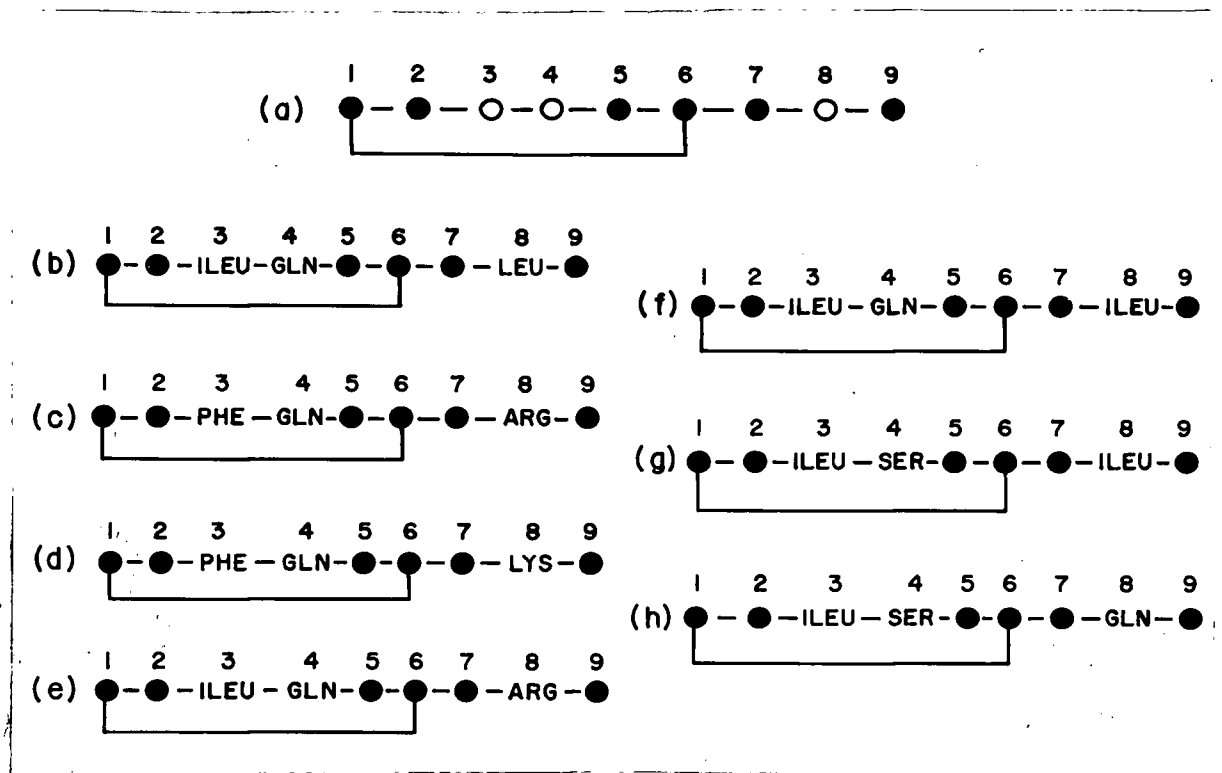
Figure 1: Neurohypophysial hormones of vertebrates

Filled circles: positions occupied by identical amino acids throughout vertebrates (1 - 1/2 Cys; 2 - Tyr; 5 - Asn; 6 - 1/2 Cys; 7 - Pro; 9 - Gly-NH$_2$). Empty circles: positions of amino acid substitutions.

(a) generalized structure; (b) oxytocin; (c) 3-phe, 8-arg oxytocin (arginine vasopressin); (d) 3-phe, 8-lys oxytocin (lysine vasopressin); (e) 8-arg oxytocin (arginine vasotocin); (f) 8-ileu oxytocin (mesotocin); (g) 4-ser, 8-ileu oxytocin; (isotocin); (h) 4-ser, 8-gln oxytocin (glumitocin).

and position 6. In all naturally occurring neurohypophysial hormones for which amino acid sequence has been determined, tyrosine, asparagine, proline, and glycinamide have been found in positions 2, 5, 7, and 9, respectively. Variations in structure involve amino acid substitutions in positions 3, 4, and 8.

(2) Characterization of Neurohypophysial Hormones

Neurohypophysial hormones are often characterized on the basis of their pharmacological activities. Comparison of the activity ratios in a number of biological assays (pharmacological profile) of an unknown hormone with the activities of known peptides has been used to predict the chemical identity of the unknown with considerable precision (5). This procedure is often used because the sensitivity of the biological assays and their specificity allows the examination of very small amounts of impure hormones. However, pharmacological studies can give erroneous or ambiguous results (5). The determination of the amino acid content of a hormone and the definition of the sequence in which the amino acids are arranged is consequently the most fundamental approach to characterization. This has been attempted less often than pharmacological characterization because it requires more hormone material and this must be in a completely pure form.

Studies by either pharmacological or chemical methods have led to the conclusion that in each vertebrate species there are two neurohypophysial hormones. Possible exceptions to this generalization are the cyclostomes in which only one active principle has been detected (6) and elasmobranchs in which the presence of three neurohypophysial hormones cannot be ruled out at the present time (7).

(3) Neurohypophysial Hormones in Vertebrates Other than Fishes

The first structural analysis of neurohypophysial hormones was done by du Vigneaud's group on 3-phe, 8-arg oxytocin (arginine vasopressin)(8) and on oxytocin (9) of ox pituitaries. Studies on four other mammals followed closely (sheep, horse, man, and whale) and revealed two substances in each species with the amino acid composition of oxytocin and 3-phe, 8-arg oxytocin (10). A pharmacological survey of representatives of most families of mammals by Ferguson and Heller confirmed the wide distribution of these hormones (11). One exception found is 3-phe, 8-lys oxytocin (lysine vasopressin) from hog pituitaries where it replaces 3-phe, 8-arg oxytocin (12). The two peptides differ in one amino acid in position 8. The survey of Ferguson and Heller indicated the presence of 3-phe, 8-lys oxytocin in order Suiformes (11).

Oxytocin has been found in birds (13), (14), but 3-phe, 8-arg oxytocin is replaced by 8-arg oxytocin (arginine vasotocin) (10, (15).

A synthetic preparation of 8-arginine oxytocin was reported before the hormone was found in nature (16). It subsequently has been found in pituitaries of representatives of all vertebrate classes with the exception of mammals (17). In mammals, however, 8-arginine oxytocin has been reported from the pineal gland of beef on the basis of chromatographic and pharmacological evidence (18). Subsequently 8-lys oxytocin was found in the pineal bodies of pigs (19). It is difficult to assess the significance of these findings, because a subsequent attempt to isolate 8-arg oxytocin from beef pineal was not successful (20).

According to the data available at present, pituitary 8-arginine oxytocin appears to have remained unchanged throughout vertebrate evolution until Mammalia. The other hormone, on the contrary, has been subject to evolutionary change. Oxytocin has been chemically identified from chicken (13) and pharmacologically from turkey (21) and two other birds (14). The possible existence of oxytocin in lower vertebrates (reptiles, amphibians, fishes) is a disputed subject. Another neurohypophysial homologue, 8-ileu oxytocin (mesotocin) has been chemically characterized in several amphibian species (22), (23), (24), (25).

However, while Acher described 8-ileu oxytocin from <u>Rana</u> <u>esculenta</u> in chemical terms (25), Munsick identified oxytocin pharmacologically from <u>Rana</u> <u>pipiens</u> (26). Acher has identified 8-ileu oxytocin on the basis of amino acid analysis from some reptilian species (27), (28), yet a recent report on cobra, <u>Naja</u> <u>naja</u>, suggests the presence of both oxytocin and 8-ileu oxytocin (29).

(4) <u>Neurohypophysial</u> <u>Hormones</u> <u>of</u> <u>Fishes</u> <u>Other</u> <u>than</u> <u>Teleosts</u>

In cyclostomes only one neurohypophysial hormone, 8-arg oxytocin, has been indicated pharmacologically (6), (17).

The hormone, 4-ser, 8-gln oxytocin (glumitocin) was characterized chemically by Acher's group in 1965 from elasmobranch fishes (30), and subsequently found by the same group in additional elasmobranch species (31), (32). The structure of 4-ser, 8-gln oxytocin was confirmed by snythesis by Klieger (33). Acher's work indicates the presence of small amounts of the second hormone, 8-arg oxytocin, in elasmobranchs. The presence of oxytocic principles other than 4-ser, 8-gln oxytocin has not yet been excluded for some elasmobranchs (7), (34). The study of Sawyer on <u>Hydrolagus</u> <u>colliei</u> failed to find 4-ser, 8-gln oxytocin in this holocephalian ratfish, and indicated that oxytocin may be present together with 8-arg oxytocin (34).

In Dipnoi, in addition to 8-arg oxytocin, both oxytocin and 8-ileu oxytocin have been characterized pharmacologically. Follet and Heller have tentatively identified 8-ileu oxytocin from a species of African lungfish (Protopterus aethiopicus) and from an Australian species, Neoceratodus forsteri (23). Pickering and McWatters, however, reported oxytocin from a South American species, Lepidosiren paradoxa (35). Sawyer and van Dyke also found oxytocin in the African P. aethiopicus (36). Sawyer's specimens were collected from a different lake than were those of Heller. Sawyer is also reported to have found 8-ileu oxytocin in another batch of glands from the same African species (35). All of the identifications of the neurohypophysial hormones of Dipnoi were on a pharmacological basis.

Sawyer and van Dyke indicate the presence of 8-arg oxytocin as well as 8-ileu oxytocin in the primitive bichir, Polypterus (36). The classification of Polypterus adopted by different authors varies. Romer classified Polypterus with the Chondrostei (37); Colbert also placed Polypterini within the Chondrostei (38); Nikolskii included them in the Actinopterygii, but separated them from Chondrostei (39); Follet and Heller separate them from the Actinopterygii as Brachiopterygii (40).

Present knowledge of the neurohypophysial hormones
of the other Chondrostei relies exclusively on the pharmaco-
logical data of Follet and Heller (40). Their studies
detected only one neurohypophysial hormone in the paddlefish,
Polyodon spathula. On the other hand, they found two hor-
mones in three Acipenser species examined. One was tenta-
tively identified as 8-arginine oxytocin, also present in
the paddlefish. The second hormone remained unidentified
since it represented only a small fraction (under 4%) of the
total activity found.

The members of Holostei included in the survey of
Follet and Heller indicated the presence, in addition to
8-arg oxytocin, of a principle indistinguishable chromato-
graphically or pharmacologically from the teleost hormone,
4-ser, 8-ileu oxytocin (isotocin, ichthyotocin)(40).

(5) Neurohypophysial Hormones of Teleost Fishes

In teleost fishes examined up to present two hormones
have been found: 4-ser, 8-ileu oxytocin, and 8-arg oxy-
tocin. The presence of the then still unidentified 4-ser,
8-ileu oxytocin was suggested by Heller et al. (41) on the
basis of biological assays of pollack pituitaries, Pollachius
virens.

The first chemical data on the structure of this new
hormone were published by Acher et al. in 1962 (42). This
group studied three marine species of the family Gadidae:

pollack *Pollachius* *virens*, hake *Merluccius* *merluccius* and bib cod *Gadus* *luscus*. The structure of 4-ser, 8-ileu oxytocin was confirmed shortly after Acher's report by the independent syntheses of Guttman (4) and Johl (44). Sawyer's study of this hormone from *Pollachius* *virens* demonstrated pharmacological identity of the pollack hormone and the synthetic standard (45). 8-Arg oxytocin has also been indicated, but not identified chemically in the above studies of neurohypophysial hormones of marine Gadidae.

In 1965, 4-ser, 8-ileu oxytocin and 8-arg oxytocin were reported from a fresh water teleost, *Cyprinus* *carpio* (Family Cyprinidae) by Acher's group. The identification of both hormones was based on quantitative amino acid analysis and on biological assays (46).

8-Arg oxytocin appears to be as ubiquitous in teleost fishes as it is in all vertebrate classes with the exception of mammals (17). Shortly after this peptide was first synthesized in the laboratory (16), it was identified in pollack by Heller and Pickering (41), (47). The first amino acid analyses of 8-arg oxytocin from teleost fish were reported in 1961 from *Gadus* *luscus* by Acher (48) and by Rasmussen from *Urophycis* *tenuis* (49), (50). Two species, *Pollachius* *virens* and *Merluccius* *merluccius* have been used as sources for amino acid sequence studies reported by Acher (51).

Thus it can be said that five species of teleost fish have so far been examined on a chemical basis with respect to neurohypophysial hormones. These species are: _Pollachius virens_, _Merluccius merluccius_, _Gadus luscus_, _Cyprinus carpio_ and _Urophycis tenuis_ (one hormone only identified from the latter species) as well as Scombridae species referred to by Acher in 1967 (32). The pharmaco- logical survey of Follet and Heller dealt with the following species of teleost fish: _Gadus callarias_, _Esox luscius_, _Anquilla anquilla_, _Cyprinus sp_. and _Salmo irrideus_. The tentative identifications of 4-ser, 8-ileu oxytocin and 8-arg oxytocin in this survey are based on bioassays of fractions obtained by paper chromatography of pituitary extracts (40).

In their survey of neurohypophysial hormones of non- mammalian vertebrates, Heller and Pickering have identified, pharmacologically, the presence of 8-arg oxytocin in the rainbow trout, _Salmo irrideus_ (52). In another survey, on bony fishes and cyclostomes, the same group of workers pharmacologically identified 4-ser, 8-ileu oxytocin from _S. irrideus_ (40).

(6) _Present Study_: _Neurohypophysial Hormones in_
Oncorhynchus tschawytscha

The position of salmonid fishes on the evolutionary tree of the teleosts makes the salmoniform order particularly

significant for study. Romer considers Salmoniformes as a primitive stock at the base of the evolutionary line leading toward more advanced forms of teleosts (37). The families of teleosts whose neurohypophysial hormones have been studied previously (Gadidae, Cyprinidae and Scombridae) are positioned by Romer moderately well along the branches of the teleost evolutionary tree (37). Because of this the characterization of neurohypophysial hormones in salmonids may be considered an important step in the study of evolution of teleost hormones.

The objective of the present work was the isolation and characterization of the neurohypophysial hormones of the Pacific chinook salmon, Oncorhynchus tschawytscha. Determination of the structure by chemical rather than pharmacological analysis was chosen, because, as indicated earlier, the method is potentially less ambiguous. Exhaustive chemical studies of teleost neurohypophysial hormones have not been found in the literature. This also made it desirable to do a chemical study in the present investigation.

The study of amino acid composition and sequence of the two neurohypophysial hormones which have been isolated in the course of this work from salmon was possible for two major reasons. First, a large number of pituitary glands was available because of the slaughtering of sexually mature fish which accompanies hatchery culture of Pacific salmon

(53). Secondly, the structural work was made possible by such recent advances in peptide sequencing methods as the Dansyl method of Gray and Hartley (54) and by the increased sensitivity of automatic amino acid analyzers which became available in the course of the last decade. It was thus possible to determine the sequence of amino acids in one to two micromoles of neurohypophysial hormones, which in terms of weight represents one to two milligrams of purified material.

The determination of amino acid sequence requires material of at least 90% purity. In the present study an extraction and purification procedure on the preparative scale was developed with the aim of minimizing losses of material at each successive step of processing. Extraction methods, molecular exclusion, ultrafiltration and cation exchange techniques were extensively examined leading to a relatively simple procedure for isolation of pure hormones in good yields.

II. EXPERIMENTAL PROCEDURES

(1) Collection of Pituitary Glands

Pituitary glands of spawning Oncorhynchus tschawytscha were collected at hatcheries in Washington State. The glands used in the first part of this work were collected during 1963, 1964, and 1965 at three locations: Green River State Hatchery (near Auburn) and Spring Creek and Little White Federal Hatcheries (near Bingen). In 1966 and 1967 glands were collected only at Green River. The collection took place in September and October, the spawning season in those areas.

The fish were killed by hatchery workers and gonadal products removed. The slaughtering techniques differed from one hatchery to another. At Green River fish were killed by severance of the spinal chord at the level of the operculum. At Spring Creek and Little White hatcheries, the fish were killed by a blow on the head, which often resulted in skull fracture and cerebral haemmorhage.

Using an electric borer, a cylindrical core was re-moved from the head of the fish in such a manner as to in-clude the pituitary glands. The details of the procedure have been described by Tsuyuki, Schmidt and Smith (53). Male and female cores were kept separately. The intact

glands were removed from isolated cores using a pointed laboratory spatula. The glands were placed into screw cap plastic jars, labelled and kept in solid carbon dioxide until arrival at Vancouver, B.C. In this manner the glands were frozen at most within two hours after the death of the fish.

The length of storage time in solid carbon dioxide varied with the site and date of collection. In the earlier years (1963 to 1965) the glands remained in solid carbon dioxide up to five days, and upon arrival at the laboratory were stored in liquid nitrogen (-196°C). In subsequent years (1966 and 1967), the glands remained in solid carbon dioxide for a maximum period of twelve hours. Upon arrival in Vancouver these glands were stored in a -80°C freezer (Revco, Ultra Low Temperature).

The number of glands collected varied from year to year. About 7,800 glands were collected in 1966 and 3,900 glands in 1967. This represented approximately 780 and 390 grams of tissue, respectively.

(2) Biological Assays

The assays, based on contraction of rat uterus in presence of oxytocic substances, were performed according to Holton (55) on virgin Wistar laboratory rats, weighing from 180 to 230 gm. The stage of the estrous cycle was determined by microscopic examination of an unstained vaginal

smear, and only animals in pro-estrus or estrus were used for the experiments (5). Rats were killed with an overdose of ether, whole uteri excised and one horn used for the assay. The tissue was suspended in the buffered physiological saline recommended by Munsick (Table 1). The cervical end of the uterine horn was anchored to a glass air lead at the bottom of the tissue bath, while the ovary was attached by means of a thread to the arm of a kymograph (C.F. Palmer Ltd.). The lever arm of the kymograph was either not weighted, or was weighted with a piece of plasticine weighing up to 0.5 gm. The temperature of the jacketed glass tissue bath (volume 10 ml) was maintained constant at 35°C by a circulating water pump (Haake, Model F).

At the end of each contraction the entire solution was removed through an outlet at the bottom of the tissue bath using the suction of a water aspirator. The uterus was rinsed immediately with Munsick's solution and then re-suspended in the same solution in preparation for the next bioassay. Munsick's solution was dispensed from a plastic squeeze bottle kept in a water bath at 35°C. The pH of both the tissue bath and the stock Munsick's solution was maintained at 7.4 - 7.5 by constant bubbling of 5% CO_2 in air mixture. This also served to mix the solutions in the tissue bath.

Syntocinon-10 (Sandoz Pharmaceuticals) was used as a standard. A one ml ampule containing 10 International

T A B L E   I

Munsick's Buffered Tissue Bath Solution (56)

A.   Stock solution: NaCl 120.67 gm

    (18 L) $NaHCO_3$ 46.62 gm

                                    KCl 8.275 gm

                                    0.02% phenol red 270 ml

B.   Phosphate buffer, stock:

    (a) 22.714 gm Na_2HPO_4 diluted in hot distilled water
         to 1,000 ml

    (b) 5.520 gm NaH_2PO_4 diluted as above

(a) and (b) were mixed in approximately equal quantities
until pH of 7.4 was obtained and the resulting solution
was refrigerated.

C.   Working solution: 988 ml of A

                                    10 ml of B

                                    1 ml of 0.5 M $CaCl_2$

                                    500 mg glucose

For the sake of convenience the stock solution A was often
made more concentrated (in three instead of eighteen liters)
and a 165 ml quantity diluted to 988 ml as required.

Units of oxytocin per ml was diluted to 100 ml with the
same buffer as for the unknown.

Qualitative assays: one point assays were used to
monitor fractionation of molecular exclusion and ion ex-
change columns, and ultrafiltration filtrates. Quantitative
assays, however, were performed on all pooled biologically
active fractions.

Quantitative assays: four point assays were per-
formed according to the method of Holton (55). When the
limit of the standard error was to be calculated, five to
ten groups of four doses were used for each unknown. For
a quick check of the activity of an unknown sample (e.g.
when the effluent peaks of chromatographic separations were
to be defined) only one to three groups of four assays were
performed.

For assays of the oxytocic effect of hormone prepar-
ations in the presence of magnesium ion (57), magnesium
chloride was added to Munsick's solution in order to make
it 0.5 mM with respect to magnesium ion. In all other res-
pects, the assay was performed in the identical manner as
the assay without magnesium.

(3) Analytical Methods

(i) Measurement of protein concentration

Protein concentration was measured by the Folin Lowry
method (58) and by absorbance at 280 nm. Absorbance at

260 nm was also measured routinely. Figure 2 illustrates elution profiles of the same column measured by these three methods. It can be seen that the elution profile based on 260 nm readings is sharper, but otherwise parallels the 280 nm profile. The profile obtained by Folin Lowry measurements is entirely different: most of the Folin Lowry positive material is eluted in the larger molecular weight fraction.

The Folin Lowry method was not used routinely, since it irrevocably uses an aliquot of the sample for measurements. The amount of hormone material lost by this method is insignificant at the start of the isolation procedure, but increases rapidly as the purification progresses.

The spectrophotometers used for measurements of absorbance in the course of this investigation were: Beckman models DU, DK and DB, and Unicam SP 820, series 2. The latter instrument was used with and without an expansion scale.

(ii) Specific conductivity measurements

Salt concentration was monitored by conductivity measurements. A Radiometer Type CDM 2d conductivity meter was used for this purpose. The cell constant of the electrode was 0.5586. Thus the conductance measurements obtained by direct reading were divided by this number to obtain specific conductivity values.
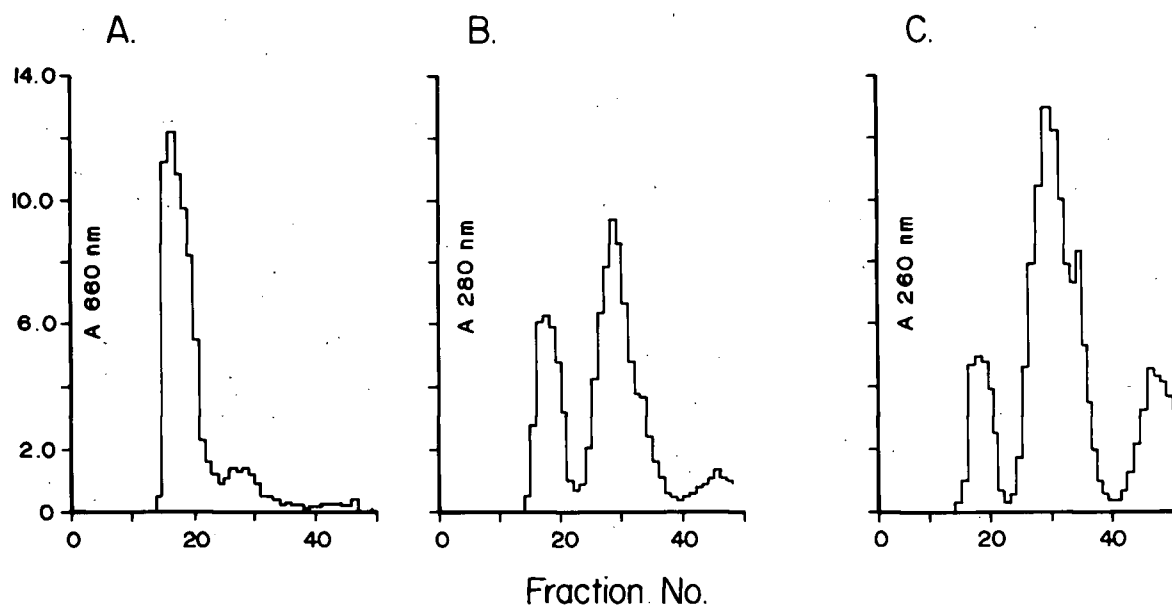
Figure 2: Monitoring of the effluent of a gel filtration column by three different methods.

Loading sample: 10 ml, 600 mg Folin-Lowry peptide; 680 A.U.$_{280 nm}$; 830 A.U.$_{260 nm}$; Biogel P-2.

A. Folin-Lowry peptide, absorbance at 660 nm

B. Absorbance at 280 nm

C. Absorbance at 260 nm

(4) Extraction of Oxytocic Activity from Salmon Pituitaries

(i) Extraction with 0.2 M acetic acid at 4°C

The 0.2 M acetic acid and the glassware to be used
for the extraction were precooled. The extraction was
carried out in a cold room (temperature approximately 4°C).
Frozen glands (100 gm) were weighed quickly and then placed
into 0.2 M acetic acid (1,000 ml, pH 2.6). The tissue was
disrupted immediately in a Waring blender at maximum speed
until the suspension was of even consistency. This required
0.5 to 1.0 minutes. Further disruption of the tissue was
carried out using an electric homogenizer of Potter-Elvehjem
type (TRI-R STIR-R, Model S-63, setting 5) for approximately
one minute.

The tissue homogenate was then centrifuged at 48,200
g for 30 min at 0°C, using a Servall RC 2B centrifuge and
SS-34 head. The supernatant liquid was decanted and kept on
ice. The sediment was resuspended in 100 - 200 ml of cold
0.2 M acetic acid and the suspension centrifuged at 48,000 g
for 45 to 60 min at 0°C, using the Servall RC 2B centrifuge
and SS-34 head. The two supernatant solutions were pooled
to yield the crude extract. An aliquot was saved for ultra-
violet absorbance measurements and for bioassay.

An attempt to prepare the crude extract by centri-
fugation at 27,300 g for 45 minutes at 0°C, using the

Servall RC 2B centrifuge and GSA head was not successful
because the resulting crude extract was opaque and required
re-centrifuging.

The supernatant solution obtained by re-extraction of
the sediment was bioassayed on several occasions, and found
to contain from 5.9 to 17.2% of the total activity of the
extract. A third extraction of sediment did not yield
detectable oxytocic activity.

(ii)   Extraction at 100°C in 0.25% acetic acid of acetone
        powder prepared from frozen pituitaries

The acetone was dried over calcium chloride for five
days with several changes of dessicant. The acetone was
then cooled in an ice bath and the frozen pituitaries placed
in the acetone. Following this, the acetone was replaced
three times at half hour intervals, and once again six hours
after the third change. The glands were then left in
acetone at 4°C for an additional 18 hours. At the end of
this period the acetone was decanted and the glands dried on
a slightly warm Petri dish. Dry glands were homogenized in
0.25% acetic acid at 25°C using 10 ml of extractant per gm
of wet tissue. An electric homogenizer, TRI-R STIR-R
(Model S-63, setting 5) was used for disruption of the tis-
sue. The tissue homogenate was then placed in a boiling
water bath for 3 minutes, cooled on ice and centrifuged at
48,200 g for 30 min at 0°C, using the Servall RC 2B centri-

fuge and an SS-34 head. The supernatant liquid was decanted and kept on ice. The sediment was resuspended in 20% of the original extractant volume, and the extraction,cooling, and centrifugation repeated. The two supernatant solutions were pooled to yield the crude extract. An aliquot was saved for ultraviolet absorbance measurements and for bio-assay.

This extraction procedure was intended to approximate the method reported by Kamm (59) and to compare the yield obtained by Kamm's procedure with the extraction procedure developed for salmon pituitaries (i).

(iii) Additional extraction methods

Other extraction methods consisted of variations on the two procedures described above. Variations on the first extraction procedure were as follows:

(a) Lengthening the time of extraction. In these experiments the tissue homogenate was left on a magnetic stirrer for the entire period of extraction at 4°C or at 25°C.

(b) Three variations in extractant were done. Glacial acetic acid, 2 M acetic acid, and 1 M sodium acetate (pH 5) were each used for the extraction of the tissue. Glacial acetic acid extracts were inconvenient to handle because the excess acid had to be removed by lyophylization. This could only be done after dilution with water to approxi-

mately 1 M acetic acid. Such dilution produced large volumes
of extract for lyophylizing and consequently consumed a great
deal of time. 2 M acetic acid extracts also required the
removal of excess acid by lyophylization, but the dilution
required for this process was two-fold rather than approxi-
mately sixteen-fold as in the case of glacial acetic acid.

(c) Omission of the Potter-Elvehjem homogenized step
from the extraction procedure, the disruption being carried
out using Waring blender only.

(d) Use of lyophylized tissue for extraction involved
freeze-drying of whole frozen glands. Lyophylization facili-
tated the tissue disruption step.

The only variant on extraction procedure (ii) con-
sisted in omission of the acetone powder step and subsequent
extraction in boiling water bath for 5 minutes rather than
3 minutes. The timing began when the temperature of the
homogenate reached a plateau at approximately 92°C.

The extraction procedures used in the course of this
study and the yields of oxytocic activity obtained are
summarized in Table III, p. 52-53.

(5) Concentration of the Crude and Partially Purified
    Extracts

Crude extracts did not have to be concentrated in
large scale (100 gm) extracts in 0.2 M acetic acid (cf.
Figure 7). Prior to this, crude extract was concentrated to

remove excess acid and to reduce the volume of the sample
for further purification steps. Concentration of crude
extracts by ultrafiltration was found unsatisfactory, be-
cause precipitation of material took place above the membrane
and substantially reduced the flow rate. Ultrafiltration of
partially purified extracts in order to concentrate and to
de-salt them was carried out routinely. Ultrafiltration is
discussed in section 6 of this chapter.

Concentration and removal of excess acid by lyophyli-
zation was done repeatedly in the course of isolation pro-
cedure. A Thermovac Industries Corp. freeze dryer and round-
bottom lyophylization flask was used for this purpose.
Losses of hormonal activity were not observed in the course
of this lyophylization procedure. It was essential to the
recovery of the hormones, however, that all of the dry
material in the flask be brought into solution by repeated
rinsing of the flask.

(6) Ultrafiltration

The Dia-flo ultrafiltration apparatus is designed to
carry out fast selective filtration of aqueous solutions.
The high rate is achieved by applying nitrogen pressure to
the chamber containing the sample, and the selectivity is
introduced by membranes of varying pore sizes through which

the solution is filtered. Three types of membranes were available: UM-1, UM-2, and UM-3, with molecular weight cut-off ranges of 10,000, 1,000, and 350, respectively. The molecular weights of neurohypophysial hormones are in the 1,000 range, while the molecular weight of salts in the extract is below 350. Thus a UM-3 membrane was expected to de-salt and to concentrate a solution of neurohypophysial hormones.

The ultrafiltration apparatus was used according to the directions of the manufacturer (60). Ultrafiltration chambers 450 and 600 ml in volume and 7.5 cm Diaflo ultrafiltration membranes were used. Pressure applied was 80 p.s.i. and flow rates ranging from 1.2 to 2.0 ml/min were obtained. By varying the pressure applied and the concentration of the solution in the chamber, it was observed that the flow rate appeared to depend on the concentration of the solute in the chamber rather than on the pressure applied to that chamber.

In order to minimize losses of hormone during ultrafiltration, a compromise was reached between lowering of the ionic strength by ultrafiltration and by dilution. This consisted in concentrating the sample above the ultrafiltration membrane and thereby reducing the salt content of the sample by a factor dependent on the ratio between the starting volume and that of the final concentrate (10 to 15 ml). The total salt content was thereby reduced, but usually

not sufficiently to permit cation exchange chromatography. The solution was adjusted later to the desired conductance for loading the cation exchange column by dilution.

(7) Gel Filtration

(i) Preparation of the gel and column packing

Two types of gel filtration media were used in the course of these experiments: crosslinked dextran (Sephadex, Pharmacia) and crosslinked polyacrylamide (Biogel, Biorad). Both gels were treated identically in respect to swelling and in packing of the column. The treatment differed slightly only at one point. More care had to be exercised in sus-pending the dry Biogel because it tended to form large aggregates. Consequently the suspension had to be stirred vigorously and the dry gel added slowly.

The swelling usually was allowed to take place over-night or for twenty-four hours, and fining of the gel was accomplished during that time. The swelling and packing was done in the same molarity of acetic acid in which the column was to be run. Occasionally the Sephadex gel was swollen by the fast method recommended by the manufacturer; for one hour in a 60°C bath (61).

Small columns (i.e. 200 mm x 12 mm diameter) were packed by pouring the thin gel slurry by hand. The column was half filled with buffer when the pouring began and the

outlet was opened slightly when the bed was packed to a depth of 20 to 30 mm. Larger columns (25 to 50 mm in diameter) were packed using a funnel in which the gel suspension was continuously stirred. A large (500 ml) glass funnel with a short wide stem was inserted into a rubber stopper fitted to the top of the column. An electric rotary blade stirrer (Gerald T. Heller Company, GT 21) was used to stir the suspension in the funnel. The funnel was kept filled with gel slurry. When the column bed reached the desired height the funnel was removed and a perlon disc placed on top of the gel bed. The column was washed with two bed volumes of eluant and was ready to use. The packing of all columns was carried out at 25°C.

Blue Dextran (Pharmacia) solution was run through newly packed Sephadex columns to monitor the quality of packing. Blue Dextran could not be used with Biogel, since it was retained by the gel. Sephadex G-10 also tended to spread the Blue Dextran band. On G-15, however, Blue Dextran behaved as described by the manufacturer (62).

(ii) Loading and elution of gel filtration columns

Experiments were generally carried out at 25°C. After removal of the liquid above the column bed, solutions of hormone preparations were loaded in the appropriate volume and then washed into the gel with 0.5 to 10.0 ml of buffer.

(10 ml was used for 450 mm x 50 mm diameter columns). The column was then eluted with the buffer and fractions collected using a mechanical fraction collector (Gilson, model V-10, equipped with timer).

In order to process a large volume of extract (approximately 1 liter), a Sephadex G-15 column (450 mm x 50 mm diameter) was used repeatedly to fractionate 225 ml batches of the extract (tandem gel filtration). When this technique was employed a fresh sample of extract was applied to the column as soon as the biologically active material was eluted and fractions collected continually. The total time required for a tandem 5-column run was between 20 and 25 hours.

The absorbance of fractions at 260 and 280 nm, their conductivity, and oxytocic activity, were determined. The biologically active fractions were then pooled and either processed further immediately or stored at 5°C.

Packed Sephadex columns were stored at 25°C with 0.02% sodium azide after washing with several bed volumes of buffer.

(8) Ion Exchange Chromatography

(i) Preparation of the exchangers and column packing

Precycling of Whatman CM-32 and of Whatman CM-22 was carried out according to the directions of the manufacturers (63). Usually the amount precycled was about 30% more than

required by the column. The dry exchanger was stirred into 0.5 N sodium hydroxide (15 ml per gm of exchanger) and left for 30 minutes. At the end of this period the liquid was decanted and the exchanger washed with distilled water in a 500 ml Buchner funnel under suction until the washings were at pH 8. The exchanger was then stirred into 0.5 N hydrochloric acid (15 ml per gm of exchanger) and again left for 30 minutes. The liquid was decanted and the exchanger washed on a Buchner funnel until the washings were neutral. The hydrochloric acid treatment was repeated once. Equilibration with the appropriate buffer was carried out at 2M ionic strength and the correct pH value (pH 5). It was found convenient to carry out this step in a 500 ml graduated cylinder. The exchanger was stirred with the buffer and allowed to settle for 10 minutes. At the end of this period the liquid was removed by suction, the cylinder refilled with buffer, inverted several times and again allowed to settle. This step was repeated 7 to 8 times. The conductance and pH of the liquid were checked for equilibration. The equilibrated exchanger was then stirred into 20 volumes of the starting buffer and allowed to settle for 30 minutes. The liquid was removed by suction and the step repeated once. Most of the removal of fine particles was accomplished during the equilibration step. A thick slurry of the prepared exchanger (approximately 20% w/v) was degassed using a water

pump. It was found to be more convenient to prepare a thick slurry and pour it into the column in one pass, because the flow rate of this exchanger is low and packing of the column would take too long when a thin slurry was used. For the same reason it was found desirable to have the starting buffer to a depth of only 20 to 30 mm at the bottom of the column prior to pouring. The exchanger was allowed to settle to a height of about 20 mm before the outlet was opened. The column was usually packed in the late afternoon and left to wash with starting buffer overnight. By morning complete equilibration was achieved with regard to both conductivity and pH.

Phosphocellulose (Selectacel) did not require special precycling. It was equilibrated in 0.2 M buffer and packed into the column in the starting (0.002 M) buffer. Because of the fibrous nature of the exchanger it was packed in a thin slurry. The column was equilibrated overnight with the starting buffer.

SE-Sephadex C-25 (Pharmacia) was precycled according to the directions of the manufacturer (64). The dry exchanger (30 gm) swelled to a volume of 300 ml while soaking in water. No appreciable shrinkage was noticed as the column was packed.

The exchanger was allowed to swell in water for at least one hour and then fine particles were removed by

decantation. The exchanger was washed on a Buchner funnel with 500 ml of 0.5 N sodium hydroxide followed with distilled water until neutrality was reached. Equilibration was carried out in 2 M buffer of desired pH. Several changes of buffer were required until complete equilibration was achieved. The column was packed and washed overnight as in the preparation of phosphocellulose columns. All cation exchange columns were precycled, equilibrated and packed at 25°C.

(ii) Buffers used in cation exchange

Sodium acetate, ammonium acetate, and ammonium formate buffers were made by titration of the acid at desired molarity with the appropriate base to the required pH. A Radiometer 26 pH meter was used for the titration. A 2 M stock solution was freshly made before use and lower ionic strength buffers obtained by dilution. In ammonium formate buffers, the pH had to be adjusted with formic acid following dilution.

(iii) Loading and elution of cation exchange columns

Loading and elution were conducted at 25°C. The fractions were collected using the Gilson fraction collector. Usually the sample was loaded onto the column from a reservoir and the collection of fractions started immediately.

The conductivity of the loading sample was adjusted by dilution with distilled water to make it lower than the conductivity needed to elute Hormone I. After all of the sample percolated into the column bed, the walls of the column above the exchanger bed were rinsed with several milliliters of starting buffer and the rinsings allowed to pass into the bed. The column was washed with starting buffer until the ultraviolet absorbance of the eluate decreased. The elution with linearly increasing salt concentrations was then carried out using a gradient forming system with equal volumes of appropriate buffers in a mixing chamber and in a reservoir of equal dimensions (65).

In most cases, bioassay and the ultraviolet absorbance measurements were made as fractions emerged from the column. The gradient was checked by measuring the conductance of the fractions.

(iv) Repeated use of the same cation column

Unless it could be re-used immediately, the exchanger was discarded after use. On a few occasions all three exchangers were re-used within a day or two of the first run. All required complete re-equilibration, which consisted in washing the column with high ionic strength (1 - 2 M) buffer followed by a two to three liter wash with the starting buffer. SE-Sephadex, phosphocellulose, and Whatman CM-22 could be re-used without re-packing. Whatman CM-32 required

re-packing because of decreasing flow rate. Phosphocellulose
in wet form was never kept longer than absolutely essential
because of possible phosphatase activity in microbial con-
taminants.

(9) Performic Acid Oxidation

Cystine and cysteine residues were converted to
cysteic acid residues by performic oxidation (66). Cysteic
acid gives better quantitative data than cysteine on the
automatic amino acid analyser. Additionally, conversion to
cysteic acid removes the intramolecular disulfide bridge in
neurohypophysial hormones thus facilitating sequence deter-
mination.

The sample of peptide intended for oxidation, the
freshly prepared performic acid solution, and the pipette
for measuring the latter were cooled in ice to 0°C. Performic
acid was added to the peptide sample, the test tube stoppered
with a ground glass stopper and the reaction allowed to
proceed at 0°C for two to three hours. Excess reagents were
removed by lyophylization. The first lyophylization was
carried out until no peroxide could be detected by smell.
The sample was then redissolved in water and relyophylized.
A large excess of oxidant was used, i.e. 0.2 ml of performic
acid to 0.05 μmoles of peptide.

Performic acid was prepared by mixing 88% formic acid and 30% hydrogen peroxide in 9 : 1 proportions and allowing the mixture to stand at room temperature for one hour. A mixture of formic acid to peroxide (9.5 : 0.5) warmed for five minutes in a 50°C bath has also been used and found satisfactory.

(10) Acid Hydrolysis of Peptides

Total hydrolysis of the sample was required in three types of experiments: (1) amino acid analysis of the whole molecule; (2) amino acid analysis following Edman degradation cycle(s) for subtractive Edman method of amino acid sequence determination; and (3) identification of dansyl-derivatives of N-terminal residues of whole or partially degraded hormones.

The sample to be analyzed was placed in a test tube and dried in a vacuum dessicator over phosphorus pentoxide and sodium hydroxide. Kimax culture tubes, 10 x 75 mm, were used for total hydrolyses and for subtractive Edmans. Hydrolysis of dansylated peptides was carried out in the same test tube, 6 x 30 mm, used for dansylation reaction.

Twice distilled 50% hydrochloric acid (20 µl for 0.01 µmole of peptide) was added to the dried sample and the test tube pulled out in a hot flame to form a 1 - 2 mm diameter neck in the middle. The hot glass was cooled in

a stream of air or at room temperature, and the contents
frozen in an acetone and solid carbon dioxide bath. The
mouth of the tube was then attached to an oil vacuum pump
and the tube evacuated. The solution was allowed to melt
and evacuation continued until bubbling ceased. When
bubbling was too intense, as was the case with the hydro-
lyzates of dansylated peptides, it was controlled by
repeated freezing and thawing of the sample. The tube was
sealed under vacuum using a small flame.

The hydrolysis was conducted in a controlled temper-
ature heating block (Temp-Blok Module Heater, Lab-Line
Instruments) which was covered with several thicknesses of
aluminium foil with a hole for a thermometer stem. The
hydrolysis was allowed to proceed for 16 hours at 105°C,
unless otherwise indicated in the text.

Following hydrolysis, the sealed tube was opened and
the hydrochloric acid evaporated under vacuum over sodium
hydroxide pellets. Dessication was allowed to proceed
longer than would be warranted for the appearance of the
sample in order to remove all traces of hydrochloric acid.
When 0.1 ml hydrochloric acid had been added for hydrolysis,
the sample was left under vacuum for 1.5 hours. The dry
sample was then ready for the next step; either amino acid
analysis or thin layer chromatography. If the next step was
not done immediately the sample was stored at -20°C.

(11) Amino Acid Analyses

On occasion during this investigation, three models
of amino acid analyzer were used. They were a Beckman
Model 120, a Technicon analyzer, and a Beckman Model 120 C,
equipped with an integrator. The bulk of total hydrolyzates
and all of the subtractive Edman results were analyzed on
the Beckman Model 120 C, and the experimental procedure
described is for this model.

(i) Procedure

The sample containing 0.01 to 0.03 μmoles of peptide
was dissolved in 100 or 200 μl of pH 2.2 sodium citrate
buffer. When only the long column was to be used, as in
sequencing of Hormone I, the sample was dissolved in 100 μl
and the total amount applied to the column. When the short
column was to be run in addition to the long column, the
sample was dissolved in 200 μl of buffer and 100 μl of
solution was applied to each column. The long column on
Beckman 120 C is used for resolution of acidic and neutral
amino acids; the short column is used for resolution of
basic residues.

The run was performed according to the directions of
the manufacturer (67), (68). The sample was applied using
nitrogen pressure and the walls of the column rinsed twice
with the starting buffer (pH 3.52 or pH 5.18). The space
above the resin was filled to the top of the column with the

starting buffer, the lead from the appropriate pump attached and the run started. The elution of basic amino acids was carried out with a pH 5.18 sodium citrate buffer and the time required was 50 minutes. The elution of acidic and neutral amino acids required a buffer change for the elution of the latter group. The buffer change was from the starting buffer to pH 4.25 buffer and was set to begin automatically 25 to 30 minutes after the run began. This difference in time depended on the length of the column used. Similarly, the duration of the total long column run was 125 to 130 minutes.

(ii) Quantitation of results

The integrated values for each amino acid were either read off the integrator tape, or calculated by multiplying the net height of each peak by its width. Quantitation for proline was obtained from the 440 nm recorder tracing, while the 570 nm line was used for integration of all other amino acids.

The numerical value obtained above was divided by the constants obtained from a standard amino acid run to give values for the amounts of amino acids in micromoles. In order to transpose the number of micromoles into residues per mole, all values for amino acids found in approximately equal amounts in the molecule were averaged and the average was equated with one residue per mole.

To express the specific activity of purified hormones in terms of oxytocic units per milligram hormone, the total number of oxytocic units contained in the aliquot used for amino acid analysis was divided by the average value of micromoles obtained from this analysis and then divided by the number of milligrams equal to one micromole of the hormone (Tables VII and VIII).

(12) Partial Acid Hydrolysis

The method of partial acid hydrolysis described by C.M. Tsung and Fraenkel-Conrat (69) was followed. This method is intended to effect preferential release of aspartic acid (and asparaginyl) residues. The peptide was dissolved in 0.03 N hydrochloric acid to a final concentration of 1 mg/ml and hydrolyzed at 105°C in a sealed evacuated tube. The half time for hydrolysis of aspartic acid residues is reported to be 5.5 hours and of asparagine residues 11 hours. In control experiments it was found that 22 hour hydrolysis of 3-phe, 8-lys oxytocin resulted in six fragments in addition to aspartic acid. For Hormone I, an 18 hour hydrolysis was used.

(13) High Voltage Electrophoresis

High voltage electrophoresis was used to monitor the purity of hormone preparations and to identify peptide fragments obtained either by partial acid hydrolysis or as a

result of Edman degradation cycles. The experiments were
carried out on Whatman 3 MM paper at either pH 6.5 (pyri-
dine : acetic acid : water; 25 : 1 : 225) or pH 3.6
(pyridine : acetic acid : water; 1 : 10 : 289). Either a
flat-plate apparatus (Savant Instruments) or a vertical
liquid cooled apparatus (Michl-Ryle type) equipped with a
high voltage D.C. power supply (Canadian Research Institute,
Model EP5K-200) was used for electrophoretic separations.

Reference amino acids (6.5 μmoles/ml in 0.1 M sodium
bicarbonate) were each applied at the origin in a streak
5 mm long. Peptide was dissolved in buffer and applied as
a streak 20 - 25 mm long at the origin in such a manner that
about one fifth of the peptide band could be cut off and
stained along with amino acid markers, while the remaining
peptide area(s) could be used for elution.

After spotting, the origin was dried in a stream of
warm air, and the paper wetted with buffer using appropriate
precautions to prevent movement or diffusion of substances
at the origin. Following the electrophoretic separation,
the paper was dried and the edge bearing the amino acid
markers and some of the unknown was cut off for staining.
The cadmium acetate/ninhydrin stain was made up immediately
prior to use from 1% ninhydrin in acetone, 15 mls, and 85
mls of a solution of cadmium acetate (5 gm) and glacial
acetic acid (250 ml) in distilled water (500 ml).

Areas marked for elution on the unstained strip were
cut out and the elution carried out using the method of
Heppel (70).

The vertical Michl-Ryle type high voltage apparatus
was found to be preferable to the flat plate type in peptide
work because considerable spreading was observed with the
latter apparatus.

(14) Edman Degradation

The Edman reaction essentially consists of two steps:
formation of a phenylthiocarbamyl peptide (PTC-peptide) and
splitting off of the N-terminal amino acid as an anilino-
thiazolinone, thus resulting in a shortening of the peptide
chain by one amino acid residue. Edman in 1950 described
the application of this reaction to the determination of
amino acid sequence in proteins (71). The method used in
the present work was that of Black, Kauffman and Dixon (72)
with some modifications.

The peptide (0.1 μmole) was dissolved in 200 μl of
50% pyridine (redistilled) in water in a conical graduated
15 ml centrifuge tube equipped with a glass stopper. 25 μl
of N-ethyl morpholine (distilled) and 10 μl of phenyliso-
thiocyanate (distilled) were added, the tube flushed with
nitrogen, stoppered, and the coupling reaction allowed to
proceed for 3 hours at 37°C.

At the end of this time, 2 drops of de-ionized water were added and the mixture extracted with an equal volume of benzene to remove excess reagents. Initially, the benzene extraction step was repeated three times, but losses of PTC-peptide were observed, and the benzene extraction step was thereafter only done once on each sample. Benzene extraction could be omitted altogether when only the subtractive Edman method was used in amino acid sequence determination.

The aqueous phase was dried in a vacuum dessicator overnight. Trifluoroacetic acid (0.1 ml) was added to the dry residue and the cleavage reaction allowed to proceed at room temperature for one hour. Trifluoroacetic acid was removed under vacuum, either at 50°C for 30 minutes or at 25°C for 90 minutes.

The dry residue was initially dissolved in water and extracted three times with 1 ml of butyl acetate. Because losses of peptide material were observed to occur, the extraction with butyl acetate was performed once on the dry residue in subsequent experiments using 0.5 ml of butyl acetate.

Excess butyl acetate was removed in a vacuum desiccator and the residue redissolved in 50% pyridine (distilled) in water.

(15) <u>Subtractive</u> <u>Edman</u> <u>Method</u> <u>in</u> <u>Amino</u> <u>Acid</u> <u>Sequence</u>

<u>Determination</u>

The subtractive Edman sequence method involved amino
acid analysis of the residual peptide at the end of each de-
gradation step described in section 14 of this chapter. A
series of amino acid analyses was thus obtained showing a
gradually decreasing peptide chain as the successive amino
acids were removed from the N-terminus by the Edman degrada-
tion reaction. The next Edman degradation cycle was not
begun until the results of the previous cycle were obtained
from amino acid analysis. It was found that the sequence of
four to five N-terminal residues could be defined by the
subtractive Edman method.

(16) <u>Dansylation</u> <u>Method</u> <u>in</u> <u>Amino</u> <u>Acid</u> <u>Sequence</u> <u>Determination</u>

While the subtractive Edman method used comparative
amino acid analysis to establish which amino acid was removed,
and hence which was the terminal, dansylation involves posi-
tive identification of each successive N-terminus as it
becomes exposed following an Edman degradation cycle (54).
Dansyl chloride (1-dimethylaminonaphthalene-5-sulfonyl
chloride) is reacted with an aliquot of the peptide following
each Edman degradation cycle to form a dansylated peptide
(dansyl chloride reacts not only with alpha amino groups, but

also with the epsilon amino group of lysine and with the phenolic hydroxyl groups). Dansylated peptide is then hydrolyzed and dansylated amino acids identified. The method followed in the present work is approximately that described by Black, Kauffman and Dixon (72).

(i) Dansylation reaction and hydrolysis

An aliquot containing 0.01 μmole of peptide was re-moved from the pyridine-water solution of the peptide at the end of each Edman degradation cycle (section 14 of this chapter). The aliquot was placed in a 6 x 30 mm test tube and dried in a vacuum dessicator over phosphorus pentoxide and sodium hydroxide. 20 μl of sodium bicarbonate was added and the peptide dried again. 10 μl of de-ionized water was added to the dry peptide and the pH of the resulting solu-tion checked with ¦pH   ¦ paper. If the pH was less than 8, the step was repeated. 10 μl of dansyl chloride (3 mg/ml in acetone) was added to the aqueous solution of peptide, the tube sealed with Parafilm, and the reaction allowed to pro-ceed at 37°C for 1.5 to 3 hours, or until the yellow colour of the reagent disappeared. The sample was then dried in a vacuum dessicator and hydrolyzed with 50% hydrochloric acid (re-distilled twice) for 16 hours at 105°C after sealing the tube under vacuum, as described in section 9 of this chapter. A shorter hydrolysis time (6 hours) was used where a dansyl-

proline residue was suspected, since dansyl-proline is destroyed by prolonged acid hydrolysis.

(ii) Identification of dansyl amino acids by thin layer chromatography

Dansyl amino acid standards were prepared by the method of Boulton and Bush (73) and checked against standards purchased from Calbiochem. Both sets of standards were used. The commercially obtained standards were free from dansyl sulfonic acid. The latter provided a useful reference compound in some thin layer systems, while in others it was found to obscure the amino acid derivatives with similar Rf values.

Three solvent systems were used routinely in identification of dansyl amino acids (72): system A (chloroform : methanol : glacial acetic acid; 95 : 10 : 1), system B (n-propanol : ammonia; 80 : 20) and system C (n-propanol : water; 80 : 20). Systems A and B separated most of the dansyl derivatives of amino acids present in neurohypophysial hormones, while system C was used to improve the separation of DNS-aspartic and DNS-cysteic acids. Because it is basic, System B enhanced fluorescence and was an excellent medium to bring out weak fluorescence of some peptide derivatives.

Kontes thin layer plates, 200 x 200 mm, were spread with Silica gel (5 gm suspended in 9.5 ml of water) using

the glass applicator rod according to the directions of the
manufacturer (74). After drying sufficiently to turn opaque,
the glass plate was transferred to an 85°C oven for one hour
and then stored in a dessicator over calcium chloride. Prior
to spotting, the plate was divided into 7 - 10 mm channels
using a sharp object to mark the coating on the plate. The
dry sample of dansylated peptide hydrolyzate was dissolved
in 1 M ammonium hydroxide (5 μl) and spotted repeatedly until
all fluorescence was transferred from the test tube as
observed in the U.V. viewer (Chromato-Vue, Ultraviolet
Products Ltd., San Gabriel, Calif.). The standard solutions
were such that one to two applications yielded a spot of
suitable intensity. Small compact spots improved separation.
For this reason, a thin capillary tube (1 mm in outside
diameter) was used for application, and the spot dried
immediately in a current of warm air.

Kontes thin layer chromatography tanks (250 x 250 mm)
were lined with Whatman No. 1 filter paper and equilibrated
with the solvent system. A fresh solvent mixture was used
for each run. When sufficient material was available two
plates were spotted and chromatographed simultaneously in
two solvent systems. When the amount of material was limited,
the same plate was re-run in a different system after a
short reactivation period. When the spots to be identified
were slow running in the first solvent system, the second

chromatography was done in the same direction as the first. When the spots were fast running in the first system, it was found convenient to reverse the plate and to do the second chromatography in the opposite direction. Chromatography in system A required approximately 1 hour; in B, 2-1/2 hours; and in C, 3-1/2 hours. Following chromatography, the plates were dried and photographed.

The photography was done using a Polaroid Model 160 camera equipped with a copy lens and U.V. filter (Tiffen Photar, U.V., 1-b series 7) and Polaroid Land Picture Roll Type 47, 3,000 speed film and No. 10 aperture on the camera. The plate was illuminated with a Mineralight UVS 11 ultra-violet light source (Ultraviolet Products, San Gabriel, Calif.). The exposure time varied from 18 seconds for the plates chromatographed in propanol : ammonia to 45 seconds for the plates run in chloroform : methanol : acetic. The time of exposure was determined in each case by the intensity of fluorescence. The development of the photograph was allowed to proceed for 10 seconds.

III. RESULTS

A. Stability of Oxytocic Activity in the
Pituitary Tissue and Tissue Extracts
of Oncorhynchus tschawytscha

(1) Stability of Oxytocic Activity During Storage of

Glands at -196°C

The oxytocic activity of pituitary glands stored in

liquid nitrogen (-196°C) for periods ranging from two to

twenty-six months was surveyed in order to determine which

group of glands would give the best yield of neurohypophysial

hormones. In the same experiment a comparison of glands

collected at three different locations were made. Approxi-

mately one gram of each tissue was extracted in identical

manner, using 2.5 ml of 0.25% acetic acid per gram and fol-

lowing the procedure prescribed by the British Pharmacopeia

(1958) (59). The bioassay was done using the method of

Holton (55). The time interval between the extraction of

the glands and the performance of the bioassay was identical.

The results are shown in Table II.

The differences in specific activities observed were

minor, considering that the average error of the bioassay

was 10%. All preparative extracts were from pituitaries

collected at Green River Hatchery. Oxytocic activity sur-

T A B L E   II

Comparison of the Specific Activities of the Pituitary

Samples of <u>Oncorhynchus</u> <u>tschawytscha</u> collected at

Three Different Locations

Site of Collection	Months Stored at -196°C	S e x	Specific Activity Oxytocic units per A.U.$_{280\ nm}$	Limit of Error of Bioassay %
Little White	26	female	0.0437	6.0
Little White	14	female	0.0471	10.9
Little White	2	female	0.0463	7.7
Spring Creek	2	female	0.0582	12.3
Green River	2	female	0.0593	14.3
Green River	2	male	0.0512	8.1

Green River Hatchery (State), near Auburn, Wash.

Spring Creek and Little White Hatcheries (Federal),

near Bingen, Wash.

vived extended cold storage of the intact glands. This method of preservation at -196°C, or alternatively, at -80°C, was adopted as the standard procedure.

(2) Stability of Oxytocic Activity in Crude and Partially

Purified Pituitary Extracts

Figure 3 demonstrates the loss of oxytocic activity in impure extracts under neutral and slightly basic conditions. It can be seen that activity is lost gradually at pH 7.6 (50% of initial value found after 80 minutes), while at pH 8.1 oxytocic activity of a crude extract is lost at a higher rate.

In the course of long term storage (21 days) of crude pituitary extracts in 0.002 M acetic acid at 4°C losses up to 68% of the initial oxytocic activity were observed. Storage in 0.05 M acetic acid under the same conditions resulted in losses of oxytocic activity up to 42%. Losses of activity were not observed when the crude pituitary extracts were stored in 0.2 M acetic acid at 4°C for 21 days. Similar losses were not observed in control experiments using Parke-Davis oxytocin.

A partially purified extract (biologically active effluent of gel filtration) containing $557^{\pm}$ 79 units of oxytocic activity was stored for two months at -20°C as a lyophylized powder. The bioassay following this period of
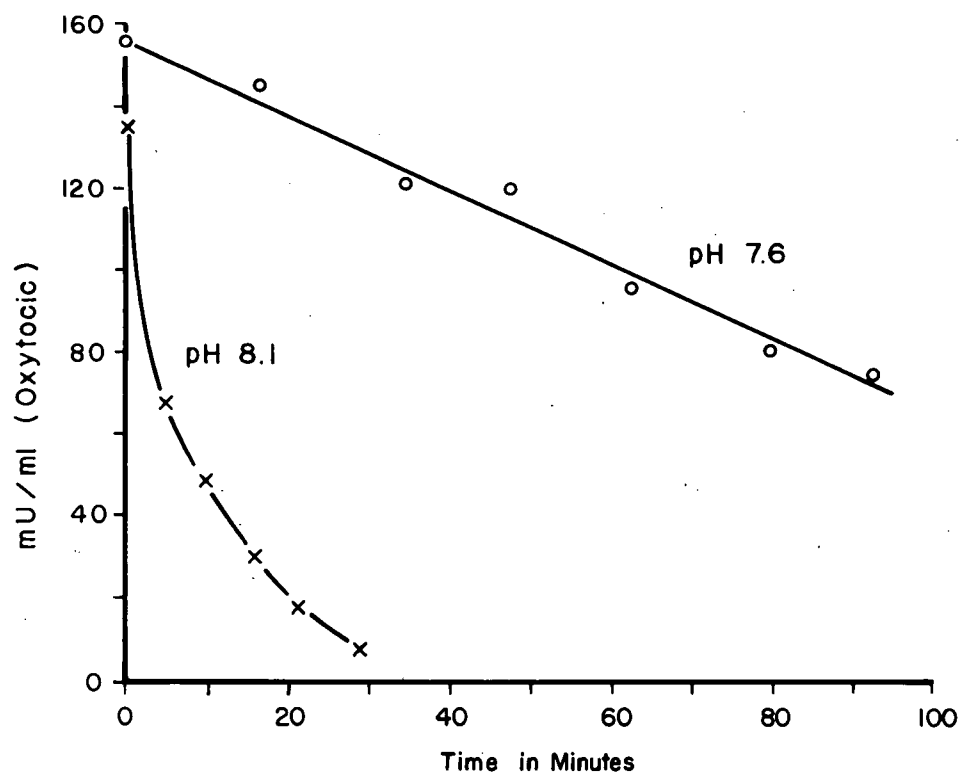
Figure 3: <u>Loss</u> <u>of</u> <u>oxytocic</u> <u>activity</u> <u>in</u> <u>crude</u> <u>salmon</u>
<u>pituitary</u> <u>extract</u> <u>at</u> <u>neutral</u> <u>and</u> <u>slightly</u>
<u>basic</u> <u>pH</u>, <u>at</u> <u>25°C</u>.

Each point on the graph represents one four point assay on
rat uterus without magnesium (55).

storage showed that $530^{\pm}57$ units of oxytocic activity sur-
vived the storage period.

B.   Extraction of Neurohypophysial Hormones
from Salmon Pituitaries

The effect on the yield of oxytocic activity of
varying several of the parameters in the extraction pro-
cedure are recorded in Table III.   Factors which were in-
vestigated included the temperature of extraction, pH,
concentration of extractant, the relative amounts of solvent
and tissue, the state of the tissue (frozen, lyophylized, or
acetone dried glands) and the method of disrupting the
tissue.   Factors which led to increased yield of oxytocic
activity included adequate disintegration of tissue, low
temperature, low pH and adequate amounts of extracting sol-
vent.   The concentration of acetic acid above 0.2 M, the
state of the tissue (frozen, lyophylized or acetone dried)
were relatively unimportant in obtaining optimum yields.
The procedure subsequently adopted for extraction of hormones
involved disruption of tissue in 0.2 M acetic acid at 4°C.

C.   Purification of Neurohypophysial
Hormones of Salmon

Twenty four pituitary extracts were made in the
course of this work.   The first twenty extracts served to

T A B L E

Extraction of Oxytocic Activity

State of tissue	Method of disruption*	Extractant	Volume of extractant (ml) per gm wet tissue wt.	Extraction Temp. (°C)	Duration
Frozen	Potter-Elvehjem	0.25% HOAc	2.5	100	5 min.
Acetone powder	Potter-Elvehjem	0.25% HOAc	5.0	100	5 min.
Acetone powder	TRI-R	0.25% HOAc	10.0	100	3 min.
Frozen	TRI-R	glacial HOAc	10.0	25	24 hr.
Lyophyl-ized	Potter-Elvehjem	2M HOAc	10.0	25	24 hr.
Lyophyl-ized	Potter-Elvehjem	2 M HOAc	10.0	4	24 hr.
Frozen	TRI-R	2 M HOAc	10.0	4	5 min.
Frozen	TRI-R	1 M NaOAc	10.0	4	5 min.
Frozen	Waring + TRI-R	0.2 M HOAc	10.0	4	5 min.
Frozen	Waring	0.2 M HOAc	10.0	4	5 min.

* Potter-Elvehjem glass tissue homogenizer. TRI-R STIR-R (model S-63) electric tissue homogenizer, setting 5. Waring blender, full speed.

I I I

from the Pituitaries of <u>Oncorhynchus</u> <u>tschawytscha</u>

Crude extract obtained			Limit of error of bioassay %
Absorbance ratio nm 280/260	Oxytocic units per A.U.$_{280nm}$	Gm wet wt.	
0.67	0.0593	1.8	6.0
0.81	0.0765	2.5	13.0
0.84	0.1040	4.5	10.2
---	0.0360	6.3	8.6
0.83	0.144	9.5	7.3
0.81	0.148	8.5	11.0
0.93	0.076	8.2	6.2
0.88	0.010	1.4	12.9
0.87	0.079	10.0	10.6
0.90	0.098	7.3	9.3

develop optimal conditions for purification steps, and
utilized from one to ten grams of tissue each. The last
four extracts were done on preparative scale each utilizing
100 gm of tissue, and provided pure hormones for the
analyses of amino acid composition and sequence. The flow
chart illustrated in Figure 4 summarizes the purification
schedules developed for large scale extracts.

(1) Gel Filtration of Crude Salmon Pituitary Extracts

Two types of gel filtration media were used in puri-
fying the neurohypophysial hormones of salmon: Biogel and
Sephadex. Only Sephadex G-15 was used in preparative work.

Figure 5 illustrates a typical elution profile ob-
tained using Biogel P-2 and 0.2 M acetic acid as the eluting
buffer. Spreading of hormonal activity was observed when
0.002 M acetic acid was used for elution. Similar spreading
of synthetic oxytocin took place under identical conditions.

A comparison of Sephadex G-25 and G-15 is shown in
Figure 6. A spreading of hormonal activity, similar to that
observed on Biogel P-2, did not take place although a low
ionic strength buffer was used. The purification on
Sephadex G-15 is almost 100% better than that obtained on
G-25 columns. This observation was made consistently, since
Sephadex G-25 columns were used in purifying the first ten
salmon pituitary extracts. Sephadex G-15 was used in all

Oncorhynchus tschawytscha, frozen pituitaries, 100 g

                  0.2 M HOAc, cold, 10:1 v/w

TISSUE HOMOGENATE

             48,200 g, 30 min.

PRECIPITATE   CRUDE EXTRACT

re-extracted   (1 L)

                                  Sephadex G-15

                                  5 tandem runs

                            ACTIVE EFFLUENT

                                  (1 L)

                                  UM-2 or UM-3
                                  ultrafiltrations
                                  (lyophylization)

FILTRATE   CONCENTRATE

                                  one of:

                                  Whatman CM-32
                           SE-Sephadex G-25
                                  Phosphocellulose

HORMONE I   HORMONE II

(ultrafiltration;   Ultrafiltration;
lyophylization)   lyophylization

              SE-Sephadex C-25 or
              Whatman CM-32

HORMONE I   HORMONE II
Spec.activity:* $1.25\text{-}1.45 \times 10^2$ $2.09\text{-}2.29 \times 10^2$

Figure 4: Purification Sequence of Large Scale Salmon
              Pituitary Extract

* Oxytocic units per mg

Procedure shown in brackets was not always employed.
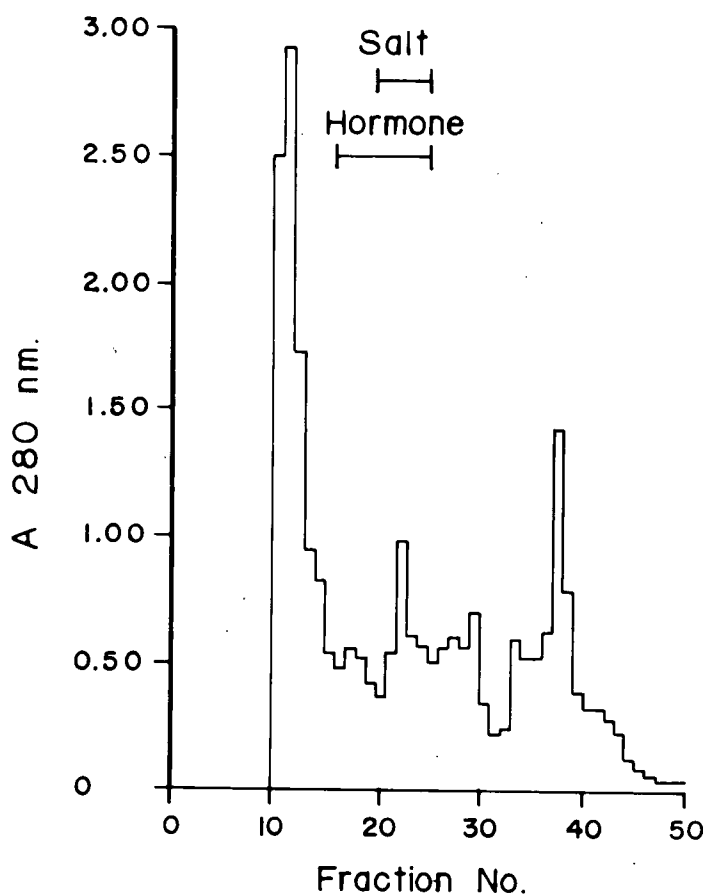
Figure 5: Gel filtration of crude salmon pituitary extract on Biogel P-2.

Column 320 mm x 30 mm diameter; eluent 0.2 M acetic acid; flow rate 1.0 ml/min; fraction volume 5.0 ml.

Loading sample: 5.0 ml; 94.5 A.U.$_{280 \text{ nm}}$; 15.9 ± 1.2 oxytocic units.

Purification 3.4 fold; recovery of oxytocic activity 89.5%.
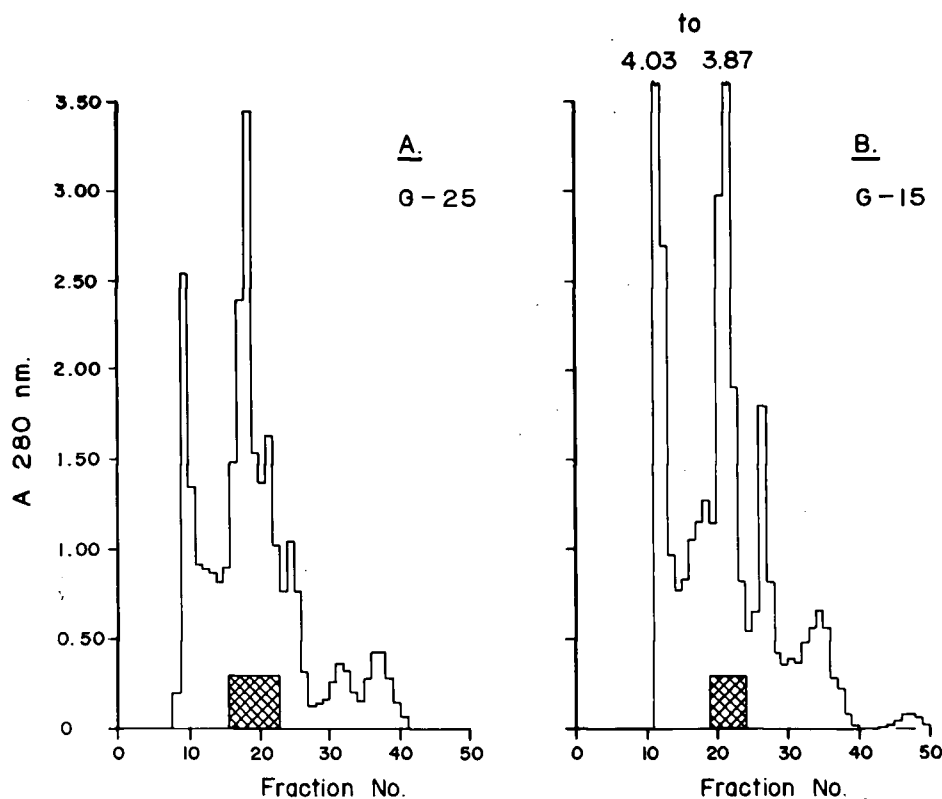
Figure 6: Comparison of gel filtration on Sephadex G-25 and Sephadex G-15.

Columns 550 mm x 10 mm diameter; eluent 0.002 M acetic acid; flow rate 0.5 ml/min (A) and 0.3 ml/min (B); fraction volume 1.5 ml (A) and 1.0 ml (B).

Loading sample (A and B): 0.8 ml; 51.0 A.U.$_{280\ nm}$; 0.76$\pm$0.051 oxytocic units.

Purification 2.6 fold (A), and 4.7 fold (B); recovery of biological activity 53% (A) and 48% (B).

Solid line: absorbance at 280 nm; cross-hatched: hormone containing effluent fractions.

later experiments, but the molarity of eluting buffer was increased to 0.2 M. This was done in order to preserve maximum oxytocic activity of the extract, as discussed in section 2 of this chapter.

Further adaptation of the Sephadex G-15 gel filtration involved using the same column repeatedly (tandem separations) to allow rapid processing of large volumes of extract (Figure 7). This permitted gel filtration immediately following the extraction of the tissue without the need for concentration. The elution of hormones coincided with the elution of salt in all gel filtration experiments. The usual yield of oxytocic activity following gel filtration on Sephadex G-15 with 0.2 M acetic acid as eluent ranged from 90 to 100%. However, in one large scale experiment, the salt peak was used instead of the bioassay to locate the hormones, and the recovery of the initial oxytocic activity was 78%.

Rechromatography of the biologically active fraction from Sephadex G-15 on Sephadex G-10 or G-15 resulted in two-fold purification. Purification afforded at this stage by G-10 columns was comparable with that of G-15 columns. An increase in length of the column had no effect on purification obtained by this rechromatography (Figure 8). A two-fold purification at this early stage of the isolation sequence was regarded to be of little value to the overall

Loaded 225 ml. (1) → Loaded 225 ml. (2) → Loaded 225 ml. (3) → Loaded 222 ml. (4) → Loaded 153 ml. (5) →

A 280 mu

8.0

6.0

4.0

2.0

0

1.5
1.0
0.5
0
mmho

0   40   80   120   160   200   240   280
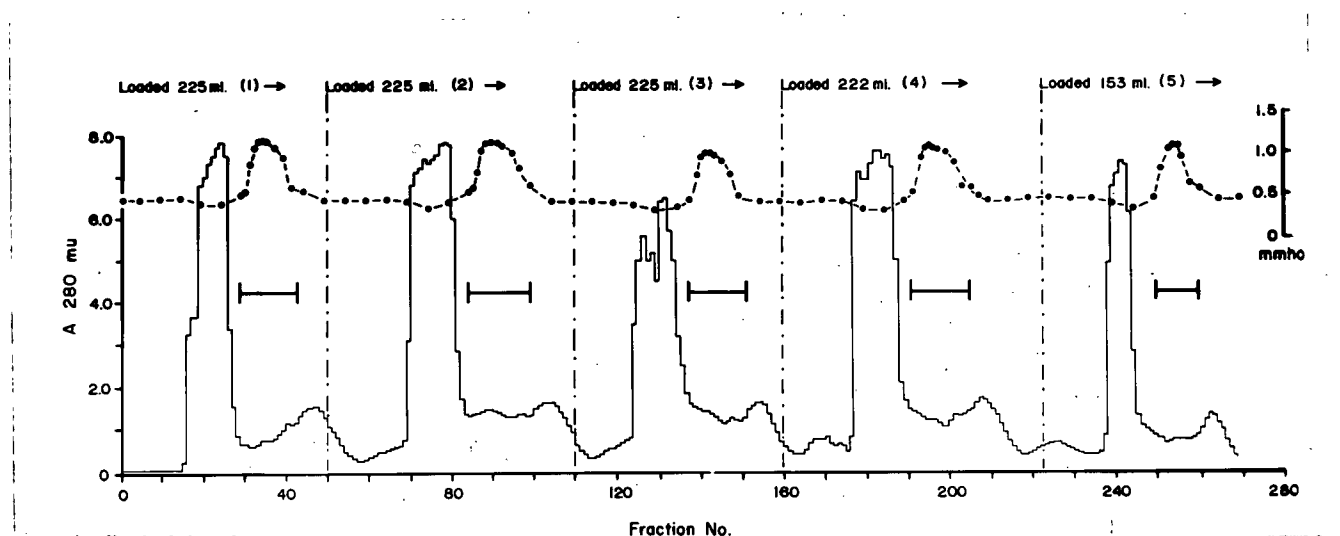
Fraction No.

Figure 7: <u>Gel filtration of crude salmon pituitary extract using a tandem series of Sephadex G-15.</u>

Column 450 mm x 50 mm diameter; eluent 0.2 M acetic acid; flow rate 4 ml/min; fraction volume 20 ml.

Total loading sample: 1050 ml; 12060 A.U.$_{280\ nm}$; 954 ± 103 oxytocic units.

Purification 4.4 fold; recovery of oxytocic activity 96%.

Solid line: absorbance at 280 nm; dotted line: specific conductivity; bars: hormone containing effluent fractions.

A. G-15, 2.5 x 92   **B.** G-15, 5 x 44
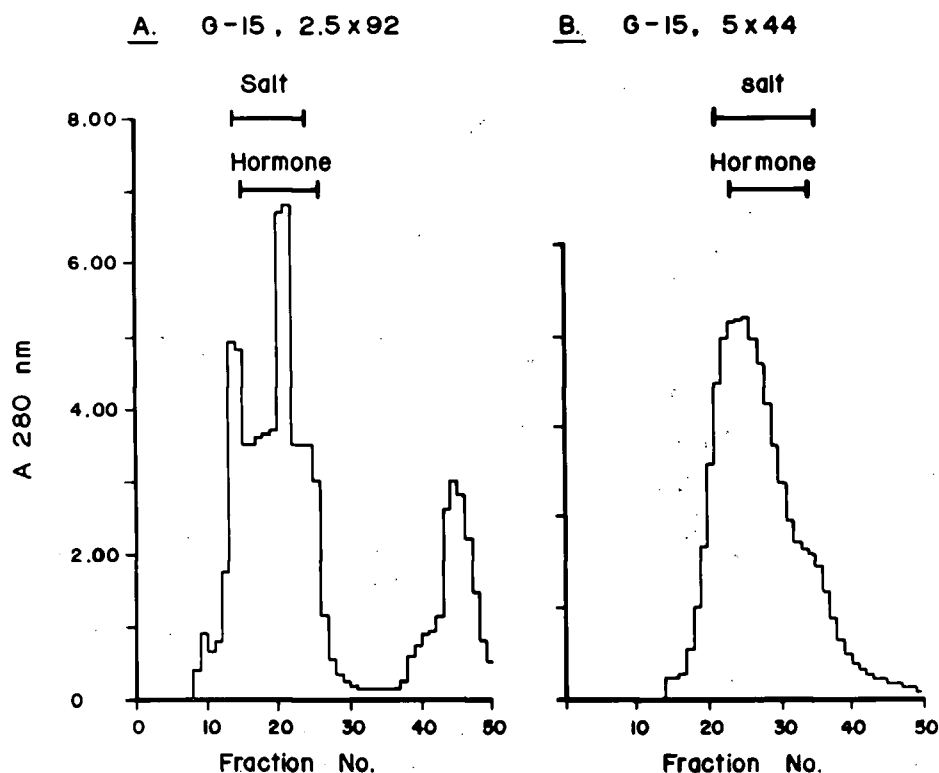
Figure 8:   Rechromatography of partially purified salmon
            pituitary extract on Sephadex G-15 columns of
            equal gel bed volumes and different dimensions.

A.   Column 920 mm x 25 mm diameter; eluent 0.2 M acetic acid;
     flow rate 0.6 ml/min; fraction volume 10.0 ml. Loading
     solution: 12.0 ml; 723 A.U.$_{280}$ nm; $435^{\pm}44$ oxytocic units.
     Purification 1.9 fold; recovery of oxytocic activity 93%.

B.   Column 440 mm x 50 mm diameter; eluent 0.2 M acetic acid;
     flow rate 4.0 ml/min; fraction volume 20.0 ml. Loading
     solution: 41 ml; 800 A.U.$_{280}$ nm; $480^{\pm}49$ oxytocic units.
     Purification 2.1 fold; recovery of oxytocic activity 94%.

purification of the extract. On the other hand, every additional step included in the isolation sequence was a potential source for loss of valuable material. For this reason rechromatography of the active eluent from the first gel filtration step on another gel filtration column was not adopted for routine use.

(2) De-salting of Neurohypophysial Hormones

De-salting of partially purified neurohypophysial hormones is a necessary preliminary to ion exchange chromatography. It was observed during gel filtration experiments on salmon pituitary extracts (cf. section 4(a) of this chapter) that the salt peak coincided with the peak of oxytocic activity on Biogel P-2, Sephadex G-25 and Sephadex G-15. Two methods of de-salting were examined in the course of this work: gel filtration on Sephadex G-10 and ultra-filtration.

(i) De-salting on Sephadex G-10

Commercial preparations of oxytocin (Sigma, synthetic powder, Grade IV) and bovine albumen (Armour, crystaline) were used for these experiments. Bovine albumen was used as the indicator of the elution front in place of Blue Dextran (Pharmacia), because the latter exhibits a high degree of spreading on Sephadex G-10 gel.

A Sephadex G-10 (1·1m x 10 mm diameter) column pro-
vides adequate separation of oxytocin and sodium chloride
(Figure 9,A). However, sodium acetate and ammonium acetate
could not be separated from oxytocin in the same system
(Figure 9,B). Attempts were made to obtain separation by
varying temperature, flow rate, and the composition of the
eluting buffer. The flow rate was varied from 0.5 to 13.7
ml/min, the temperature from 9.5°C to 73.0°C, and the
eluting buffer was varied by increasing the concentration
of acetic acid from 0.002 M to 0.200 M and by addition of
20% methyl alcohol. None of the conditions tried, singly
or in combination, achieved the desired separation.

(ii) De-salting by ultrafiltration

Diaflo UM-2 and UM-3 membranes were used for de-
salting of pituitary extracts of salmon. The biologically
active eluent from Sephadex G-15 was subjected to ultra-
filtration prior to the separation of hormones on a cation
exchanger. Following this separation and before rechroma-
tography of individual hormones on another cation exchanger,
de-salting was also required. The degree to which the sample
was de-salted by ultrafiltration at each step was deter-
mined by the ionic strength at which the desired substance
was expected to be eluted from the ion exchange column.
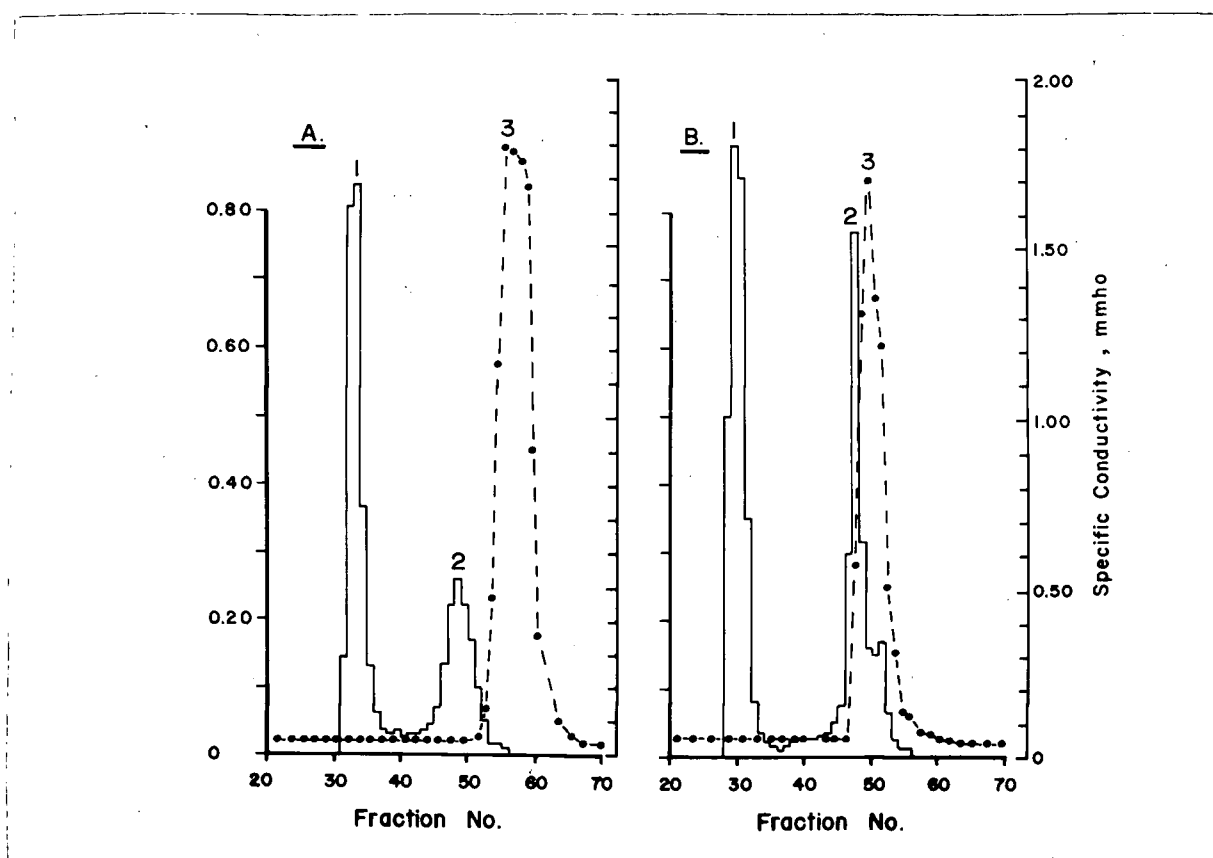
Figure 9: De-salting of oxytocin on Sephadex G-10.

Column 1.1 m x 10 mm diameter; eluent 0.002 M acetic acid; flow rate 0.3 ml/min; fraction volume 1.0 ml.

Peak 1 (solid line) bovine albumen, Armour crystaline; peak 2 (solid line) oxytocin, Sigma synthetic powder, Grade IV; peak 3 (dotted line) - salt; Left-hand scale: A 280 nm.

A: peak 3 is sodium chloride.   B: peak 3 is sodium acetate.

Tables IV and V illustrate the recoveries of material following ultrafiltration. The results obtained appeared to be independent of the contents or of the volume of the sample, and subject to individual peculiarities of the membranes used. The recoveries ranged from the very good to the very poor. In former cases, a considerable concomitant purification of the sample was observed as can be seen from the absorbance measurements. It can be seen from these results that two types of losses of material were experienced: passing of the hormones into the filtrate and complete loss of the hormones. The second type of loss could not be explained since the details of membrane structure are not reported by the manufacturers (60).

Some experiments with UM-1 membranes, directed at removing high molecular weight contaminants were largely negative. Sandoz "Syntocinon" was passed quantitatively into the ultrafiltrate, as was expected from the molecular weightscut-off range of this type of membrane (10,000) and the molecular weight of oxytocin (1,000). Oxytocic activity of crude extracts of salmon pituitaries, on the other hand, was not passed by the UM-1 membranes.

(3) Separation of Neurohypophysial Hormones of Salmon by Ion Exchange Chromatography

Following gel filtration and de-salting, the two neurohypophysial hormones of salmon were separated by ion

T A B L E IV

Ultrafiltration of Partially Purified Pituitary Extracts of <u>Oncorhynchus</u>

<u>tschawytscha</u> Using Diaflo UM-2 Membranes

Volume of sample (ml)	% of initial oxytocic activity in concentrate	in filtrate	% of initial absorbance @ 280 nm in concentrate	in filtrate
100 (Syntocinon)	97.5	nil	-- (a)	-- (a)
70	92.0	7.2	70.5	26.8
105	107.8	nil	-- (b)	10.5
225	41.2	13.7	44.6	50.4
300	80.4	15.7	49.4	54.4
100	83.8	12.5	72.8	30.4

(a) absorbance too low for measurement

(b) not tested: precipitation of material in concentrate

All samples were filtered until 10 - 15 ml remained above the membrane. All samples, with the exception of Syntocinon, were purified by passage through a Sephadex G-15 column.

Pressure of 80 p.s.i. was used in all experiments.

T A B L E   V

Ultrafiltration of Partially Purified Pituitary Extracts of

Oncorhynchus tschawytscha Using Diaflo UM-3 Membranes

Loading Solution Contents	Volume, ml	% initial oxytocic activity		% initial absorbance @ 280 nm	
		concentrate	filtrate	concentrate	filtrate
Sandoz 'Syntocinon' pH 4.8	160	22.8	23.0	-- (a)	-- (a)
Sandoz 'Syntocinon' pH 2.1	160	25.6	23.2	-- (a)	-- (a)
Sephadex G-15 effluent	600	77.8	20.6	92.0	10.6
Ultrafiltration concentrate of Sephadex G-15 effluent	250	89.1	12.6	80.3	18.8
Sephadex G-15 effluent	1335	83.0	7.9	-- (b)	55.0
Sephadex G-15 effluent	1050	103.0	nil	66.8	45.5
Hormone I after ion exchange separation	70	90.5	5.8	65.2	28.0

(continued)

99

Loading Solution Contents	Volume, ml	% initial oxytocic activity		% initial absorbance @ 280 nm	
		concentrate	filtrate	concentrate	filtrate
Hormone I after ion exchange separation	57	56.4	5.4	50.0	55.0
Hormone II after ion exchange separation	600	103.0	nil	31.0	-- (a)
Hormone II after ion exchange separation	250	32.4	68.4	-- (a)	-- (a)
Hormone II after ion exchange rechromatography	350	96.0	15.8	-- (a)	-- (a)

(a)   absorbance too low for measurement.

(b)   not tested: precipitation of material in concentrate.

All samples were filtered until 10 - 15 ml remained above the membrane.

exchange chromatography (cf. Figure 4). Three types of
cation exchangers were used for this purpose: Whatman
carboxymethylcellulose (CM-32, microgranular), Selectacel
phosphocelluose and Sulfoethyl Sephadex (SE-Sephadex,
CM-25). The ion exchange media were first standardized
with known synthetic neurohypophysial hormones (Sigma
oxytocin, Grade IV or Sandox Syntocinon, and N.B.C. 3-phe,
8-arg oxytocin). The neurohypophysial hormones of salmon
were eluted from these cation exchangers at the ionic
strengths corresponding to those at which oxytocin and
3-phe, 8-arg oxytocin were eluted, respectively. This
indicated that at a given pH the charge borne by the salmon
neurohypophysial hormones were identical with the charges
of oxytocin and 3-phe, 8-arg oxytocin, respectively.

Initially the separation of hormones on Whatman CM-32
and on phosphocellulose was achieved using an elution method
reported by Sawyer (45). The method involves the elution
of the less basic hormone using 0.02 M ammonium acetate
buffer, pH 5, followed by a gradient to 0.2 M buffer of
pH 7.5 to elute the more basic hormone. Subsequently this
method was substituted by a continuous salt gradient from
0.002 M to 0.200 M sodium or ammonium acetate at constant
pH (pH 5) for elution of both hormones. Representative
elution profiles for Whatman CM-32 and phosphocellulose,
using a continuous salt gradient at pH of 5, are illustra-
ted in Figures 10 and 11 respectively.
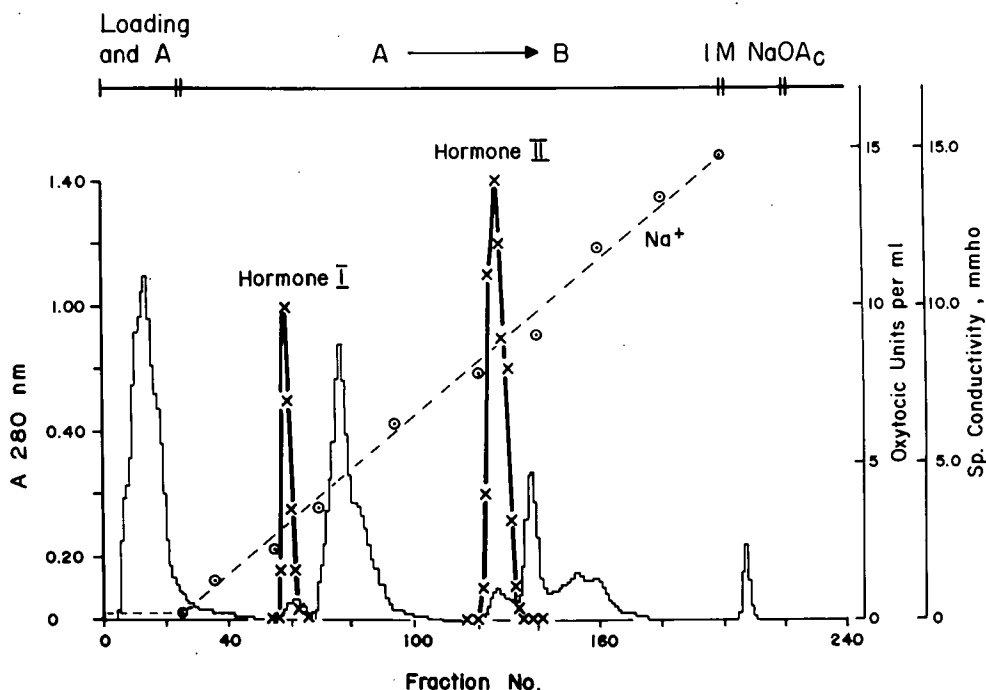
Figure 10: Separation of salmon neurohypophysial hormones on Whatman CM-32.

Column 390 mm x 12 mm diameter; flow rate 0.6 ml/min; fraction volume 5.0 ml; sodium acetate, pH 5.

Buffer A (to fraction 30) 0.196 mmho, 0.002 M.
Buffer B (A -- B gradient, fractions 31 -- 200) 14.8 mmho, 0.200 M.

Loading solution: 42 ml; specific activity (oxytocic units per $A.U._{280}nm$) 1.4.

Hormone I, specific activity 25.8; Hormone II, specific activity 65.0.

Recovery of oxytocic activity: 98%.

Light black line: absorbance at 280 nm; heavy black line: oxytocic activity (each point = one four-point assay); dashed line: specific conductivity.
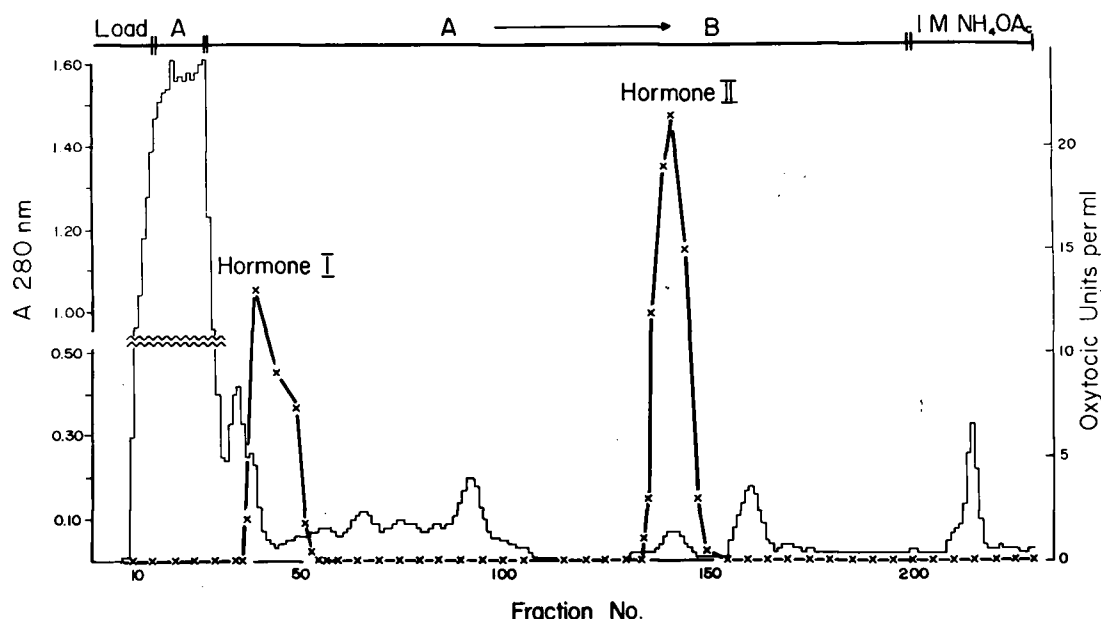
Figure 11: Separation of salmon neurohypophysial hormones on Selectacel phosphocellulose.

Column 420 mm x 12 mm diameter; flow rate 1.7 ml/min; fraction volume 5.0 ml; ammonium acetate, pH 5.

Buffer A (to fraction 30) 0.59 mmho, approx. 0.004 M; Buffer B (A -- B gradient, fractions 31 -- 200) 9.3 mmho, 0.200 M.

Loading solution: 97 ml; specific activity (oxytocic units per $A.U._{280}$ nm) 1.6.

Hormone I, specific activity 8.9; Hormone II, specific activity 93.0.

Recovery of oxytocic activity: 88%.

Light black line: absorbance at 280 nm; heavy black line: oxytocic activity (each point = one four-point assay).

Chromatography on SE-Sephadex at low pH (2.45) was

developed in the course of this work in an effort to purify

Hormone I.   Subsequently the system was scaled up and

applied to the separation of the hormones.   An eluent pro-

file of a large scale separation on SE-Sephadex is shown

in Figure 12.   While the elution of Hormone I gives a sym-

metrical narrow peak, the elution of Hormone II results in

a broad band.   In a subsequent experiment the elution of

Hormone II was done in a stepwise manner with 2 M ammonium

acetate at pH 5, and oxytocic activity was eluted in three

fractions.

The choice of cation exchanger to be used for the

initial separation of hormones was governed by the purpose

for which the separated hormones were intended.   Toward the

end of this investigation additional amounts of Hormone I

were required for structural studies, and separation on the

sulfoethyl column provided a fast method for obtaining this

hormone.   Table VI illustrates the ionic strengths at which

the hormones were eluted from the three cation exchange

media used.   The results indicate that Hormone I was best

retained by SE-Sephadex at a pH of 2.45, and successively

less well by SE-Sephadex at pH 5, by phosphocellulose, and

by Whatman CM-32.   Increase in length of Whatman CM-32

columns and a change from sodium to ammonium acetate buffer

did not appear to affect the ionic strength at which Hormone
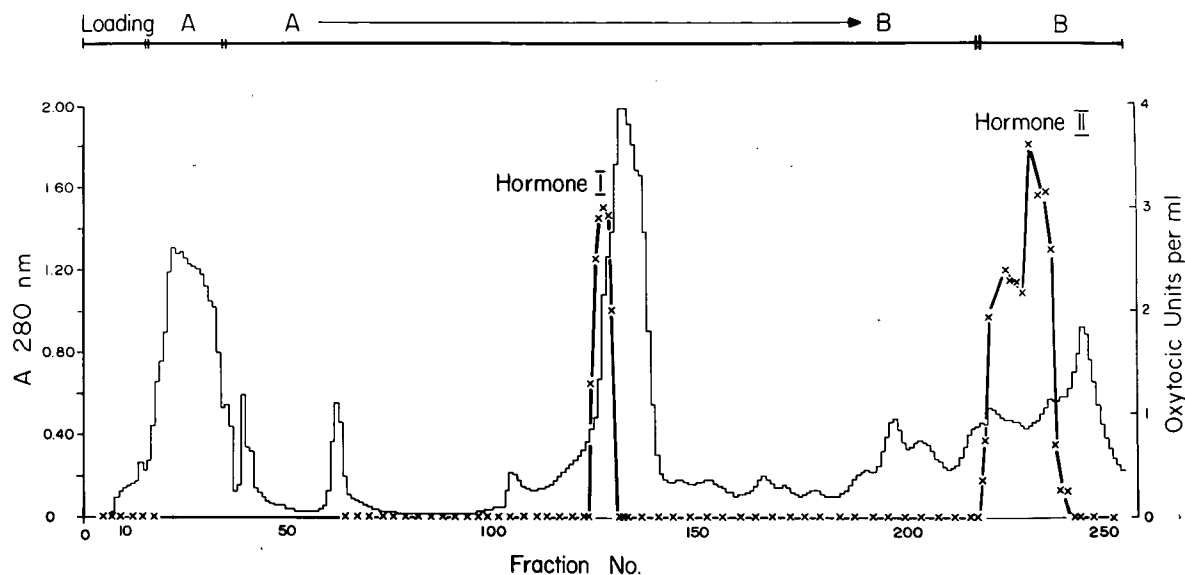
Figure 12: Separation of salmon neurohypophysial hormones on SE-Sephadex.

Column 430 mm x 25 mm diameter; flow rate 0.7 ml/min; fraction volume 10 ml; ammonium formate pH 2.45.

Buffer A (to fraction 40) 1.75 mmho, 0.085 M. Buffer B (A -- B gradient, fractions 41 -- 220; B, fractions 221 -- 250) 22.0 mmho, 0.2 M.

Loading solution: 130 ml; specific activity (oxytocic units per $A.U._{280}nm$) 0.42.

Hormone I, specific activity 2.7; Hormone II, specific activity 3.3.

Recovery of oxytocic activity: 92%.

Light black line: absorbance at 280 nm; heavy black line: oxytocic activity (each point = one four-point assay).

T A B L E   VI

Ion Exchange Chromatography of Neurohypophysial Hormones of <u>Oncorhynchus</u> <u>tschawytscha</u>:   Specific Conductivities for Eluted Hormones

Cation Exchanger	Column length x diameter mm	Eluting buffer used		Specific conductivity		% initial oxytocic activity recovered
		Salt	pH	Hormone I	Hormone II	
Whatman CM-32	90 x 12	sodium acetate	5.0-7.5	0.8 - 1.7	8.2 - 9.2	87
Whatman CM-32	90 x 12	sodium acetate	5.0	0.8 - 1.0	7.1 -11.2	70
Whatman CM-32	190 x 12	ammon. acetate	5.0	0.8 - 1.9	----	89
Whatman CM-32	390 x 12	ammon. acetate	5.0	0.9 - 1.0	5.7 - 6.7	98
Phosphocellulose	420 x 12	ammon. acetate	5.0	1.3 - 2.0	8.0 - 8.8	88
SE-Sephadex	120 x 12	ammon. acetate	5.0	2.2 - 2.5	10.7 -11.2	77
SE-Sephadex	120 x 12	ammon. formate	2.45	3.7 = 4.0	----	68
SE-Sephadex	430 x 25	ammon. formate	2.45	9.6 -11.1	20.0	92

Mixing chamber/reservoir volumes used were as follows: 250 : 250 cc for 90 x 12 columns; 450 : 450 cc for 420 x 12 columns; 950 : 950 cc for 420 x 25 columns.

Ammonium and sodium acetate gradients were from 0.002 M to 0.2 M;   ammonium formate from 0.085 M to 2.0 M.   A pH gradient was applied only in the first experiment in the Table.

I was eluted. The recoveries of initial oxytocic activity did not appear to vary with the cation exchange media used, and fluctuated from 68 to 98 percent.

At this stage of the purification, the extent of potentiation with magnesium ion (57) was measured in an effort to predict the hormones' chemical identity. The potentiation values ranged from 2.1 to 3.8 for Hormone I and from 1.5 to 2.5 for Hormone II. It was not found possible to predict the identity of the hormones on the basis of these results and the potentiation measurements were not carried on routinely. On the other hand, the ratio of total oxytocic activity of Hormone II compared to that of Hormone I was consistently observed to lie between 2.0 and 3.0 in each of the cation exchange separations of the two hormones.

(4) Purification of Salmon Neurohypophysial Hormone I by Ion Exchange Chromatography

In order to determine the sequence of amino acids within the molecule it was necessary to prepare a sufficient amount of the salmon hormone of 90%, or greater, purity. Quantitative amino acid analysis of the hormone preparation was used as a criterion of purity. An amino acid analysis of the Hormone I was not attempted from the initial separa-

tion of the two hormones by cation exchange chromatography (cf. preceeding section), because neither the specific activity of the hormone at this stage of purification, nor the appearance of the elution profile warranted an analysis.

Rechromatography of Hormone I on Whatman CM-32 or on phosphocellulose did not yield a pure preparation as judged by amino acid analyses. A better purification was obtained by rechromatography on SE-Sephadex at pH 5, yet the level of contaminating amino acids was still too high to attempt sequence studies: glu, lys, ala, val, leu, phe, his, and arg were present in amounts ranging from 0.1 to 0.4 residues per molecule.

A reduction in pH of the buffer used for rechromatography (pH 2.45 ammonium formate) yielded a pure preparation of Hormone I. Figure 13 illustrates this rechromatography step. The absorbance readings corresponding to the peak of biological activity approached the limit of error of the spectrophotometer and, as such, could not be used for estimation of specific activity of the purified Hormone I. The specific activity was calculated using the data obtained from amino acid analysis of the preparation (cf. Experimental Procedures).

Further studies on the isolation of Hormone I showed that the sequence in which the chromatographies were con- ducted at pH 5 and pH 2.45 respectively was not relevant to
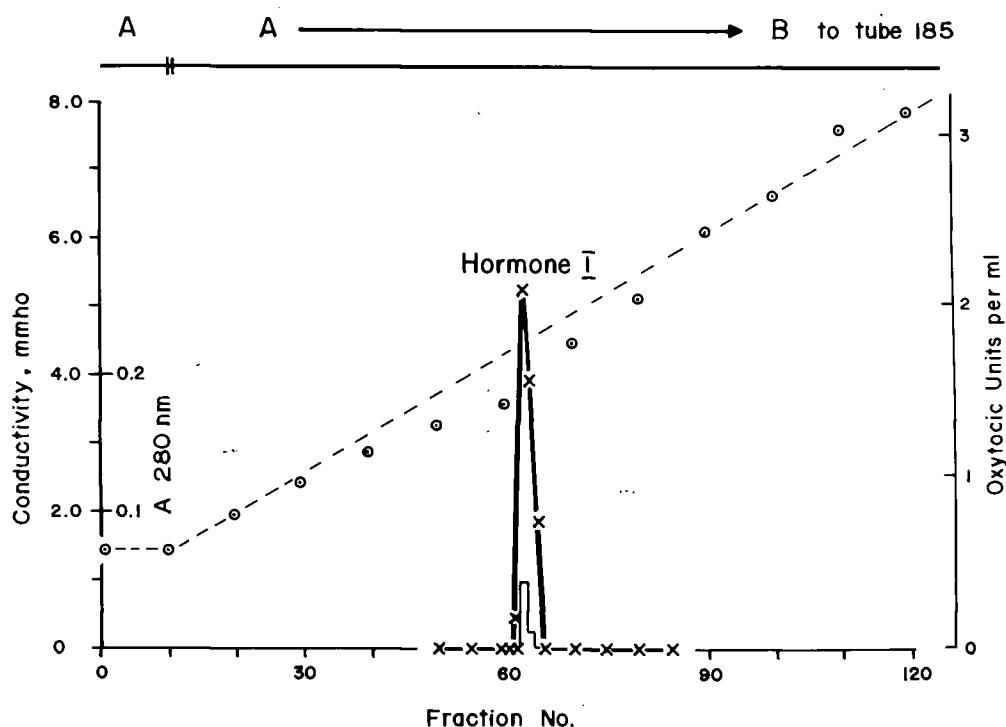
Figure 13: Rechromatography of Hormone I on SE-Sephadex.

Column 120 mm x 12 mm diameter; flow rate 2.5 ml/min; fraction volume 5.0 ml; ammonium formate pH 2.45.

Buffer A (to fraction 10) 1.72 mmho, 0.085 M.   Buffer B (A -- B gradient, fractions 11 -- 185) 11.4 mmho, 1 M.

Loading solution: 4 ml; specific activity (oxytocic units per A.U.$_{280}$nm) 20.0.

Hormone I (purified), specific activity 145 oxytocic units per milligram.

Recovery of oxytocic activity: 68%.

Light black line: absorbance at 280 nm; heavy black line: oxytocic activity (each point = one four-point assay); dashed line: specific conductivity.

the purification. Thus the initial separation of Hormone I and Hormone II could be done on SE-Sephadex at pH 2.45, and the subsequent rechromatography of Hormone I on Whatman CM-32 at pH 5. Figure 14 illustrates a rechromatography on Whatman CM-32 and which resulted in preparation of pure hormone, following the initial separation of hormones on an SE-Sephadex column.

Amino acid analyses of purified Hormone I from three separate isolation procedures are given in Table VII and are compared with the theoretical values for 4-ser, 8-ileu oxytocin which have been reported from other teleosts. On the basis of quantitative amino acid analysis data in this table, Hormone I of O. tschawytscha and 4-ser, 8-ileu oxytocin appear to have identical amino acid compositions. The specific activity calculated for Hormone I of salmon was 125, 138 and 145 oxytocic units per mg, and the specific activity value reported in the literature using the same assay method on the synthetic preparation of the hormone is 150 oxytocic units per milligram (Table VII).

(5) Purification of Salmon Neurohypophysial Hormone II
    by Ion Exchange Chromatography

Amino acid analyses of Hormone II following only one cation exchange chromatography (the initial separation of Hormone I and Hormone II on Whatman CM-32) appeared to be

Figure 14: Rechromatography of Hormone I on Whatman CM-32.

Column 190 mm x 12 mm diameter; flow rate 0.8 ml/min;
fraction volume 5.0 ml; ammonium acetate pH 5.

Buffer A (to fraction 20) 0.215 mmho, 0.002 M; Buffer B
(A -- B gradient, fractions 21 -- 214) 6.9 mmho, 0.1 M.

Loading solution: 53 ml; specific activity (oxytocic
units per A.U.$_{280}$nm) 3.0.

Hormone I (purified), specific activity 125 oxytocic units
per milligram.

Recovery of oxytocic activity: 89%.

Light black line: absorbance at 280 nm; heavy black line:
oxytocic activity (each point = one four-point assay).

T A B L E   VII

Amino Acid Composition (Residues per Mole) of

Neurohypophysial Hormone I of <u>Oncorhynchus</u> <u>tschawytscha</u>

Amino Acid	4-ser, 8-ileu oxytocin theoretical		P r e p a r a t i o n		
			No. 24 (a)	No.23 (b)	No. 22 (b)
Ammonia	2.00		2.19	3.38	2.34
Cysteic acid	2.00	(b)	--	1.59	1.64
Aspartic acid	1.00		1.04	0.96	1.03
Serine	1.00		1.02	1.01	1.00
Proline	1.00		0.90	1.03	0.90
1/2 Cystine	2.00	(a)	1.69	--	--
Glycine	1.00		1.21	1.22	1.34
Isoleucine	2.00		1.87	1.71	1.80
Tyrosine (c)	1.00		0.83	0.21	0.42
Other amino acids:					
Lysine	nil		0.06	0.14	nil
Valine	nil		0.04	0.04	trace
Glutamic acid	nil		0.16	trace	trace
Leucine	nil		trace	trace	trace
Oxytocic units per μm	--		133	121	140
Oxytocic units per mg	150 (75)		138	125	145
Limit of bioassay error, %	8.0 (75)		10.2	14.0	8.0

(a) Hydrolyzate of unoxidized hormone.

(b) Hydrolyzate of the hormone following performic acid
     oxidation.

(c) Loss of tyrosine was observed frequently, in both the
     oxidized and the un-oxidized hydrolysates, of both the
     salmon hormones and the standards.

Oxytocic assays according to Holton. Molecular weight of
4-ser, 8-ileu oxytocin was calculated as 965.
Hormone I from salmon extracts 23 and 24 was purified on
Whatman carboxymethylcellulose (CM-32, microgranular) at
pH 5 following the initial separation of hormones on
sulfoethyl Sephadex (SE-Sephadex C-25), at pH 2.45. The
reverse of this procedure was used in purification of
Hormone I from extract 22.

warranted on the basis of three criteria: specific activity of the pooled hormone peak, appearance of the elution profile, and behaviour of the molecule on high voltage electrophoresis. The specific activity of Hormone II obtained by separation of neurohypophysial hormones of salmon on Whatman CM-32 was in the range of 100 oxytocic units per milligram, while the highest values reported in the literature for 8-arg oxytocin were 115 and 125 oxytocic units per milligram (75), (6). The 280 nm absorbance peak corresponding to the oxytocic activity peak of Hormone II appeared symmetrical. High voltage electrophoresis of the preparation (pH 3.6, 3,000 volts, 35 min) revealed the presence of only one ninhydrin positive substance and this, upon elution from the paper, was found to have oxytocic activity. Amino acid analysis, however, revealed an impure preparation: lys (0.43); his (0.52); ammonia (3.53); arg (1.36); asp (0.95); thr (0.21); ser (0.53); glu (1.52); pro (1.30); 1/2 cys (1.74); gly (1.55); ala (0.26); val (0.31); met (0.13); ileu (1.07); leu (0.20); tyr (0.93); phe (0.09). The underlined amino acid residues represent those residues later found to constitute the molecule of Hormone II.

Further purification of Hormone II by rechromatography was next investigated. Following an initial separation on SE-Sephadex at pH 2.45 an attempt was made to purify

Hormone II using the same system as for Hormone I, that is, by rechromatography on Whatman CM-32. However, a pure preparation of Hormone II was not achieved by this method: ammonia (6.12); arg (0.80); CySO$_3$ (1.55); asp (0.94); glu (1.10); pro (2.44); gly (1.11); ileu (0.86); tyr (0.40); lys (1.43); val (1.69). The remaining material was subjected to rechromatography on phosphocellulose and a pure preparation of hormone was obtained.

Further studies on the rechromatography of Hormone II showed that phosphocellulose at pH 5.0 provided the best purification, irrespective of whether the initial fractionation was done at pH 5.0 or at pH 2.45. Satisfactory purification was also obtained on Whatman CM-22 (fibrous) at pH 5.0 after initial fractionation under similar conditions (Figure 15).

The amino acid analyses obtained for three separate preparations of Hormone II are comparable to the theoretical values for 8-arg oxytocin (Table VIII). However, the specific activities of the preparations of Hormone II were almost twice those reported for 8-arg oxytocin (Table VIII).
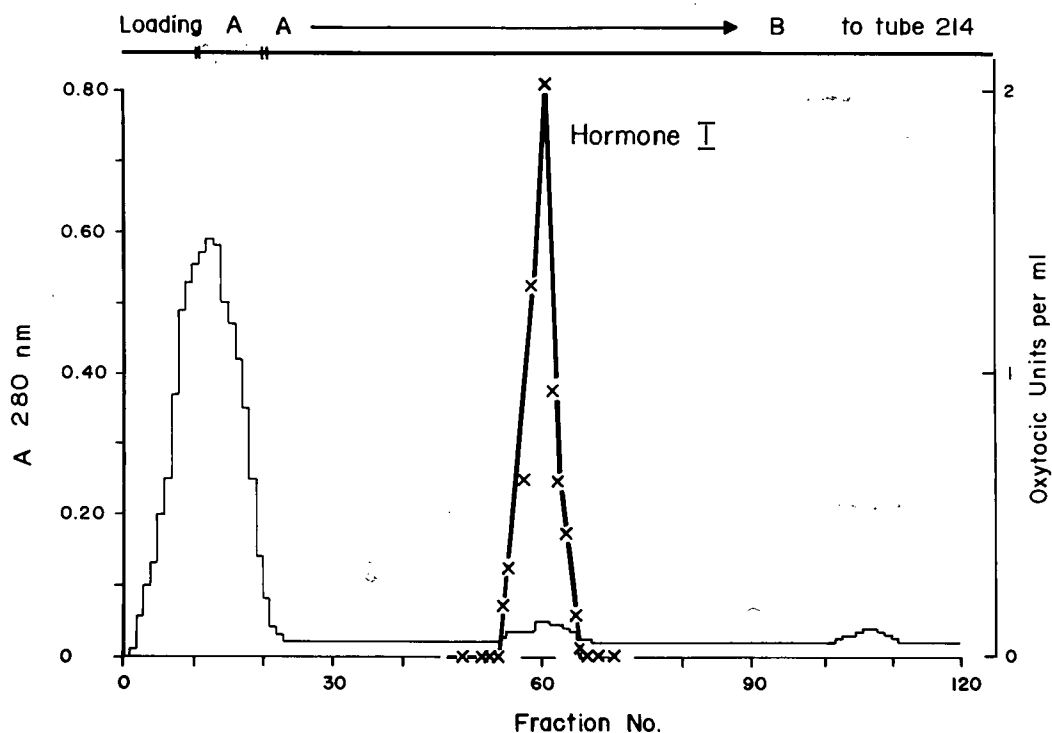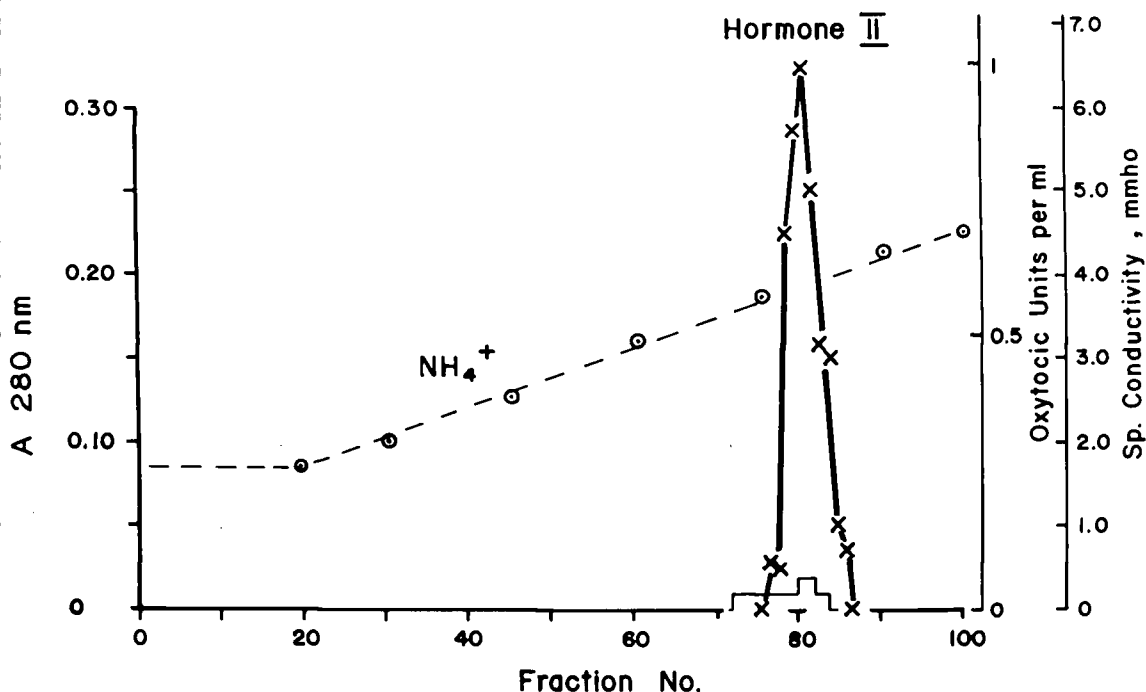
Figure 15: <u>Rechromatography of Hormone II on Whatman CM-22.</u>

Column 440 mm x 12 mm diameter; flow rate 2.8 ml/min;
fraction volume 5.0 ml, ammonium acetate pH 5.

Buffer A (to fraction 10) 1.65 mmho, 0.03 M; Buffer B
(A -- B gradient, fractions 11 -- 200) 8.5 mmho, 0.2 M.

Loading solution: 16.5 ml; specific activity (oxytocic
units per A.U.$_{280}$nm) 12.3.

Hormone II (purified), specific activity 201 oxytocic units
per milligram.

Recovery of oxytocic activity: 63%.

Light black line: absorbance at 280 nm; heavy black line:
oxytocic activity; dashed line: specific conductivity.

T A B L E   VIII

Amino Acid Composition (Residues per Mole) of

Neurohypophysial Hormone II of <u>Oncorhynchus</u> <u>tschawytscha</u>

Amino Acid	8-arginine-oxytocin theoretical		Preparation			
			No. 23 (b)	No. 22 (b)	No. 22 (a)	No. 21 (a)
Ammonia	3.00		5.20	3.98	3.68	2.98
Arginine	1.00		0.79	0.83	0.82	0.68
Cysteic acid	2.00	(b)	1.71	1.96		
Aspartic acid	1.00		1.02	1.09	1.06	0.96
Glutamic acid	1.00		1.04	1.00	1.08	1.02
Proline	1.00		1.05	0.80	0.84	0.92
1/2 Cystine	2.00	(a)			1.54	1.40
Glycine	1.00		0.94	1.14	1.27	1.01
Isoleucine	1.00		0.90	0.87	0.92	0.65
Tyrosine (c)	1.00		0.25	0.43	0.55	0.40
Other Amino Acids:						
Lysine	nil		nil	nil	nil	0.25
Histidine	nil		nil	nil	nil	0.18
Serine	nil		trace	nil	nil	0.20
Alanine	nil		nil	nil	nil	0.16
Valine	nil		nil	nil	nil	0.14
Leucine	nil		trace	nil	nil	nil
Oxytocic units per μm	--		220	241	238	192
Oxytocic units per mg	125 (6)		209	229	227	201
Limit of bioassay error, %	115 (75) 13.0 (75)		13.0	6.0	6.0	6.5

(a)   Hydrolyzate of unoxidized hormone.
(b)   Hydrolyzates of oxidized hormone (performic acid)
(c)   Loss of tyrosine observed frequently.

Oxytocic assays according to Holton (55).
Molecular weight of 8-arg oxytocin was calculated as 1050.

Hormone II from salmon extract 23 was purified by rechromato-
graphy on phosphocellulose (Selectacel) following the initial
separation of hormones on sulfoethyl Sephadex (SE-Sephadex
C-25).   Hormone II from salmon extract 22 was purified by
rechromatography on phosphocellulose (Selectacel) following
the separation of hormones on Whatman CM-32.   Hormone II
from salmon extract 21 was purified by rechromatography on
Whatman CM-22 (carboxymethylcellulose, fibrous) following
separation of hormones on Whatman CM-32 (carboxymethylcellulose,
microgranular).

D. Amino Acid Sequence of Neurohypophysial
Hormones of <u>Oncorhynchus</u> <u>tschawytscha</u>

The sequence of amino acids of both salmon neurohypophysial hormones was determined following performic acid oxidation to convert cystine and cysteine residues into cysteic acid (66).

(1) <u>Amino</u> <u>Acid</u> <u>Sequence</u> <u>of</u> <u>Hormone</u> <u>I</u> <u>of</u> <u>Salmon</u>

(i) Determination of N-terminal pentapeptide

The N-terminal amino acid of Hormone I was identified as a dansyl derivative of cysteic acid by thin layer chromatography in two solvent systems (propanol : water, and chloroform : methyl alcohol : acetic acid). Confirmation of this result, and the sequence of the next three amino acids was obtained by the subtractive method of Edman. Table IX presents the results obtained by the subtractive Edman method. It can be seen that after the first degradation cycle one of the two cysteic residues is lost, tyrosine is lost after the second, isoleucine after the third, and half of the serine residue after the fourth degradation cycle. Using this method, the sequencing has been repeated four times on the N-terminal tripeptide and twice on the N-terminal tetrapeptide. The sequence of $CySO_3H$-Tyr-Ileu-Ser was thus assigned to the N-terminus.

T A B L E   IX

The Amino Acid Sequence of N-terminal Tetrapeptide

of Hormone I of <u>Oncorhynchus</u> <u>tschawytscha</u>

Results of subtractive Edman degradation method:

Amino Acid	Amino acid analysis (residues per mole) after step no.:				
	0	1	2	3	4
Ammonia	2.19				
Cysteic acid	1.69	<u>1.00</u>	0.97	0.91	0.91
Aspartic acid	1.04	1.00	1.00	0.90	0.94
Serine	1.02	0.91	0.85	0.84	<u>0.49</u>
Proline	0.90	0.89	0.90	0.95	1.00
Glycine	1.21	1.20	1.22	1.18	1.22
Isoleucine	1.87	1.69	1.63	<u>1.14</u>	1.10
Tyrosine	0.83	0.78	<u>0</u>	0	0

These are results of duplicate analyses.

Following the fifth Edman degradation cycle, 22% of
the aspartic residue was lost from the peptide, indicating
that aspartic residue probably occupied position 5 in the
molecule. However, due to the rising baseline of incom-
pletely reacted N-termini it was not possible to continue
with the Edman degradation cycles without purification of
the material. An attempt to separate the completely re-
acted material, represented at this stage by the C-terminal
tetrapeptide, from the unreacted peptide species, on a
DEAE-cellulose column (18 mm x 12 mm diameter in pyridinium-
acetate, pH 5) failed to recover the desired material.

In a subsequent experiment use was made of partial
acid hydrolysis in order to obtain a fragment for further
sequence determination. The peptide following five Edman
degradation cycles again revealed only a 20% loss in aspar-
tic residues. Partial acid hydrolysis of this material
using 0.01 N hydrochloric acid at 105°C, for 18 hours, was
carried out to cleave the aspartyl (or asparaginyl) residue
at the adjoining N- and C-terminal peptide bonds (69).
Table X illustrates the peptide series which could be en-
visaged to be present in the partial hydrolyzate on the basis
of the results obtained for the N-terminal sequence of the
hormone. Cleavage was expected to release the remaining 80%
of the inaccessible C-terminus, as well as to confirm the
presence of aspartic acid in position five. Table X (2)
illustrates the results obtained by amino acid analysis of

T A B L E   X

Partial Acid Hydrolysis of Hormone I Following

Five Edman Degradation Cycles

(1) Peptide species and free amino acids in partial hydrolyzate
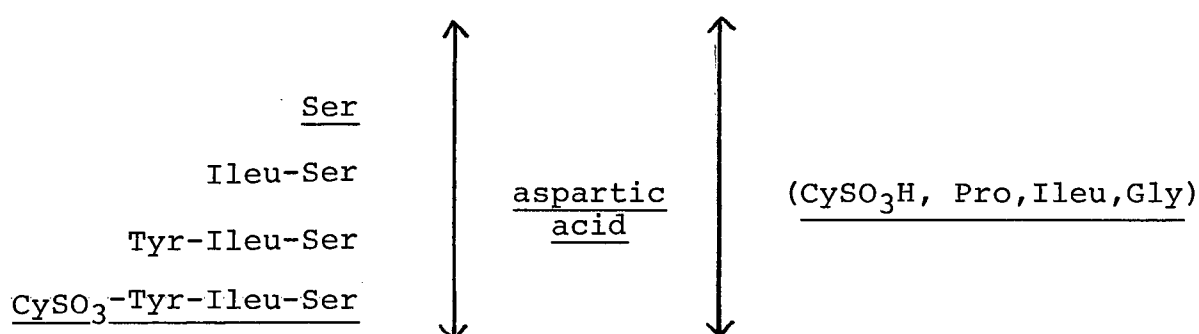
Peptides and free amino acids represented above are those which are expected to be produced by partial acid hydrolysis of partly degraded molecule of Hormone I. Vertical arrows represent hydrolytic cleavage sites expected. Underlined amino acids and peptides are those which have been identified by electrophoretic separation and amino acid analyses following partial hydrolysis.

(2) Free amino acids (residues per mole of hormone) found on

amino acid analysis of partial hydrolyzate

Cysteic acid	0.19
Aspartic acid	1.00
Serine	0.20
Glycine	0.39
Isoleucine	0.17

Liberation of aspartic acid was 85% of the theoretical value.

the partial hydrolyzate. It can be seen that free aspartic acid was released by the reaction as expected, together with smaller amounts of free cysteic acid, serine, glycine, and isoleucine. The release of free serine was also expected from the partially reacted hexapeptide series (Table X (1)), but the release of other amino acids indicated that the hydrolysis was more extensive than anticipated from pilot studies on synthetic 3-phe, 8-lys oxytocin. The release of aspartic acid was calculated to be 85% of that remaining after five Edman degradation cycles.

Electrophoretic separation of partial hydrolyzate (pH 6.5, 4,000 volts, 35 min) confirmed the presence of aspartate in position 5. Aspartic acid, cysteic acid, and one unidentified neutral amino acid were revealed by ninhydrin, in addition to two minor peptides and one major peptide. The peptides were eluted from the paper and sub-jected to amino acid analyses. The first minor peptide, identified as 'O' (from the neutral amino acid region) did not provide detectable amounts of amino acids. The second minor peptide, identified as '1' (mobility 100 mm toward the anode) contained equimolar amounts of cysteic acid, isoleucine, tyrosine and serine, which suggested that it was derived from the N-terminus of the hormone. The re-maining 50% of the eluted material from '1' was subjected to three consecutive Edman degradation cycles and placed

on the amino acid analyzer to reveal the presence of only one free amino acid, serine, thus re-confirming the placement of serine into position 4. The major peptide '2' (mobility 116 mm toward anode) was found on amino acid analysis to contain equimolar amounts of cysteic acid, proline, glycine and isoleucine, and a trace of serine. Thus '2' was identified as the C-terminal tetrapeptide.

(ii) Characterization of C-terminal tetrapeptide

The material remaining after the amino acid analysis of peptide '2' contained enough material (approximately 0.015 µmoles) for two dansyl-Edman cycles. The N-terminus of this tetrapeptide (position 6 in oxidized hormone molecule) was identified as dansyl-cysteic acid in two thin layer chromatography systems. Table XI (1) lists the mobilities obtained in this separation.

Position 7 (second amino acid in '2') was identified after one intervening Edman cycle as dansyl-proline in two thin layer chromatography systems used. The separation is shown in Figure 16. Table XI (2) lists the mobilities of the first chromatography system; the same thin-layer plate was re-run in the second system in the opposite direction, therefore the mobilities of the second separation are not given.

Thus positive identification was obtained for seven out of nine amino acids in the sequence of Hormone I. The

T A B L E XI

Identification of Dansyl Derivatives of Amino

Acids 6 and 7 of Hormone I

(1) Determination of the N-terminus of the C-terminal
tetrapeptide: amino acid 6 of Hormone I.

Mobility of dansyl amino acids in solvent systems

	A		B	
DNS-NH$_2$	1.00		1.00	
DNS-pro	0.99		1.00	
DNS-CySO$_3$H	0		0.13	
Unknown	0		0.12	
DNS-OH	0.03	(blue)	0.56	(blue)
DNS-ser	0.05		0.40	
DNS-gly	0.42		0.71	
DNS-ileu	1.00		1.00	

(2) Determination of the second amino acid from the
C-terminal tetrapeptide: amino acid 7 of Hormone I.

Mobility of dansyl amino acids in solvent system

DNS-NH$_2$	1.00
DNS-ser	0.05
DNS-pro	1.00
Unknown	1.00
DNS-ileu	1.00
DNS-gly	0.42

Mobilities are expressed relative to dansyl-ammonia.

Solvent systems: A - chloroform : methanol : acetic

acid (95 : 10 : 1); B - n-propanol :
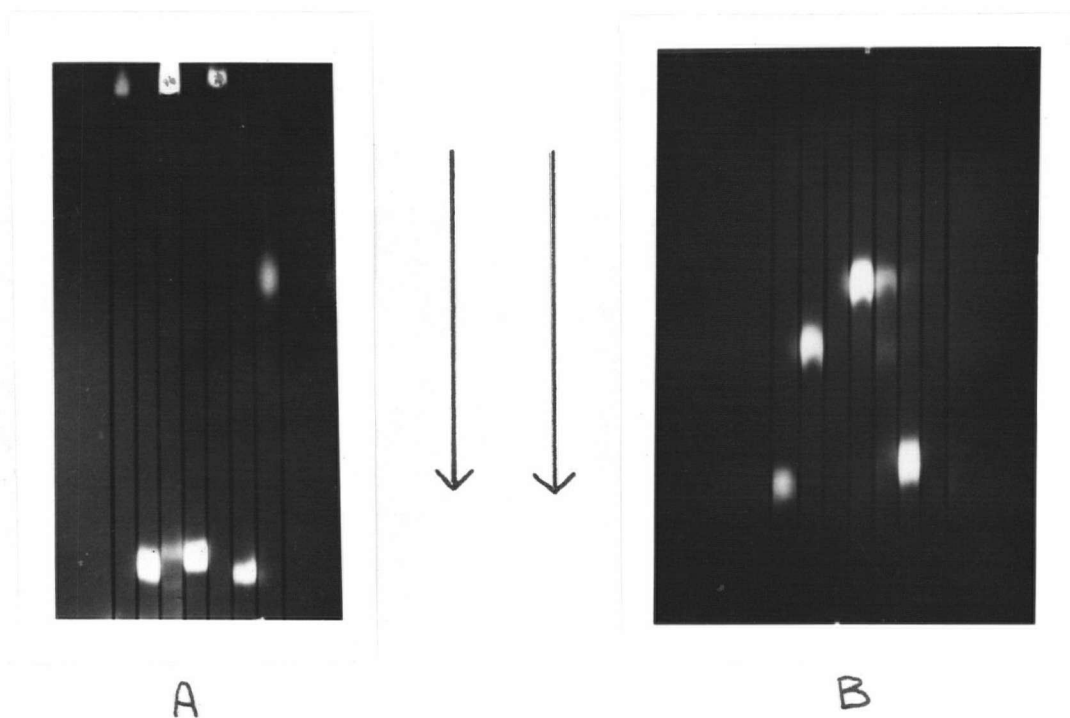
ammonia (80 : 20).

Figure 16: <u>Identification of amino acid 7 of Hormone I as DNS-proline</u>.

A: chloroform : methanol : acetic acid (95 : 10 : 1)
      left to right: DNS-ser; DNS-NH_2; DNS-OH and DNS-a.a.$_7$;
      DNS-pro; DNS-OH; DNS-ileu; DNS-gly.

B: n-propanol : ammonia (80 : 20), Plate reversed.
      left to right: DNS-gly; DNS-ileu; nil; DNS-pro;
      DNS-a.a.$_7$; DNS-NH_2; nil.

assignment of the last two amino acids (isoleucine and glycine) was by homology with the other neurohypophysial hormones which universally contain glycine as residue 9. The complete sequence of Hormone I following the oxidation with performic acid was thus determined as:

$$CySO_3H-Tyr-Ileu-Ser-Asp-CySO_3H-Pro-Ileu-Gly$$
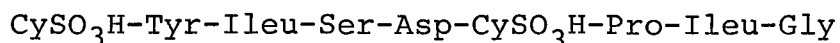
(2) Amino Acid Sequence of Hormone II of Salmon

(i) Determination of N-terminal pentapeptide

The N-terminal tripeptide was identified as $CySO_3$-Tyr-Ileu using the dansyl-Edman technique. The identification of dansyl derivatives of tyrosine and isoleucine was found to be difficult as the spots obtained were of faint fluorescence and the experiments had to be repeated twice, thus losing the advantage of the sensitivity of the method. Identification of the N-terminal pentapeptide using the Edman subtractive method, on the other hand, presented no difficulties. The results of the Edman subtractive analysis are summarized in Table XII. It can be seen from this table that one cysteic acid residue was lost after the first Edman cycle, tyrosine after the second, isoleucine after the third, glutamic acid after the fourth, and aspartic acid after the fifth cycle. The background of unreacted N-termini, similarly to the degradation of Hormone I, began

T A B L E   XII

The Amino Acid Sequence of N-terminal Penta-

peptide of Hormone II of <u>Oncorhynchus</u> <u>tschawytscha</u>

Results of subtractive Edman degradation method:

Amino Acid	Amino acid analysis (residues per mole) after step no.:					
	0	1	2	3	4	5
Ammonia	3.98	3.20	--	3.19	2.27	2.04
Arginine	0.83	0.79	0.85	0.75	0.69	0.68
Cysteic acid	1.96	<u>1.18</u>	0.99	0.93	1.02	0.94
Aspartic acid	1.09	1.06	1.03	1.02	1.00	<u>0.59</u>
Glutamic acid	1.00	1.03	1.03	1.00	<u>0.45</u>	0.47
Proline	0.80	1.00	0.99	1.01	0.89	0.97
Glycine	1.14	1.04	0.99	1.05	0.91	1.03
Isoleucine	0.87	0.86	0.92	<u>0.41</u>	0.20	0.26
Tyrosine	0.43	0.44	<u>0.13</u>	--	0.05	0

These are the results of duplicate analyses except

for residue 5 which was a single analysis.

to rise after the third degradation cycle. The N-terminal pentapeptide was identified from these experiments as $CySO_3H$-Tyr-Ileu-Glu-Asp.

(ii) Determination of C-terminal tetrapeptide

Amino acid analysis of the C-terminal sequence of Hormone II was carried out on the material which remained after five Edman degradation cycles used for subtractive method analysis. The next three amino acids (positions 6, 7, and 8) were identified using the dansyl-Edman technique. The mobilities of dansyl derivatives in thin layer chromatography systems are listed in Table XIII and the identification of amino acid 7 illustrated in Figure 17.

On the basis of the experiments, amino acids 6, 7, and 8, were identified as cysteic acid, proline, and arginine, respectively.

The identification of the amino acid in position 8 was not entirely satisfactory. At this point of sequence analysis only two amino acids remained to be placed: arginine and glycine. While in the chromatographic system A the unknown behaved like the dansyl arginine standard, a separation of the unknown into two fluorescent bands, one of which corresponded to dansyl-arginine, became evident in system B, and even more pronounced in C (Table XIII (3).

T A B L E   XIII

Identification of Dansyl Derivatives of Amino

Acids 6, 7, and 8 of Hormone II

(1) Determination of amino acid 6 of Hormone II:
Mobilities of dansyl amino acids in solvent system.

	A	C
DNS-NH$_2$	1.00	1.00
DNS-pro	0.95	0.64
DNS-gly	0.46	0.74
DNS-arg	0	0.52
DNS-CySO$_3$H	0	0.60
Unknown	0	0.59
No.6 of LVP[a]	0	0.59

(2) Determination of amino acid 7 of Hormone II:
Mobilities of dansyl amino acids in solvent systems

	A	B
DNS-NH$_2$	1.00	1.00
DNS-pro	1.00	0.64
Unknown	1.00	0.64
DNS-gly	0.41	
DNS-arg	0	

(3) Determination of amino acid 8 of Hormone II:
Mobilities of dansyl amino acids in solvent systems

	A	B	C
DNS-NH$_2$	1.00	1.00	1.00
DNS-arg	0	0.37	0.74
Unknown	0	0.39 + 0.41	0.76 + 0.79
DNS-gly	0.50	0.67	0.89

C: same plate re-run through three systems in (3)

(a) Synthetic 3-phe, 8-lys oxytocin (lysine vasopressin)
Mobilities are expressed relative to dansyl-ammonia.

Solvent systems: A - chloroform : methanol : acetic acid
(95: 10 : 1); B - n-propanol : ammonia (80 : 20); C -
n-propanol : water (80 : 20).
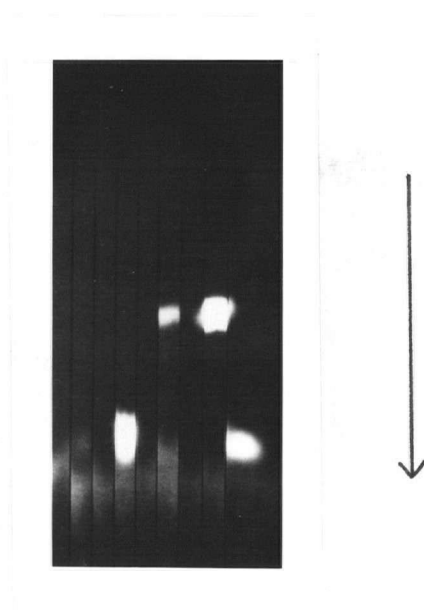
Figure 17: <u>Identification of amino acid 7 of Hormone II</u>
<u>as DNS-proline</u>.

Left to right (starting in channel 4): DNS-gly; nil;
DNS-a.a.$_7$; nil; DNS-pro; DNS-NH$_2$.

However, the second band did not have the mobility of
dansyl-glycine or of a dansyl-derivative of any amino acid
in the hormone.

The placement of arginine into position 8 was sub-
stantiated by positive identification of the C-terminal
amino acid. The residue of 8 Edman degradation cycles was
subjected to electrophoresis (pH 6.5, 15 minutes, 4,000
volts) with glycinamide and arginine standards. No free
arginine was revealed in the residue of Hormone II, while
a strong glycinamide band was observed (212 mm toward
cathode).

Positive identification was thus obtained for the
complete amino acid sequence of Hormone II. Following oxi-
dation with performic acid the hormone molecule was found
to have the following amino acid sequence:

$$CySO_3H-Tyr-Ileu-Glu-Asp-CySO_3H-Pro-Arg-GlyNH_2 \; .$$
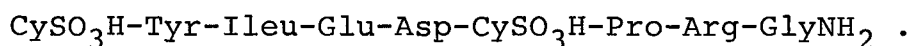
(3) Reaction Yields Encountered in the Course of Amino
      Acid Sequence Work

In the course of successive Edman reactions the total
amount of material remaining for the next degradation cycle
was found to diminish in excess of the amounts that could
be accounted for, through the removal of aliquots for amino
acid analyses in the subtractive Edman degradation, and for

chromatographic identification of dansyl derivatives in the dansyl-Edman method. The losses may be ascribed to the two extraction steps that the mixture undergoes with each successive Edman degradation cycle: the benzene extraction following the coupling step and the butyl acetate extraction following the cleavage step of the Edman reaction.

During dansyl-Edman sequence analysis of Hormone II and of synthetic 3-phe, 8-lys oxytocin, benzene extraction and butyl acetate extraction were repeated three times. Hormone II (0.2 μm) became exhausted after three, and 3-phe, 8-arg oxytocin (1.0 μm) after six, degradation cycles. Attempts to omit the benzene extraction entirely resulted in large amounts of extraneous violet-blue fluorescence on chromatography of dansyl derivatives which obscured the fluorescence of the dansyl amino acid.

The butyl acetate extraction was found to be responsible for a considerable loss. When the subtractive Edman method was applied to sequence determination of Hormone II, the benzene extraction was omitted entirely, while butyl acetate extraction of aqueous peptide solution was performed three times after each cyclization step. In this experiment, the amount of material left after second, third, and fourth Edman degradation cycle was found to be 63.5, 20.3 and 15.6% of theoretical value respectively. In

subsequent experiments on Hormone II one benzene extraction
was again introduced after the coupling step (to allow
identification of dansyl derivatives), but the butyl acetate
extraction step was performed only once. This improved the
yield of remaining peptide: 62% of peptide remained after
four Edman degradation cycles of Hormone II. When the
butyl acetate extraction step was modified so as to extract
the cyclization residue in dry rather than in aqueous form,
the yield of basic peptide after four Edman cycles was
found to be 65%.

Degradation of Hormone I resulted in higher losses
than that of Hormone II, possibly because the polar side
chain of the arginine residue found in Hormone II makes it
less susceptible to extraction with organic solvents. The
highest yield obtained after performing four degradation
cycles on Hormone I was 50% of the theoretical value, even
though benzene extraction was omitted altogether and butyl
acetate extraction carried out only once on dry residues.

IV. DISCUSSION

(1) Neurohypophysial Hormone Content of Salmon Pituitaries

A major difficulty in comparing the amounts of hor-
mones isolated from O. tschawytscha with those isolated
from other teleosts by other workers lies in the variation
in experimental conditions. The content of neurohypophysial
hormones of teleost pituitaries may be expressed in units
of biological activity per unit body weight (76), per gland
(40), or per unit of gland weight (10). The biological
activity is expressed in terms of units of oxytocic, pressor,
or antidiuretic activity (5). Also a variety of species of
animals can be used for a given type of bioassay. Thus oxy-
tocic activity is estimated using the uterus of guinea pig,
rat, or cat in vivo or in vitro (5). The bioassay on rat
uterus in vitro can be done with or without magnesium ion
(57). Thus, the values obtained by Lederis in his work
with Salmo irideus were given in milliunits of oxytocic
activity per 100 gm of body weight of the trout, and the
oxytocic activity was measured on rat uterus in vitro in
the presence of magnesium ion (76). For this reason, his
data were not included in the present comparison. Addition-
ally, a distinction must be made between the biological

activities reported for whole tissue powders, as in the work of Acher and of Chauvet (46), (48), (77), and the biological activities of the extracts of those powders or of fresh tissues, as in the work of Follet and Heller (40). Table XIV attempts to summarize oxytocic activities of teleost pituitaries reported in the literature and to compare them with those of the present study.

The hormone content per gland of Oncorhynchus tschawytscha is relatively high. A practical advantage of this was that approximately 4,000 fish provided sufficient pure hormone for chemical studies. Ten thousand fish glands were used by Acher et al. for determination of amino acid composition of 8-arg oxytocin of Gadus luscus (48). On the other hand, the activity per unit weight of salmon gland was low and consequently relatively large amounts of tissue had to be processed to obtain milligram quantities of pure hormones.

Perks mentions that in the elasmobranch fishes neurohypophysial hormone per pituitary does not appear to vary with the size of the fish, and that care must be taken not to overestimate differences observed as unknown factors, such as seasonal variation, may be involved (7). The apparent variation in the amounts of hormone per pituitary in different species of fish (Table XIV) may reflect differences due to variations in the extraction procedure,

T A B L E XIV

Comparison of Neurohypophysial Hormone Content of

Teleost Pituitaries

Species	Oxytocic activity[a] mµ/fish	mµ/mg dry wt.	Extraction	Author
Salmo irideus (trout)	81	--	hot 0.25% HOAc of acetone powder	Follet and Heller (40)
Esox lucius (pike)	81	--	hot 0.25% HOAc of acetone powder	Follet and Heller (40)
Anguilla anguilla (eel)	114	--	hot 0.25% HOAc of acetone powder	Follet and Heller (40)
Gadus callarias (cod)	5	--	hot 0.25% HOAc of frozen glands	Follet and Heller (40)
Gadus luscus (bid cod)	185[b]	500	whole acetone powder	Acher (48)
Merluccius merluccius (hake)	97[b]	200	whole acetone powder	Chauvet (77)
Cyprinus carpio (carp)	--	70	whole acetone powder	Acher (46)
Oncorhynchus tschawytscha (Pacific chinook salmon)	750 - 1,000	50	cold 0.2 M HOAc of frozen glands	present study

(a) Oxytocic activity measured using rat uterus without magnesium method of Holton(55).
(b) Activity per fish calculated from the data of the authors on the number of fish glands used to obtain a given weight of acetone powder. Such value was not given for C. carpio.

or may be biologically significant. Further study of
neurohypophysial hormones in elasmobranchs is required to
clarify this point.

(2) Biological Assays Used in Isolation of Neurohypophysial
    Hormones of Salmon

Several criteria were used in choosing the assay for
isolation of salmon hormones. An oxytocic assay was desir-
able since all neurohypophysial hormones of animals have some
oxytocic properties, while not all of them have pressor or
antidiuretic activity (75). The assay method had to lend
itself to accurate quantitation in order that isolation
methods could be compared and examined in terms of yields
of hormones. A rapid method was required so that the
effluent fractions of chromatographic columns could be
monitored without delay. The method of Holton using a rat
uterine horn in vitro (55) fulfilled these requirements.
A variant of the Holton method, rat uterus contractility
in presence of magnesium ion, was used in several experiments
following the stage of separation of the two hormones. The
assay in presence of magnesium has been described by Munsick
as an additional tool for pharmacological characterization
of neurohypophysial hormones, because the magnesium ion
appears to potentiate the action of all neurohypophysial
hormones, with the exception of oxytocin, on the uterine

tissue. The potentiation ratio is different for different neurohypophysial hormones (57). The potentiations obtained for the two salmon hormones were within the range of those reported for 4-ser, 8-ileu oxytocin and 8-arg oxytocin.

(3) Extraction of Neurohypophysial Hormones

Fresh frozen glands of fishes were extracted by Follet and Heller (40), lyophylized glands were extracted by Sawyer (45), but the majority of workers appear to use acetone powders of pituitary glands (10), (40). The widely used extraction method of Kamm recommended by the British Pharmacopeia (1958) (59) involves heating of pituitary acetone powder in 0.25% acetic acid. In addition to Kamm's method, other extraction procedures have been successfully used by some investigators. Among them are the dilute sulfuric acid extraction of Acher et al. (78) and the pyridinium acetate extraction of Lindner et al. (79).

The extraction method of Acher was first designed for extraction of mammalian neurohypophyses. It involves extraction with cold 0.01 N sulfuric acid in order to preserve the neurophysin-hormone complex found in the extracts of mammalian neurohypophyses (10), (78). Following the extraction, the neurophysin complex is separated from the small molecules in the extract by salt precipitation and

dialysis. The next step involves disruption of the complex
and separation of the small molecular weight neurohypophy-
sial hormones from the hormonally inert neurophysine (78).
Acher was unable to detect a neurophysin complex in the
pituitary extracts of lower vertebrates, and adapted his
method to this group of animals by introducing mammalian
neurophysin into the extract (15). It has been previously
shown by him that inter-specific neurophysin-hormone com-
plexes can indeed be formed (80). The addition of 'foreign'
neurophysin can produce an erroneous result if the puri-
fication of neurophysin is incomplete (26). For this
reason Acher's extraction method was not attempted in the
course of this work.

The method of Lindner also involves the neurophysin
hormone complex. The extraction of acetone dried neurohy-
pophyses is carried out at 4°C using pyridinium acetate
buffer. Under these conditions the mammalian neurophysin-
hormone complex is preserved. The high molecular weight
complex is separated by gel filtration and then disrupted
using 1 N formic acid (79). This method was attempted on
two occasions in the early part of this investigation
using frozen salmon glands, but a high molecular weight
hormone complex was not detected.

It has been shown by du Vigneaud's group that acetone
forms a reversible adduct with neurohypophysial hormones

which results in de-activation of the hormones (81), (82).
The adduct, believed to be a monoisopropylidene derivative,
forms at room temperature in 48 hours. The loss of bio-
logical activity is almost total (3% of lysine vasopressin
activity was detected after the acetone treatment), but can
be restored by heating the isopropylidene derivative to
90°C for 30 minutes in 0.25% acetic acid. In view of these
results, acetone /dried pituitary powders were not used routinely
in the course of the present work.

The extraction method developed for salmon pitu-
itaries involved disruption of the tissue immediately upon
removal from the freezer (-80°C) in 0.2 M acetic acid at
4°C. It has been shown in the course of this work that
oxytocic activity of salmon pituitaries survived long per-
iods of cold storage. The yield of oxytocic activity
obtained by this extraction method was somewhat better than
that obtained by the method of Kamm. The molarity of the
resulting crude extract permitted the next step of iso-
lation procedure to follow immediately after centrifugation
of tissue homogenate.

(4) Purification of Neurohypophysial Hormones

A number of purification methods have been applied,
singly or in combination with each other, in neurohypophy-
sial hormone work. These included salt precipitation,

counter-current distribution, adsorption on silica, electro-
phoresis on a cellulose column, partition chromatography,
gel filtration, paper chromatography, and ion exchange
chromatography (10).

Chemically pure neurohypophysial hormones have been
obtained from pituitaries by du Vigneaud using counter-
current distribution (83), by Acher using salt precipitation
followed by ion exchange chromatography (10), by Rasmussen
using counter-current distribution and gel filtration (49),
and by Vliegenthart using gel filtration, ion exchange,
and paper chromatography (84). Criteria applied in develop-
ing the purification schedule used for the present work
were adaptability of the purification sequence to work on
a preparative scale, and potential for maximum overall
yields coupled with maximum purification of both hormones.

The purification procedure which was adopted for
routine use originated from the work of Sawyer and van
Dyke on pollack pituitary (45), which was modified and ex-
tended in the course of present studies. Sawyer's iso-
lation procedure consisted of gel filtration on Sephadex
G-25 and subsequent separation of two pollack hormones on
a carboxymethylcellulose column (CMC). In a subsequent
report on _Hydrolagus colliei_ Sawyer substituted partition
chromatography on Sephadex for gel filtration, and ion ex-
change on CM-Sephadex for ion exchange on CMC (34).

The purification procedure used repeatedly by
Acher's group to isolate neurohypophysial hormones of
teleosts, has been adapted from the original procedure for
beef glands by Acher, Light and du Vigneaud (78) by addi-
tion of foreign neurophysin, as discussed in the preceeding
section. The details of the procedure evolved by Acher,
Light and du Vigneaud consisted of the following steps: a
precipitation of neurophysin complex with sodium chloride,
repeated dialysis against water, dissociation of the com-
plex with TCA, chromatography on Amberlite IR-45 to remove
TCA, and separation of the two hormones on Amberlite
IRC-50 (78).

The procedure developed for the purification of
salmon neurohypophysial hormones in the present study con-
sisted of gel filtration, ultrafiltration, separation of
the two hormones by ion exchange, ultrafiltration, and
rechromatography of the individual hormones. Gel filtration
was used to separate the hormone-containing low molecular
weight fraction from the high molecular weight substances
in the crude extract. Sephadex G-25 has been used by
other workers successfully for this purpose (45), (85).
In the present investigation Sephadex G-15 and Biogel P-2
were examined, because their molecular weight exclusion
limit is lower than that of Sephadex G-25. On the basis
of these preliminary experiments Sephadex G-15 was used in
large scale isolations of salmon hormones. The use of a
tandem series of separations on a Sephadex G-15 column

was found to be a highly efficient purification step for processing large volumes of extract. The yield of hormones was good, the experimental time short, and direct application of crude extract was possible.

Cation exchange has been proved an effective technique for separating two neurohypophysial hormones on the basis of their different ionic charges by Acher's group using acrylic resins (10) and by Sawyer using carboxymethylcellulose (45) or carbomethyl-Sephadex columns (34). In the present study cellulose-based and dextran-based cation exchange media such as phosphocellulose, SE-Sephadex and Whatman CM-32 were used in preference to carboxymethyl-cellulose. Prior ion exchange separations of neurohypophysial hormones on cation exchangers by Acher's and Sawyer's group utilized both changes in salt concentration and in pH (from pH 5.0 to pH 7.5 or 7.7) for eluting the more basic of the two hormones (10), (34). Published work on disulfide interchange (86) suggests that neutral pH is not appropriate for preservation of integrity of -S-S-containing peptides such as neurohypophysial hormones. In the present work it was found possible to omit a change to neutral pH and to elute the more basic of the two hormones (Hormone II) at pH of 5, using only a salt gradient.

Salmon hormones obtained by separation on a cation exchange column were not pure, as judged by the amino acid analyses, specific activities, and elution profiles of the

hormones. For this reason, rechromatography of individual hormones was carried out. For this purpose it was found advantageous to use a different cation exchanger from that used for the separation of hormones. Thus, if the hormones were first separated on a carboxymethyl ion exchanger, purification of the more basic salmon hormone (Hormone II) was achieved best by rechromatography on phosphocellulose. Purification of the less basic hormone (Hormone I) required rechromatography at a different pH from that used in the separation of hormones. The sequence in which the two chromatographies were done appeared not to matter: if the initial separation was done at pH 2.45 on SE-Sephadex, then the subsequent rechromatography of the less basic hormone was on Whatman CM-32 at pH 5 or vice versa.

In summary, rechromatography of the two individual hormones only had to be performed once to obtain pure preparations providing that the conditions outlined above were observed: a pH change from 5 to 2.45 or vice versa for the less basic hormone (Hormone I) and a rechromatography on phosphocellulose of the more basic of the two hormones (Hormone II) irrespective of whether the separation of the two hormones was done on SE-Sephadex or on Whatman CM-32.

A complete list of yields per step of purification and of overall yields has been reported by Acher, Light and du Vigneaud on their work with beef pituitaries (78). Table

XV presents a comparison between the yields they reported and the yields obtained in the course of purifying pituitary extracts of O. tschawytscha. The procedure employed in the present investigation resulted in preparation of two pure hormones at an overall yield level comparable to that of Acher, Light and du Vigneaud.

The losses experienced in purifying salmon hormones can be largely attributed to the ultrafiltration steps. It should be possible to improve yields by either reducing the extent of ultrafiltration or by using alternate procedures to de-salt and to concentrate the material. The requirement for ultrafiltration may be possibly reduced by separation of the hormones on SE-Sephadex at pH 5, and re-chromatography of the less basic hormone on the same column at pH 2.45. The ionic strengths at which this hormone is eluted from various cation exchange columns are given in Table VI. Concentration of material could be achieved by lyophylization. Losses due to lyophylization were not observed during the present work. On the other hand, a de-salting procedure such as has been reported by Porath and Lindner on Sephadex G-25 (87) could be substituted for ultrafiltration. The disadvantage of the method used by Porath and Lindner is that the hormones are eluted from the column using a mixture of acetic acid, pyridine and water (15 : 60 : 25) which is toxic to tissues and would have to be removed prior to conducting the bioassay.

Comparison of Yields Obtained at Different Stages of

Purification of Neurohypophysial Hormones

Acher, Light and du Vigneaud (78): beef				Present study: <u>Oncorhynchus tschawytscha</u>			
Purification step	Specific activity per A.U. Folin(a)	Hormone yield %		Purification step	Specific activity per A 280nm	Hormone yield %	
		per step	overall			per step	overall
Extraction	1	85-90	85-90	Extraction	0.08 - 0.10		100 (b)
Precipitation of complex and dialysis	2.5	70-80	60-70	Gel filtration tandem series	0.4 - 1.4	78-97	98-97
After removal of protein	10.0	75-90	45-63	Ultrafiltration and separation by chromatography	10 - 100	69-91	54-88
Separation by chromatography	oxytocin: 50 vasopressin: 100	70-90	30-55	Ultrafiltration Re-chromatography	per mg: Hormone I 125-145 Hormone II 209-229	63-74	34-65

(a) calculated from data of Acher, Light and du Vigneaud (78).
(b) oxytocic activity measured by method of Holton (55).
(c) no oxytocic activity remaining in the acid insoluble fraction of tissue homogenate.

(5) Structural Analyses of Neurohypophysial Hormones
     of Teleosts

Chemical analyses of neurohypophysial hormones of
lower vertebrates, with one exception, have been done by a
single group of workers, Acher, et al. from Laboratoire de
Chimie Biologique, Faculte des Sciences, Paris. The excep-
tion is the quantitative amino acid analysis of 8-arg
oxytocin from Urophycis tenuis (50) published by Rasmussen
(49).

(i) Quantitative amino acid analyses

To date neurohypophysial hormones of five teleost
fishes have been identified on a chemical basis; however,
a quantitative amino acid analysis has been published for
only two of the five species: Cyprinus carpio (46) and
Urophycis tenuis (49). These data are compared with those
obtained in the present study of Oncorhynchus tschawytscha
in Table XVI. It can be seen from this Table that quanti-
tative amino acid analyses of the salmon neurohypophysial
hormones are comparable to those obtained by other workers.

(ii) Amino acid sequence studies of the less basic
      teleost hormone

The amino acid sequence of 4-ser, 8-ileu oxytocin
isolated from the pituitary glands of three marine teleosts

T A B L E   XVI

Comparison of Quantitative Amino Acid Analyses of Teleost Neurohypophysial Hormones

| Amino Acid	C. carpio 4-ser,8-ileu oxytocin(46)	O.tschawytscha Hormone I	Amino Acid	C. carpio 8-arg oxytocin(46)	U.tenuis 8-arg oxytocin(49)	O.tschawytscha Hormone II
Ammonia	--	2.19	Ammonia	--	3.80	3.96
Cys	1.46*	1.69	Arg	1.06	0.82	0.83
Asp	1.00	1.04	Cys	2.04*	1.65	1.96*
Ser	0.90	1.02	Asp	1.00	1.00	1.09
Pro	1.06	0.90	Glu	0.96	0.89	1.00
Gly	1.14	1.21	Pro	0.89	1.15	0.80
Ileu	1.73	1.87	Gly	1.37		1.14
Tyr	0.61	0.83	Ileu	0.92	1.12	0.87
			Tyr	0.85	0.77	0.43
Lys	--	0.06				
Val	--	0.04	Leu	0.15	--	--
Glu	0.20	0.16	Ser	0.19	0.19	--
Leu	0.13	--				
Arg	0.08					

* value for Cys obtained following performic oxidation.

was first postulated by Acher in 1962, although quantitative
amino acid analyses of the new hormone were not given. The
amino acid sequence of the first four N-terminal amino acid
residues was assigned on the basis of degradation with the
enzyme leucine-aminopeptidase. The sequence of the re-
maining five amino acids was assigned by analogy with known
hormones. The new hormone was named 'isotocin' to indicate
the replacement of 8-leucine with isoleucine (42).

Additional structural work on 4-ser, 8-ileu oxytocin
was reported by Acher. The experiment consisted in sub-
jecting the hormone, previously oxidized with performic
acid, to the action of subtilisin. The enzyme released two
fragments whose amino acid composition provided confir-
mation for the assigned structure: one fragment was
(Tyr, $CySO_3H$, Ileu, Ser), and the other (Asp, Pro, Ileu,
$CySO_3H$, Gly) (10).

The present work on Hormone I of salmon employed
chemical rather than enzymic degradation methods. The
Edman degradation method was employed for stepwise degra-
dation of the molecule, and dilute hydrochloric acid was
used for partial hydrolysis. The sequence of the N-terminal
tetrapeptide $CySO_3H$-Tyr-Ileu-Ser, was established by the
subtractive method of Edman (72); position 5, aspartic, by
partial acid hydrolysis and additional data from a fifth
Edman degradation cycle; and positions 6 and 7, $CySO_3$-pro
by dansyl-Edman technique (72). Thus positive identifi-

cation was obtained for seven out of nine amino acid residues. The placement of amino acids 8 and 9, ileu and gly can be done on the basis of analogy. The sequence of Hormone I of salmon (following oxidation with performic acid) was thus established to be as follows:

CySO$_3$H-Tyr-Ileu-Ser-Asp-CySO$_3$H-Pro-Ileu-Gly
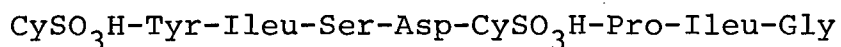
Reports on chemical degradation of neurohypophysial hormones in lower vertebrates have not been found in the literature. The subtractive method of Edman has been used by du Vigneaud in confirming the sequence of the four N-terminal amino acid residues of beef oxytocin (9). Du Vigneaud's data on stepwise Edman degradation of oxytocin show the increasingly incomplete removal of subsequent amino acid residues observed in the course of the present study of salmon hormones. In the degradation of oxytocin, the removal of N-terminal cysteic resulted in a decrease from 1.99 to 1.12 residues per mole; the removal of tyrosine produced a decrease from 0.69 to 0.17 residues per mole; the removal of isoleucine after the third Edman degradation cycle resulted in reduction from 0.74 to 0.34 residues per mole, and the removal of glutamic after the fourth degradation step produced a drop from 0.90 to 0.55 residues per mole. Edman reports almost 100% completion of degradation cycles in his experiments (88), probably because

of the purity of the reagents used and because degradations were done under nitrogen atmosphere.

(iii) Amino acid sequence studies of the more basic teleost hormone

The structure of 8-arginine oxytocin from teleost fish was studied by Acher (51). Amino acid analysis revealed the presence of stoichiometric quantities of the eight constituent amino acids: cystine, tyrosine, isoleucine, aspartic acid, glutamic acid, proline, arginine, and glycine. Leucine aminopeptidase released tyrosine and isoleucine while the N-terminal half-cystine remained attached to the molecule by the disulfide bridge. It was concluded from this experiment that the N-terminal tripeptide of 8-arginine oxytocin was Cys-Tyr-Ileu. In order to study the C-terminus of the molecule, trypsin was allowed to act on the hormone. The release of glycinamide suggested that the C-terminal dipeptide was arginyl-glycinamide (51).

The present study of the more basic hormone (Hormone II) of salmon utilized chemical degradation of the molecule only. The subtractive Edman method was applied to the N-terminal pentapeptide, $CySO_3H$-Tyr-Ileu-Glu-Asp. Positions 6, 7, and 8 $CySO_3H$-Pro-Arg, were identified using the dansyl-Edman method, and the C-terminal glycinamide was identified by electrophoretic separation of the residue re-

maining after eight Edman cycles. Thus positive identifi-
cation of all nine amino acid residues contained in the
molecule of Hormone II was obtained.

(iv) Structure of the O. tschawytscha neurohypophysial
      hormones

        The foregoing structural determination has led to
complete amino acid sequences for the performic oxidized
salmon hormones. The oxidized Hormone I has the sequence

    H_2NCySO_3H-Tyr-Ileu-Ser-Asp-$CySO_3H$-Pro-Ileu-Gly
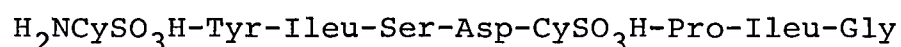
where the sequence of amino acids 8 and 9 (Ileu and Gly)
is assigned by analogy with other neurohypophysial hormones.
Oxidized Hormone II has the sequence

    H_2NCySO_3H-Tyr-Ileu-Glu-Asp-$CySO_3H$-Pro-Arg-$GlyNH_2$
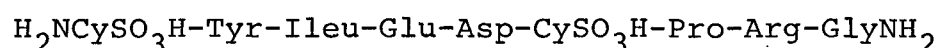
        By analogy with other neurohypophysial hormones, the
unoxidized hormones can be considered to have intramolecular
disulfide bonds. From the ammonia analyses, and more par-
ticularly from the behaviour on cation exchangers, it is
reasonable to assume that the aspartic acid and glycine
residues in Hormone I are in the form of amides. Hence the
structure 4-ser, 8-ileu oxytocin can be assigned to this
hormone. By analogous reasoning the structure 8-arg oxy-
tocin can be assigned to Hormone II.

(v)   Comments on the analytical methods

Until the present work, the dansyl-Edman sequence method had not been applied to neurohypophysial hormones. It made possible the sequencing of salmon hormones past the fourth or fifth N-terminal amino acid when the subtractive Edman method could not be continued either because of high background of unreacted residues or because of scarcity of remaining material.

The partial acid hydrolysis procedure used in this study on 4-ser, 8-ileu oxytocin does not seem to have been used previously on neurohypophysial hormones. It differed from the partial hydrolysis used by du Vigneaud (89) in that it preferentially released the aspartic acid residue (69). Because of the central position (aspartic acid occurs at position 5 in all known neurohypophysial hormones), this method represents a potentially powerful tool for complete sequence studies of these hormones. The two tetrapeptides obtained by this method should be separable on high voltage electrophoresis, and have both N- and C-termini available for analysis.

(6)   Specific Activities of Salmon Neurohypophysial Hormones

The specific activity of 4-ser, 8-ileu oxytocin purified from the pituitaries of Oncorhynchus tschawytscha ranged from 125 to 145 oxytocic units (assay of Holton) per milli-

gram (Table VII). The highest value obtained was from a preparation which contained practically no contaminant amino acids. The result agrees with the reported value of 150 oxytocic units per milligram (assay of Holton)(75).

The specific activity of 8-arg oxytocin isolated from salmon glands was almost twice as high as the values previously reported for this hormone obtained by synthesis. A specific activity value of 115 units per milligram (method of Holton) has been quoted repeatedly (5), (75). A slightly higher value, 125 oxytocic units per milligram (assay of Holton), has been reported by Sawyer (6). The values obtained in the present work range up to 229 oxytocic units per milligram on the basis of the same assay. The highest values were obtained from the preparation of hormone which did not contain any contaminant amino acids (Table VIII). The difference in specific activities of the 8-arg oxytocin from Pacific salmon and those of synthetic preparations of this peptide could be ascribed to the presence of biologically inactive dimers (90) in the latter.

(7) Relative Proportions of Neurohypophysial Hormones in Teleosts

Separation of the two neurohypophysial hormones of salmon by ion exchange chromatography consistently showed that the oxytocic activity was higher in the 8-arg oxytocin

peak than in the 4-ser, 8-ileu oxytocin peak. The actual ratio varied between 2.0 and 3.0. In separations of the two teleost hormones described in the literature, the relative amount of the first hormone is either greater than or equal to that of the second hormone. Since the neurohypophysial hormones of salmon do not appear to differ in structure from those of other teleosts, the variations in activity reflect differences in the amounts of hormones.

For example, in separation of neurohypophysial hormones of Cyprinus carpio, a freshwater teleost, the oxytocic activity contained in the 4-ser, 8-ileu oxytocin peak was approximately two times higher than that of the 8-arg oxytocin peak (46). The profile of oxytocic activity obtained in the separation of neurohypophysial hormones of Gadus luscus, a marine teleost, shows that the amounts of the two hormone activities are approximately equal (48). Two independent separations of neurohypophysial hormones of Merluccius merluccius, another marine teleost, show the oxytocic activity of the 4-ser, 8-ileu oxytocin peak as several times greater than the activity associated with 8-arg oxytocin (77), (91). A similar separation for a third marine teleost, Pollachius virens, shows the 4-ser, 8-ileu oxytocin activity to be approximately twice that of 8-arg oxytocin (91). In the five separations described above polyacrylic acid cation exchangers were used and the

elution of both hormones was carried out at pH 7.7. Sawyer
and van Dyke also have reported the separation of neurohypo-
physial hormones of <u>Pollachius</u> <u>virens</u>. The 4-ser, 8-ileu
oxytocin peak was eluted at pH 5, and the 8-arg oxytocin
peak at pH 7.5, from a carboxymethylcellulose column. Approxi-
mately equal amounts of oxytocic activity were present in
these hormone peaks (45).

In view of the present findings of 2 : 1 to 3 : 1 ratio
of oxytocic activity of 8-arg oxytocin to 4-ser, 8-ileu oxy-
tocin in the spawning Pacific salmon, it is of interest to note
the work of Lederis on the exposure of <u>Salmo</u> <u>irideus</u> to sea
water (76). The measurements of Lederis of neurohypophysial
hormones in the pituitaries of experimental trout were based
on oxytocic and pressor assays of the gland extracts. The
ratios of pituitary 8-arg oxytocin to 4-ser, 8-ileu oxytocin
obtained by this method (3.0 for control fish, decreasing
to 1.5 for the fish exposed to sea water) were similar to
the ratios observed in the present study following separa-
tion of the two salmon hormones on cation exchangers.
Oxytocic assays carried out by Lederis, however, were done
using rat uterus in the presence of magnesium ion, while
those of the present investigation were performed by the
standard method of Holton (55) without the addition of
magnesium. For this reason, the value of the above com-
parison is limited.

There are three possible explanations for the
comparatively large amount of 8-arg oxytocin obtained from

salmon pituitaries in the present work. The first, and
the simplest, explanation is that isolation of this hormone
at an acidic pH preserves its full biological activity.
Loss of biological activity of a neurohypophysial hormone
at neutral pH could be due to either the presence of a con-
taminating peptidase active in the neutral pH region, or
to the de-activation of the molecule as a result of
disulfide interchange. One pituitary peptidase has been
described as a contaminant of bovine, ovine, and human
pituitary gonadotrophins. However, this peptidase is
active at pH 4 (92). While the presence of peptidase(s)
cannot be ruled out without experimental evidence, it can
be considered unlikely at this stage of purification of
the hormones. Disulfide interchange is known to occur at
neutral pH (86). The result of disulfide interchange can
be the formation of a biologically inactive dimer (90).
The dimer formation would result in a decrease in biological
activity.

In pilot experiments on separation of the salmon
hormones a gradient to pH 7.5 was used for the elution of
8-arg oxytocin. The oxytocic activity associated with
this peak, however, was at least twice as great as the
activity associated with the 4-ser, 8-ileu peak. Care was
taken in these experiments not to leave the hormone at
neutral conditions any longer than was absolutely necessary.

Thus, assaying of the effluent fractions took place imme-
diately upon elution, and the hormone containing fractions
were pooled and acidified immediately afterwards.

The second explanation for the high 8-arg oxytocin
to 4-ser, 8-ileu oxytocin ratio obtained in the present
experiments is that there was loss of the less basic hor-
mone. If the neurophysin or a neurophysin-like complex
does exist in salmon, and if the binding of 4-ser, 8-ileu
oxytocin to that carrier molecule is stronger than that of
8-arg oxytocin, it could be envisaged that the extraction
technique did not complete dissolution of the complex. There
is some evidence of a preferential binding of oxytocin to
neurophysin in weak (0.25%) acetic acid solution at 2°C (84).
Alternatively, the less basic hormone may have a low solu-
bility in cold aqueous acetic acid.

The third explanation is the possibility of a seasonal
variation in the ratios of neurohypophysial hormones as
observed by Sawyer and Pickford in Fundulus heteroclitus (50).
These authors report a disappearance of 4-ser, 8-ileu oxy-
tocin from the pituitary glands of female fish during the
reproductive period in June, and the return of this hormone
after completion of the reproductive period. The observa-
tions were based on the pharmacological characteristics of
crude pituitary extracts.

In the present study pituitary glands were obtained from spawning salmon and consequently the results may be interpreted as confirming the studies on F. heteroclitus. The reproductive stages of the fishes used for separations of hormones mentioned at the beginning of this section were not reported. In the studies on Pacific salmon, four preparative scale (100 gm) extracts, three using pituitaries collected from males and the fourth from females, showed no difference in hormone content of the glands, the relative proportions of the two hormones, or in their chemical composition.

8. Evolutionary Considerations

Evidence exists that 3-phe, 8-arg oxytocin (vasopressin) in mammals may be synthesized by the conventional pathway of protein synthesis (93). Hence the structure of the peptide is coded by DNA base triplets. This evidence has been extrapolated to other vertebrates and has led to speculation on evolution of neurohypophysial hormones. There appears to be general agreement with the concept that the two hormones evolved from one ancestral molecule. The identity of this molecule is considered uncertain by Acher (17), Sawyer (34), and Munsick (26), while Vliegenthart and Versteeg consider it to be 8-arg oxytocin (94). The ancestral molecule concept stems from the fact that

only one hormone has been found in cyclostomes. Chemical identification of the cyclostome hormone has not been reported in the literature; it was tentatively identified as 8-arg oxytocin on the basis of pharmacological studies (6), (17), (40). Acher proposes gene duplication, occuring between cyclostomes and the other fishes, as the origin of two separate hormones (6).

Vliegenthart and Versteeg postulate single point mutations leading to amino acid substitutions in positions 3, 4, and 8 (94). In a later paper, they discuss the codons involved in these mutations (95). Isoleucine-phenylalanine interchange in position 3 can be achieved by one base change, that is, UUU (or UUC) to AUU (or AUC) (Table XVII). Phenylalanine is only found in 3-phe, 8-arg oxytocin and 3-phe, 8-lȳs oxytocin in mammals. In all other neurohypophysial hormones found in nature isoleucine occupies position three.

3-Phe, 8-lys oxytocin could have arisen by one base change in the gene for 3-phe, 8-arg oxytocin, i.e., AGA (or AGG) to AAA (or AAG). 8-Arg oxytocin could have given rise to 3-phe, 8-arg oxytocin by one base change involving the codon for amino acid 3, and the latter could also have, in turn, given rise to 3-phe, 8-lys oxytocin by one base change in the codon of amino acid 8.

T A B L E   XVII

The Genetic Code*

1st	2nd	U	C	A	G	3rd
		phe	ser	tyr	cys	U
		phe	ser	tyr	cys	C
U		leu	ser	**	**	A
		leu	ser	**	try	G
		leu	pro	his	arg	U
		leu	pro	his	arg	C
C		leu	pro	gln	arg	A
		leu	pro	gln	arg	G
		ileu	thr	asn	ser	U
		ileu	thr	asn	ser	C
A		ileu	thr	lys	arg	A
		met	thr	lys	arg	G
		val	ala	asp	gly	U
		val	ala	asp	gly	C
G		val	ala	glu	gly	A
		val	ala	glu	gly	G

* adapted from Crick (96)

** termination

The oxytocin to 8-ileu oxytocin transition would involve only one base change in the leucine or isoleucine codon (Table XVII).

In addition to the 8-arginine or 8-lysine containing hormones, the ancestral molecule also would have to give rise to a peptide series with ileu or leu in position 8. Acher regards 8-ileu oxytocin rather than oxytocin to be a more primitive molecule, preceded by 4-ser, 8-ileu oxytocin (6). Munsick considers that oxytocin and 8-ileu oxytocin may have originated at about the same time and that the latter gave rise to 4-ser, 8-ileu oxytocin (26). Sawyer considers oxytocin to be either a very ancient molecule or one that has arisen several times independently in evolutionary lines (34).

The same arginine triplet (AGA) which gives rise in subsequent mammalian evolution to the lysine triplet (AAA) for the amino acid position 8, can also become a codon for isoleucine (AUA) with only one base change (Table XVII). On this basis 8-ileu oxytocin is more likely to be older than oxytocin, because leucine substitution would have required two base changes.

The arginine codon (CGX) with one base change can become codons for leucine (CUX), (Table XVII). On this basis oxytocin can be considered older than 8-ileu oxytocin, since two base changes are required to produce the latter. The arginine codon (CG$_G^A$) can yield a glutamine

codon (CA_G^A) with one base change. Glutamine is found in

position 8 of 4-ser, 8-gln oxytocin (elasmobranchs).

However, a lysine codon cannot be derived from CG_G^A on the

basis of one base change. This suggests that an inter-

change of the arginine codons CG_G^A and AG_G^A could have occurred

during the evolution of the genes for the neurohypophysial

hormones.

Position four is occupied by serine in 4-ser, 8-ileu

oxytocin (teleosts) and in 4-ser, 8-gln oxytocin (elasmo-

branchs). In all other naturally occuring neurohypophysial

hormones glutamine is found in position 4. Serine-glutamine

substitution requires two base changes (Table XVII). One

route is via a 4-proline intermediate, the other is via a

nonsense triplet. The presence of proline would confer new

steric properties onto the ring of the molecule, and pro-

found changes in biological activity and other properties

of the hormone could be expected. The second possible

route is via the nonsense triplet UA_G^A . The nonsense

triplet could, however, give rise by one base change to

either serine (UC_G^A) or to glutamine (CA_G^A) . Such a mutation

could also account for the absence of one hormone in cyclo-

stomes.

At this stage we have to conclude that much more

information is required for a full understanding of the

interrelationship and evolution of the genes for neurohypo-

physial hormones throughout the vertebrates. The present investigation has not uncovered any new structures to help elucidate this problem.

The evolutionary relations of the teleosts are outlined in Figure 18, which also shows the groups of teleosts for which the structures of the neurohypophysial hormones have been established chemically. In addition to the salmon, representatives of the Gadiformes and Cypriniformes have been studied and found to have 4-ser, 8-ileu oxytocin and 8-arg oxytocin. Because of the position of the salmonoids in the evolutionary tree of the teleosts, it suggests that these two hormones are widely characteristic of the teleosts. It also indicates that the search for new structures should be directed at other lines of evolution.
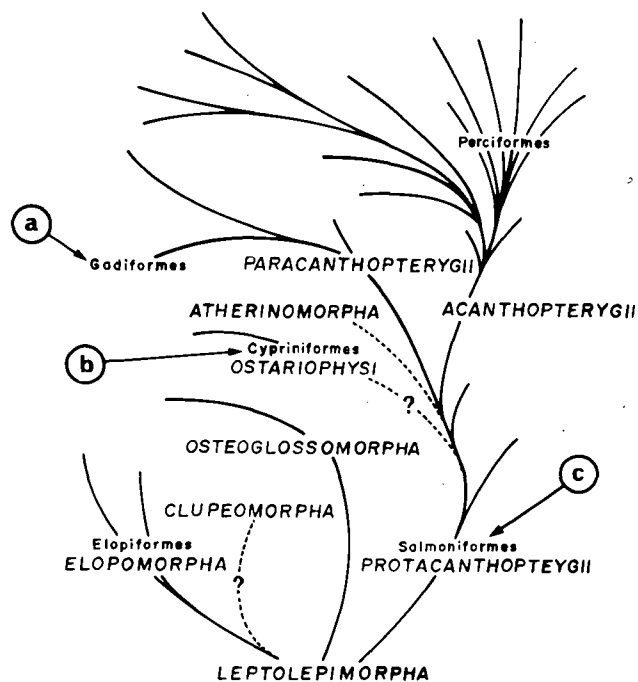
Figure 18: Family tree of teleost orders

(according to Romer (37))

Arrows indicate teleost groups for which chemical identifications of neurohypophysial hormones are available.

(a) Gadiformes: (10), (42), (48), (49), (51).
(b) Cypriniformes: (46). (c) Salmoniformes: present study.

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