Biosynthesis and axoplasmic transport of neurophysin in the hypothalamo-neurohypophysial system of the grass frog Rana pipiens

Alice Chien Chang

Iowa State University
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BIOSYNTHESIS AND AXOPLASMIC TRANSPORT OF NEUROPHYSIN IN
THE HYPOTHALAMO-NEUROHYPOPHYSIAL SYSTEM OF THE GRASS
FROG RANA PIPIENS

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Biosynthesis and axoplasmic transport of neurophysin in the hypothalamo-neurohypophysial system of the grass frog *Rana pipiens*

by

Alice Chien Chang

A Dissertation submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Major: Molecular, Cellular, and Developmental Biology

Approved:

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In Charge of Major Work

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For the Major Department

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For the Graduate College

Iowa State University

Ames, Iowa

1981
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>iv</td>
</tr>
<tr>
<td>GENERAL INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td><strong>PART I. THE BIOSYNTHESIS AND AXOPLASMIC TRANSPORT OF</strong></td>
<td></td>
</tr>
<tr>
<td><strong>NEUROPHYSIN IN THE HYPOTHALAMO-NEUROHYPOPHYSIAL</strong></td>
<td></td>
</tr>
<tr>
<td><strong>SYSTEM OF THE FROG, RANA PIPIENS</strong></td>
<td>11</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>12</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>16</td>
</tr>
<tr>
<td>RESULTS</td>
<td>20</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>47</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>54</td>
</tr>
<tr>
<td><strong>PART II. LIGHT AND ELECTRON MICROSCOPIC OBSERVATIONS IN</strong></td>
<td></td>
</tr>
<tr>
<td><strong>THE HYPOTHALAMO-NEUROHYPOPHYSIAL SYSTEM OF THE FROG, RANA PIPIENS</strong></td>
<td></td>
</tr>
<tr>
<td><strong>AFTER MICROIONTOPHORESIS OF</strong></td>
<td></td>
</tr>
<tr>
<td><strong>VINBLASTINE INTO THE MEDIAN EMINENCE</strong></td>
<td>59</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>60</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>63</td>
</tr>
<tr>
<td>RESULTS</td>
<td>65</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>125</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>134</td>
</tr>
<tr>
<td><strong>PART III. PREPARATION OF ANTIBODY AGAINST PURIFIED BRAIN</strong></td>
<td></td>
</tr>
<tr>
<td><strong>TUBULIN AND THE VISUALIZATION OF IN VITRO ASSEMBLED</strong></td>
<td></td>
</tr>
<tr>
<td><strong>MICROTUBULES BY THE UNLABELED PEROXIDASE</strong></td>
<td></td>
</tr>
<tr>
<td><strong>ANTI-PEROXIDASE METHOD</strong></td>
<td></td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>138</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>139</td>
</tr>
<tr>
<td>RESULTS</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>148a</td>
</tr>
</tbody>
</table>
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AER</td>
<td>axonal endoplasmic reticulum</td>
</tr>
<tr>
<td>AF</td>
<td>aldehyde fuchsin stain</td>
</tr>
<tr>
<td>CAH</td>
<td>chromalum haemotoxylin</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopic</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>IEF</td>
<td>isoelectric focusing</td>
</tr>
<tr>
<td>LM</td>
<td>light microscopic</td>
</tr>
<tr>
<td>MAP</td>
<td>microtubule associated proteins</td>
</tr>
<tr>
<td>M.W.</td>
<td>molecular weight</td>
</tr>
<tr>
<td>NL</td>
<td>neural lobe</td>
</tr>
<tr>
<td>PAP</td>
<td>peroxidase anti-peroxidase complex</td>
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<tr>
<td>PC</td>
<td>phosphocellulose</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>PON</td>
<td>preoptic nucleus</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus</td>
</tr>
<tr>
<td>Rf</td>
<td>relative mobility</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SON</td>
<td>supraoptic nucleus</td>
</tr>
<tr>
<td>2xMT</td>
<td>two-times polymerized microtubules</td>
</tr>
</tbody>
</table>
GENERAL INTRODUCTION

The first description of groups of specialized neurons with combined neural and presumed secretory functions dates back to 1928 when Scharrer observed the presence of glandular nerve cells in the teleost hypothalamus (Scharrer 1928). However, these specialized neurons were accorded little attention until the 1950s. Bargmann (1949, 1951) and Bargmann and Scharrer (1951) were able to show intense staining of secretory material in the neuronal cell bodies in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the hypothalamus using the chromalum haematoxylin (CAH) stain (Gomori 1940) and the more sensitive aldehyde fuchsin (AF) stain (Gomori 1950, Landing et al. 1956). This neurosecretory material is also present within axons of the hypothalamo-neurohypophysial tract and as dense aggregates in the neural lobe of the hypophysis (for review, see Bern and Knowles, 1966). The intense staining indicates the presence of protein-bound cysteine (Sloper 1955) and is now known to be due to neurophysins, the cysteine-rich carrier proteins of the peptide hormones vasopressin and oxytocin (for review, see Watkins 1975a).

Bargmann and Scharrer (1951) and Scharrer and Scharrer (1954) identified the three projections of the hypothalamo-neurohypophysial tract: First, to the neural lobe, where axon terminals contact perivascular spaces and release their hormones into the systemic circulation rather than synapse with other neurons; second, to the primary capillary plexus of the hypophysial portal system in the zona externa of the
median eminence; and third, to the cerebrospinal fluid (CSF) via the third ventricle. The major route of release of the peptide hormones and neurophysins into the general circulation is the neurohypophysis. As a consequence of these investigations, the emerging concept of neurosecretion was that the hormones vasopressin and oxytocin are synthesized in neurons within their perikarya in the SON and PVN of the hypothalamus and then transported along the axons of these neurons for storage in and release from their terminals in the neural lobe of the pituitary gland (Scharrer and Scharrer 1954).

In contrast to mammals, birds and reptiles where neurosecretory cells are located in both the SON and PVN, the neurosecretory cells of fish and amphibia are localized in one nucleus of the rostral hypothalamus, the preoptic nucleus (PON) (Hild 1951, Diepen 1962). In the toad (Bufo arenarum hensel), the preoptic nucleus gives origin to two different neurosecretory tracts: one from the ventral PON to the neural lobe, and the other one from the dorsal PON to the median eminence (Rodriguez et al. 1970). Biochemical studies have shown that the amphibian magnocellular neurosecretory system produces vasotocin and mesotocin, which differ from vasopressin and oxytocin respectively in a single amino acid residue (Acher et al. 1964, Acher 1974, Moens 1972). Vandesande, Dierickx and their co-workers, using the unlabeled antibody peroxidase anti-peroxidase technique, demonstrated that vasotocin and mesotocin are synthesized in separate neurons (Vandesande and Dierickx 1976) and stored in separate axon terminals in the hypophysis of Rana temporaria (Dierickx and Vandesande 1977,
Van Vossel-Daeninck et al. 1979) just as their mammalian counterparts (Vandesande and Dierickx 1975).

The presence of neurophysin in association with the peptide hormones vasopressin and oxytocin was first discovered in the bovine neural lobe (Van Dyke et al. 1942). Later, it was isolated and characterized in many species of mammals (Acher et al. 1955, for review, see Acher 1974). All the mammalian species examined so far have at least two different neurophysins, with the exception of the guinea pig, which has only one (Hollenberg and Hope 1968, Rauch et al. 1969, Uttenthal and Hope 1970, Cheng and Friesen 1971, Cheng and Friesen 1972, Burford and Pickering 1972, Watkins 1973, Watkins and Ellis 1973, for review, see Pickering and Jones 1978). After Dean and co-workers first showed in the cow, that two neurophysins are differentially compartmentalized with the two hormones (Dean et al. 1968), considerable evidence has been obtained in support of the view that one class of neurophysin is associated in vivo with vasopressin and the other one with oxytocin (Burford et al. 1971, De Mey et al. 1974, Valtin et al. 1974, Valtin et al. 1975, Vandesande et al. 1975). In the amphibian hypothalamo-neurohypophysial system, Moens (1974) reported the presence of cysteine-rich proteins in the neural lobe of *Rana temporaia*, and tentatively identified them as neurophysins. The presence of neurophysin in the neurosecretory neurons has been demonstrated indirectly using antibody against porcine neurophysin II (Watkins 1975b) or bovine neurophysin I (Vandesande and Dierickx 1976). Evidence obtained by the latter authors also suggests that the amphibian hypothalamo-neurohypophysial system produces two different neurophysins, one associated with vasotocin.
and the other one with mesotocin.

The biosynthesis of the two neural lobe peptide hormones and neurophysins in the dog hypothalamus has been studied intensively by Sachs and co-workers. They have shown that 1) the synthesis of vasopressin occurs only at the neuronal perikarya in the hypothalamus; the neurohypophysis, containing the axons and terminals of these same neurons, is incapable of vasopressin synthesis (Sachs 1960, Sachs and Takabatake 1964, Takabatake and Sachs 1964). 2) the application of a protein synthesis inhibitor, puromycin, during a 1.5 hour pulse-label period, inhibits the incorporation of $^{35}$S-cysteine into vasopressin. If puromycin is applied after the pulse, the incorporation of the label into the peptide is not inhibited. The authors then hypothesized that puromycin inhibits the synthesis of the precursor, but that once the precursor is synthesized, the inhibitor does not affect the cleavage of the precursor into vasopressin (Takabatake and Sachs 1964, Sachs and Takabatake 1964, Sachs et al. 1969). 3) neurophysin and vasopressin appear to be synthesized with similar kinetics. Moreover, incubation in the presence of analogues of amino acids found in neurophysin but not in vasopressin not only inhibits the synthesis of neurophysin but that of vasopressin as well (Fawcett et al. 1968, Sachs et al. 1969). Therefore, Sachs and co-workers have hypothesized that vasopressin and neurophysin are derived from a common precursor, which is synthesized by a ribosomal mechanism. Consistent with that hypothesis is the fact that Brattleboro rats with genetic defects in vasopressin synthesis are also deficient in vasopressin-neurophysin (Valtin et al. 1974, Pickering et al. 1975). More recently, Gainer and Brownstein have
demonstrated in the SON of the rat that two polypeptides with molecular weights of about 20,000 are synthesized prior to the appearance of the two lower molecular weight (12,000 dalton) neurophysins (Gainer et al. 1977a). The results of their conversion studies are consistent with the notion that the precursors are packed within the neurosecretory granulated vesicles (NGVs) and undergo post-translational, intragranular cleavage during axonal transport (Gainer et al. 1977b, 1977c). Similar studies in Brattleboro rats further allow the identification of one putative precursor for vasopressin-neurophysin and another one for oxytocin-neurophysin (Brownstein and Gainer 1977). In addition, Brownstein et al. (1977) suggest that sequence homology exists between the putative precursor and neurophysin, since these precursors specifically bind to antibody against neurophysin. Furthermore, in the dog hypothalamus, a cysteine-rich protein (20,000) MW.) has recently been isolated and identified as the precursor for vasopressin (Gruber and Morris 1980). Therefore, evidence thus far supports the concept that the neural lobe peptide hormones and their respective carrier proteins, the neurophysins, are synthesized via the ribosomal mechanism. This is in contrast to the "synthetase" mode of synthesis recently proposed for other smaller neuropeptides (e.g. TRH, GRH, and PRH) (for review, see Reichlin et al. 1976). Since the organelles for protein synthesis appear to be confined to the neuronal perikarya (Lasek et al. 1974), the biosynthesis of these neuropeptides must occur at the level of the perikaryon. Therefore, the axonal transport of these products to the distant terminals in the neural lobe for storage and release is of great functional significance in these peptidergic neurons.
Evidence for the phenomenon of axoplasmic transport was first provided by the classical experiments of Weiss and Hiscoe (1948). They observed that if the sciatic nerve is ligated at some point along its length, there is a progressive distension of the nerve proximal to the constricted region and a marked narrowing just distal to it. If the ligature is removed subsequently, a "bulb" of material can be followed centrifugally down the nerve at a rate of about 1 to 2 mm per day. In addition to the discovery that a substantial amount of material is continuously being synthesized in the perikarya of neurons for transport along their axons (Weiss 1967), evidence also exists for a retrograde axonal transport, i.e. movement of material from the axon terminal to the cell body (Dalhström 1965, Lasek 1967, Kirkpatrick et al. 1972). Recent studies using exogenous horseradish peroxidase uptake (Kristensson and Olsson 1971, La Vail and La Vail 1972) have clearly confirmed the existence of retrograde transport. The functional significance of anterograde axonal transport in terms of the intraneuronal distribution of the newly synthesized materials is obvious in view of the fact that the physiologically most active region of a neuron, the axon terminal, is so distant from the biochemically most active region, the perikaryon. Retrograde transport, on the other hand, has been reported to be related to the returning of worn-out materials from the axons and terminals to the cell body for degradation or restoration (Nagatsu et al. 1976, Hensler and Reese 1974, Holtzman 1977), or to carry trophic substance from neighboring cells and target organs to the perikaryon (Stoeckel and Thoenen 1975, Paravicini et al. 1975, Stoeckel et al. 1976).
The rate of axonal transport has been studied extensively. There is evidence for at least two distinct rates, a fast component (100-400 mm/day) and a slow component (1-5 mm/day) (Lasek 1970, Ochs 1972a, Heslop 1975, Schubert 1976, for review see Grafstein 1977). The materials transported via the fast component are mostly particulates; the characteristic constituents include glycoprotein, an important component of membranes, and transmitter synthesizing enzymes (Forman et al. 1971, Bennet et al. 1973, Sabri and Ochs 1973, Karlsson 1976, Levin 1977). The materials transported via the slow component are mostly soluble proteins including cytoskeleton elements like tubulin, actin, myosin-like proteins and the subunit proteins of neurofilaments (Sjostrand 1970, Grafstein et al. 1970, Hoffman and Lasek 1975, Willard 1977, Willard et al. 1979). The existence of an intermediate rate for distinct classes of molecules has been more controversial. The best evidence to date supporting the intermediate rates of transport comes from the studies of Willard et al. (1974) and Willard and Hulebak (1977). Gradient gel autoradiography of labeled proteins from the visual system reveals 5 ranges of transport rate which are 240, 34-68, 4-8, 2-4, and 0.7-1 mm/day.

Anterograde axoplasmic transport in the hypothalamo-neurohypophysial system has been demonstrated by the method of ligation and transection (Hild and Zetler 1953, Dellmann and Owsley 1969, Rodriguez and Dellmann 1970). In addition, the pathway of transport from nuclei (SON and PVN) in the hypothalamus to the endings in the neural lobe has been demonstrated by autoradiography (Sloper 1966, Nishioka et al. 1970, Kent and Williams 1974). The rate of transport for vasopressin, oxytocin and neurophysins
has been reported to be 24-48 mm/day in the rat (Pickering and Jones 1971, Norström and Sjöstrand 1971, Jones and Pickering 1972, Burford and Pickering 1973). A rate of 14.4 mm/day for isotocin has been reported in the goldfish (Carassius auratus) (Jones et al. 1973).

In contrast to the directionality, rate and composition of each component of the axoplasmic transport, much less is known about the mechanism of axonal transport. All the hypotheses proposed so far suffer from a lack of adequate experimental evidence (for review, see Wilson and Stone 1979, Schwartz 1979). There are several hypotheses concerning the mechanism of axonal transport. Weiss (1970) postulates that materials are moved between tracks by "intratubular convection". Specific channels within the axon are thought to be involved, through which components are translocated by peristalsis. The motive force in this hypothesis has later been suggested to be derived from interaction between axolemma and contractile proteins (Marchisio et al. 1975, Bray 1978). The smooth endoplasmic reticulum (SER) has been suggested as the channel for the fast transport (Droz et al. 1975). Alternatively, Schmitt (1968) suggests that microtubules in the axoplasm provide an essentially stationary and passive track on which specific organelles could move in a step-wise fashion. Translocation would be the result of a local energy-dependent reaction. ATP and Ca^{++} have been found to be required for fast axonal transport (Ochs and Ranish 1969, Ochs 1971, Ochs et al. 1977, Hammerschlag et al. 1975). Ochs (1971, 1972a) has proposed a model in which organelles are attached to microtubules through antinomyosin cross-bridges or "sliding filaments" of unspecified nature, which would provide the motive force by contraction in a
manner analogous to muscle contraction. The spectrum of transport rates observed may be attributed to different relative affinity between the transported materials and the transport filaments (Ochs 1971, 1972a). Since both microtubules and contractile proteins have been shown to be polar (Huxley 1963, Allen and Borisy 1974, Snell et al. 1974), Ochs' model has the potential of operating in either the anterograde or retrograde direction, provided some of the polymers are oriented in one polarity while the rest are in the opposite polarity (Ochs 1975, Kerkut 1975, Schwartz et al. 1976). Other evidence supporting the view that microtubules are involved in axoplasmic transport include: 1) there is a critical temperature characteristic of the species examined, below which translocation stops abruptly. Homeotherms usually have a higher critical temperature (11-13°C) than poikilotherms (4-7°C). It has been suggested that the stopped transport is due to the disassembly of microtubules (Ochs and Smith 1975, Coseus et al. 1976, Edström and Hanson 1973, Takenaka et al. 1978, Gross and Beidler 1973, Heslop and Howes 1972), since microtubules are known to be cold-sensitive (Weisenberg 1972); 2) morphological studies at the electron microscopic level show that vesicles and other membranous organells are often associated with microtubules (Smith et al. 1975, La Vail and La Vail 1974); 3) the amount of labeled proteins transported seems to correlate well with the density of microtubules in the axon (Ochs 1972b, Barker et al. 1976, Komiya and Kurokawa 1978); 4) a strong correlation has been observed between the effect of antimitotic drugs (e.g. colchicine and vinblastine) on microtubule assembly in vitro and their potency in inhibiting axonal transport
(Paulson and McClure 1975, Banks and Till 1975, Hanson and Edström 1977, for review, see Hanson and Edström 1978). However, there is no conclusive evidence for a direct role of microtubules since fast axoplasmic transport may be blocked without obvious changes in the microtubules (Fernandez et al. 1971, Byers 1974).

In this dissertation, experiments designed to study some specific aspects concerning the biosynthesis and axoplasmic transport of neurophysin in the hypothalamo-neurohypophysial system of Rana pipiens are described, and the obtained results are presented in three parts; Part I is concerned with the determination of the number of neurophysins present in the hypothalamo-neurohypophysial system of the frog, the detection of putative precursors for the neurophysins and the time course of their axonal transport. Part II describes the sequence of fine structural changes in the hypothalamo-neurohypophysial system after the axoplasmic transport was perturbed by vinblastine. Part III deals with the visualization of microtubules assembled in vitro by the unlabeled peroxidase anti-peroxidase technique. This method can be employed to study the role of tubulin and microtubules in axonal transport and axonal regeneration.
PART I. THE BIOSYNTHESIS AND AXOPLASMIC TRANSPORT OF NEUROPHYSIN IN THE HYPOTHALAMO-NEUROHYPOPHYSIAL SYSTEM OF THE FROG RANA PIPIENS
A class of cysteine-rich, low molecular weight (approximately 10,000) proteins was first discovered by Van Dyke et al. (1942) in extracts of the bovine neural lobe. Referred to as the "van Dyke protein", this protein was found to be complexed with the two peptide hormones, vasopressin and oxytocin. Later it was isolated and named "neurophysin" by Acher et al. (1955). Much of the work since has been directed toward the isolation and biochemical characterization of this protein in various species of mammals. There are at least two neurophysins in all of the species examined (with the exception of the guinea pig, which has only one neurophysin), one associated with vasopressin and the other with oxytocin (for review, see Pickering and Jones 1978). Vasopressin and oxytocin, together with their respective neurophysins, are synthesized in separate neurons of the supraoptic nucleus (SON) and the paraventricular nucleus (PVN) in the hypothalamus (Vandesande and Dierickx 1975, 1976a, Vandesande et al. 1975, Aspeslagh et al. 1976).

The work presented here is a study of the biosynthesis and axonal transport of neurophysin proteins in the hypothalamo-neurohypophysial system of the frog, Rana pipiens.

In the amphibian hypothalamo-neurohypophysial system, vasotocin and mesotocin are produced in the magnocellular neurosecretory neurons of the preoptic nucleus (PON) (Acher et al. 1964, Bentley 1969, Moens 1972, Acher 1974). The presence of neurophysin in these neurons has
been demonstrated indirectly using antibody against porcine neurophysin II (Watkins 1975) or bovine neurophysin II (Vandesande and Dierickx 1976b). This is possible because extensive sequence homology has been revealed in neurophysins isolated from many species (Capra et al. 1972, 1975, Wuu and Crumm 1976, Chauvet et al. 1975, 1976, Schlesinger et al. 1977, for review, see Pickering and Jones 1978, and Seif and Robinson 1978). In addition, Vandesande and Dierickx (1976b) have demonstrated that they are synthesized in different neurons. Moens (1974) has isolated a cysteine-rich protein fraction from the neural lobe of Rana temporaria and tentatively identified it as neurophysin, with a relatively large molecular weight of 25,000. However, no conclusions were drawn as to the number of neurophysins present in the frog neural lobe, because it was uncertain that the fraction was homogeneous.

The biosynthesis and intraneuronal transport of these neuro-secretory peptides (i.e. vasopressin, oxytocin and neurophysins) have also been extensively studied in mammals. Sachs (1969, 1970, 1971) and co-workers (Sachs et al. 1969) have found evidence for the existence of an inactive intermediate polypeptide in the biosynthetic pathway leading to the synthesis of vasopressin. Both in vivo and in vitro they have shown that following the intracisternal administration of $^{35}$S-cysteine, there is a lag of $1\frac{1}{2}$ hours prior to the appearance of $^{35}$S-cysteine labeled vasopressin in the neural lobe. The events during this pulse label period are sensitive to puromycin, a protein synthesis inhibitor, while those afterwards are not (Sachs and Takabatake 1964, Takabatake and Sachs 1964). Using
the same approach, these authors have also presented evidence that neurophysin is very likely synthesized via a precursor and that the "maturation" of this precursor is insensitive to puromycin (Sachs et al. 1969, 1971), thus leading them to hypothesize that vasopressin and neurophysin are derived from a common precursor, which is synthesized by a ribosomal mechanism (Sachs et al. 1969). Consistent with the above hypothesis, more recently, Gainer and Brownstein in a series of investigations have identified the presence of precursor proteins (20,000 MW) for both vasopressin-associated and oxytocin-associated neurophysin in the rat (Gainer et al. 1977a,b, Brownstein and Gainer 1977, Brownstein et al. 1977). Furthermore, a cysteine-rich protein (20,000 MW) has also been isolated and identified as the precursor for vasopressin in the dog hypothalamus (Gruber and Morris 1980). Therefore, ample evidence exists supporting the notion that the two neural lobe peptide hormones and the neurophysins are synthesized via the ribosomal mechanism, in contrast to the "synthetase" mode of synthesis recently proposed for other small neuropeptides (i.e. TRH, GRH, and PRH) (for review, see Reichlin et al. 1976). Since the organelles for protein synthesis appear to be confined to the neuronal perikaryon (Lasek et al. 1974), the biosynthesis of these neuropeptides would necessarily occur at that level of the perikaryon; therefore, the axonal transport of these secretory products to the distant terminals in the neural lobe for storage and release is of great functional significance in these peptidergic neurons.

The pathway of transport for these neurosecretory products from nuclei in the hypothalamus to the endings in the neural lobe has been
demonstrated by autoradiography (Sloper 1966, Nishioka et al. 1970, Kent and Williams 1974). The rate of transport has been reported to be about 1-2 mm/hr (24-48 mm/day) in the rat, since newly synthesized, thus labeled hormones and neurophysins both arrived at the neural lobe between 1 and 2 hours after intracisternal injection of the radioisotope (Pickering and Jones 1971, Jones and Pickering 1972, Norström and Sjöstrand 1971, Burford and Pickering 1973). The parallel changes in radioactivity associated with the hormones and the neurophysins in the neural lobe further lends support to the concept that their synthesis and transport occur within an integral unit i.e. the neuro-secretory granulated vesicle (NGV) (Swann and Pickering 1976). A transport rate of 0.6 mm/hr for isotocin has also been reported in the goldfish (Jones et al. 1973), suggesting that the transport rate in the poikilotherms is similar to that in the homeotherms.

In the present study, $^{35}$S-cysteine labeled proteins were extracted from preoptic nucleus, infundibulum and neural lobe of the frog (Rana pipiens). Analysis by three types of polyacrylamide gel electrophoresis was carried out in order 1) to determine the number of neurophysins, 2) to provide evidence for the existence of precursor proteins of neurophysins, and 3) to determine the time course of axoplasmic transport of neurophysins from the preoptic nucleus to the neural lobe.
MATERIALS AND METHODS

Animals and operative procedures

Male or female grass frogs (Rana pipiens) were anesthetized by immersion in 0.1% Finquel solution. Following paraoral exposure of the ventral brain surface rostral to the optic chiasma three ul of frog Ringer solution (2 mM HEPES buffer, pH 7.4, 114 mM NaCl, 2 mM KCl, and 1.8 mM CaCl$_2$) (Hammerschlag et al. 1975) containing 6 uCi of $^{35}$S-cysteine (specific activity: 300 Ci/mM) and 10 mM dithiothreitol (DTT) were injected into the preoptic recess over a period of 5 minutes. The needle remained in position for 10 minutes before removal. Animals usually regain consciousness 15 to 20 minutes later. At selected time intervals (specified in the RESULTS section) post injectionem, groups of 5 animals were killed and pooled samples of preoptic areas, infundibula, and neural lobes were homogenized in 0.1N HCl, kept at 4°C for 18 hours (Dean et al. 1967) before storage at -70°C.

Polyacrylamide gel electrophoresis of labeled proteins

The three types of polyacrylamide gel electrophoresis (PAGE) used to separate $^{35}$S-cysteine labeled proteins from the isolated tissues are: basic gels (with a running pH 9.5), electrophoresis in the presence of sodium dodecyl sulfate (SDS), and isoelectric focusing (IEF) gels. To prepare tissue samples for gel electrophoresis, the frozen acid extracts were thawed and centrifuged at 24,000 g. The supernatant was treated with 10% TCA and the precipitated proteins were separated from the TCA soluble fraction by centrifugation. The TCA
precipitants were washed with ether (Gainer et al. 1977a) and redissolved in sample buffer (see below) appropriate for the gel system to be used. The basic gels (7% or 10%) were prepared as described by Davis (1964) (Table A.1.1) to separate proteins on the basis of their charge and size. The TCA precipitated samples were redissolved in the sample buffer (2.5 mM Tris, 0.2 M glycine, pH 8.0) with bromophenol blue included as tracking dye. Electrophoresis was carried out either in a Buchler polyanalyst electrophoresis cell or a vertical slab gel electrophoresis cell prepared in our own laboratory. Current was maintained at 2-3 mA/tube (0.6x8 cm) or 11-15 mA/slab (0.1x9.14 cm) till the tracking dye has migrated to about 1 cm from the bottom of the gel. SDS-gels (10% or 12.5%) were prepared as described by Laemmli (1970) and modified by Takács (1979) (Table A.1.2). Samples were dissolved in sample buffer (62.5 mM Tris-HCl, 2% SDS and 5% 2-mercaptoethanol, pH 6.8) and heat-denatured. Pharmacia's low molecular weight protein calibration kit was used in co-run gels as standards. Molecular weights were determined according to the method of Shapiro et al. (1967). Electrophoresis was carried out at 4 mA/tube gel, or 12 mA/slab till the tracking dye has migrated to about 1 cm from the bottom of the gel. IEF gels with broad or narrow range pH gradient were prepared according to Winter et al. (1977), but riboflavin was used instead of ammonium persulfate as the catalyst for polymerization (Table A.1.3). Complete polymerization in tubes was achieved by exposing to fluorescent light for at least 10 hours before use. Ethanolamine (0.4%) was used as cathode(-) buffer in the upper chamber and sulfuric acid (0.6N)
was used as anode (+) buffer in the lower chamber. Samples were dissolved in solution containing 9.5M urea, 0.1% Triton X-100, 2% ampholine (LKB), loaded on the cathode end, and overlayed with 200 ul of 10% sucrose. A constant voltage of 300 was maintained during the entire electric focusing period of 20 hours. For visualization of the protein bands, the gels were removed, fixed in 12.5% TCA for at least 30 minutes, stained either with 0.05% coomassie brilliant blue R-250 in 12.5% TCA, or, with 0.25% coomassie B blue R-250 in solution containing 9% acetic acid, and 45% methanol for 2 to 10 hours before diffusion destained in 7% acetic acid.

Analysis of radioactivity

Polyacrylamide gels in tubes were sliced into 2 mm thick discs by a gel slicer. Each disc was put into a scintillation vial, to which 0.5 ml of Protosol was added and incubated at 37°C overnight. Econofluor (10 ml) was added to each scintillation vial the next day before counting in a Tricarb liquid scintillation spectrometer (model 2425, Parkard). Counting efficiency was estimated to be 38% with less than 20 cpm background. Slab gels were dried onto 3MM Whatman paper (5x7), contacted with Kodak X-Omat film (XR-5) and exposed at -70°C for 10 to 20 days before processing in an automated X-ray film processor. Fluorography was carried out according to the procedure of Bonner and Laskey (1974). Total tissue homogenate, i.e. pooled samples of the preoptic areas, infundibula, and neural lobes homogenized in 0.1N NCl (0.2-0.5 ml) were first solubilized in 0.5 ml Protosol, incubated at 37°C for at least 12 hours, then added with 10 ml of Econofluor and
counted as above.

**Light microscopy**

To localize the preoptic nucleus (PON) in the hypothalamus of the frog brain, the preoptic area was dissected out and fixed in 5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4), post-fixed in 1% OsO₄ in 0.03M veronal acetate buffer (pH 7.4), dehydrated through graded alcohol and acetone, and embedded in an Epon-Araldite mixture (Table A.1.4). Serial cross sections (3 μm) were cut and stained with Gomori's aldehyde fuchsin method using a modified procedure of Coates and Teh (1978).

The preoptic nucleus was also localized by light microscopic autoradiography. ³⁵S-cysteine (6 μCi/frog) was injected into the preoptic recess, and the animals were killed at 2, 4, 6 hours and 1, 3 and 7 days post injectionem. The preoptic area and neural lobe were fixed and processed as described above. Serial cross or sagittal sections (3 μm) mounted on slides were dipped in Kodak nuclear track emulsion (NTB-2) diluted with distilled water (v/v:22/17) at 37°C. The emulsion coated slides were allowed to dry at 37°C and 90% relative humidity for 30 minutes, then at 29°C and 90% relative humidity for 2 hours, before being sealed in slide boxes containing Drierite, and exposed at -70°C for 4-6 weeks. At the end of the exposure, the slides were developed in D-19 developer for 2 minutes, rinsed in distilled water for 10 seconds, fixed in Kodak fixer for 6 minutes at 17°C, rinsed in running tap water for 30 minutes, further rinsed in distilled water and air-dried.
RESULTS

Localization of neurosecretory neurons in the preoptic nucleus (PON)

The Gomori-positive neurosecretory cell bodies of the preoptic nucleus are found in the medio-ventral and latero-dorsal regions of the hypothalamus (Fig. 1.1C). In animals killed 2 hours after the injection of $^{35}$S-cysteine into the preoptic recess, the neurosecretory neurons are specifically labeled (Fig. 1.1 A,B,D-G), while other neurons and the ependymal cells lining the third ventricle and preoptic recess are not. Labeled neurosecretory neurons are present at 4 and 6 hours, but they are not as heavily labeled. However, 3 days or longer after the injection, the labeling is insufficient to allow a clear differentiation of these cells from the background.

Incorporation of $^{35}$S-cysteine into neurohypophysial proteins and their axoplasmic transport into the neural lobe

The time course of appearance of $^{35}$S-cysteine labeled proteins in the hypothalamo-neurohypophysial system was analyzed by basic gel electrophoresis (7%) and autoradiography. The coomassie blue staining patterns of proteins extracted from PON (Fig. 1.2A), infundibulum (Fig. 1.2C) or neural lobe (Fig. 1.2E) are similar throughout all time points examined. While many proteins are present in the PON and infundibulum, the protein profiles of the neural lobe are relatively simple. In addition to several minor bands with relatively slower mobilities, one major band with fast mobility (Rf=0.81) is found in the neural lobe.
Fig. 1.1 Preoptic area of a frog killed 2 hours after the injection of $^{35}$S-cysteine into the preoptic recess.

(A) Sagittal section showing the general distribution of PON cells. Darkfield, (x32).

(B) Sagittal section at higher magnification. Darkfield, (x 160).

(C) Preoptic neurosecretory cells (arrow) as stained by the aldehyde fuchsin stain of Gomori, (x320).

(D) Transverse section showing a few PON cells (arrow) at the latero-dorsal region of the hypothalamus. Brightfield, (x75).

(E) Darkfield image of an area in (D), (x160).

(F) Transverse section of the preoptic area, showing PON labeled cells in the medio-ventral region. Brightfield, (x75).

(G) Darkfield micrograph of an adjacent section showing that the nucleus of the PON cells and the ependymal cells lining the preoptic recess are not significantly labeled, (x235).
Fig. 1.2 Polyacrylamide slab gel electrophoresis in a pH 9.5 system of $^{35}\text{S}$-cysteine labeled proteins extracted from various regions of the hypothalamo-neurohypophysial system of the frog at selected times after injection of $^{35}\text{S}$-cysteine into the preoptic recess.

(A), (C), and (E) are the coomassie blue-stained protein banding pattern of the preoptic nucleus, infundibulum and neural lobe.

(B), (D), and (F) are the respective autoradiograms of the three stained slabs to reveal the time course of appearance of labeled proteins in the above 3 areas.

Arrow indicates the position migrated by the Np protein band.
A protein band with the same mobility is also present in the PON and the infundibulum, and thus designated as Np protein.

In the respective autoradiogram of the PON (Fig. 1.2B), at least 5 labeled protein bands including the Np band are present as early as 1 hour after the injection. These labeled bands decrease in intensity with time and are no longer detectable after 24 hours.

In the infundibulum (Fig. 1.2D) the Np band is clearly labeled by 4 hours, persists through 12 hours and is no longer detectable 24 hours after the injection. On the contrary, in the respective autoradiogram of the neural lobe (Fig. 1.2F), no labeled proteins are present at 1 and 2 hours after the injection. The labeled Np band first appears at 4 hours, and persists throughout the entire observation period of 5 days. A faint band (Rf=0.66) is also labeled. The results thus indicate that it takes at the most 4 hours for $^{35}$S-cysteine to be incorporated into the Np protein and transported to the neural lobe.

Since the average length of the hypothalamo-neurohypophysial tract of the frog is 3.6 mm, the minimum rate of transport is 0.9 mm/hr (22 mm/day).

Additional evidence that the labeled proteins are synthesized by the neurons of the PON rather than by the pituicytes in the neural lobe is also provided. Experiments in which the hypothalamo-neurohypophysial tract was either transected or microiontophoretically ejected with vinblastine at the level of median eminence at the time of isotope injection into the preoptic recess demonstrate that in control animals 5 days after the injection of isotope, the radioactivity of the neural lobe (total tissue homogenate) represents 3% of that in the PON region. It increases to 5.6% in animals stimulated by salt-loading for
7 days, whereas in animals with transected stalk, the radioactivity in the neural lobe is only 0.1% of that in the PON (Table 1.1). Light microscopic autoradiography also shows that 3 days after the isotope injection, the median eminences and the neural lobes of control animals are heavily labeled (Fig. 1.3A), whereas in vinblastine treated animals, the neural lobe is free of any significant amount of labels (Fig. 1.3b).

**Analysis of proteins transported to the neural lobe**

For further analysis of the labeled proteins transported to the neural lobe, the acid extracted, TCA precipitated proteins were separated by three different PAGE systems. Fig. 1.4 shows the pattern of labeled proteins on the basic gel (10%, pH 9.5). One major peak of radioactivity was detected. Separation of the labeled neural lobe proteins on the SDS-PAGE in cylindrical gels (12.5%) however, revealed two peaks of radioactivity (Fig. 1.5). Alternatively, when SDS-PAGE (10%) was used, the resulting fluorogram (Fig. 1.6) also reveals the presence of two labeled bands, designated at NP I and Np II. The molecular weights of Np I and Np II were estimated to be about 23,000 and 20,100 respectively (Fig. 1.7). Further evidence for the existence of two labeled proteins in the neural lobe is provided by data obtained from IEF-PAGE (pH 4-6). Two radioactivity peaks were resolved with pI=4.6 and 4.9 (Fig. 1.8).

**Biosynthesis of $^{35}$S-cysteine labeled proteins in the hypothalamo-neurohypophysial system**

The labeling profiles of proteins synthesized in the PON, transported through the infundibulum and stored in the neural lobe were analyzed
Table 1.1  Total tissue homogenate collected from various regions of the hypothalamo-neurohypophysial system were counted 5 days after the injection of 35-S-cysteine (5uCi/frog) into the preoptic recess

<table>
<thead>
<tr>
<th></th>
<th>PON</th>
<th>ME</th>
<th>NL</th>
<th>TOTAL COUNTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Control animals&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPM</td>
<td>73,320</td>
<td>47,590</td>
<td>2,310</td>
<td>123,220</td>
</tr>
<tr>
<td>% TOTAL</td>
<td>60%</td>
<td>30%</td>
<td>2%</td>
<td></td>
</tr>
<tr>
<td>% PON</td>
<td>--</td>
<td>64%</td>
<td>3%</td>
<td></td>
</tr>
<tr>
<td>B. Animals salt-loaded with 1% NaCl for 7 days before injection</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPM</td>
<td>50,480</td>
<td>36,210</td>
<td>2,860</td>
<td>89,550</td>
</tr>
<tr>
<td>% TOTAL</td>
<td>56%</td>
<td>41%</td>
<td>3.2%</td>
<td></td>
</tr>
<tr>
<td>% PON</td>
<td>--</td>
<td>71%</td>
<td>5.6%</td>
<td></td>
</tr>
<tr>
<td>C. Animals with median eminence transected at the time of isotope injection</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPM</td>
<td>54,580</td>
<td>40,010</td>
<td>90</td>
<td>94,580</td>
</tr>
<tr>
<td>% TOTAL</td>
<td>58%</td>
<td>42%</td>
<td>0.1%</td>
<td></td>
</tr>
<tr>
<td>% PON</td>
<td>--</td>
<td>71%</td>
<td>0.1%</td>
<td></td>
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<sup>a</sup> Data obtained by pool of 3 animals treated alike.
Fig. 1.3  Three days after the injection of $^{35}\text{S}$-cysteine (5uCi/frog) into the preoptic recess of the:

(A) Control animal, the neural lobe is heavily labeled. (x288)

(B) Vinblastine treated animals, labeled materials accumulated (arrow) proximal to the ejection site. Noted the absence of any appreciable labels in the neural lobe.

PI: Pars Intermedia, PD: Pars Distalis, NL: Neural Lobe, INF: Infundibulum. (x288)
Fig. 1.4  Separation of $^{35}\text{S}$-cysteine labeled proteins extracted from neural lobe 3 days after the injection on 10% basic gel electrophoresis.
Fig. 1.5 Separation of $^{35}$S-cysteine labeled proteins extracted from neural lobe 3 days after the injection on SDS-PAGE (12.5%). Upper abscissa shows the slice positions of marker proteins of known molecular weights in a co-run gel. Phosphorylase B (94,000), Serum albumin (68,000), Ovalbumin (43,000), Carbonic anhydrase (30,000), Trypsin Inhibitor (20,100), $\alpha$-Lactoalbumin (14,400).
SDS-PAGE (12.5%)

MOLECULAR WEIGHT × 10⁻³

94 68 45 30 20.1 14.4

CPM × 10²/GEL SLICE

SLICE NUMBER

20.1K

23K
Fig. 1.6 Slab SDS-PAGE (10%) fluorography and coomassie blue staining of gel containing labeled proteins of the neural lobe.

track 1: Coomassie blue staining pattern of molecular weight standards.

track 2: Coomassie blue staining pattern of labeled proteins extracted from the neural lobe.

track 3: The respective fluorogram of track 2, showing 2 labeled bands.

Molecular weight of NpI and NpII are derived from the standard curve constructed by plotting the molecular weights of standards vs. their respective relative mobilities (Rf) on a semi-log scale. PHb=phosphorylase b; BSA=bovine serum albumin; OVA=ovalbumin; CA=carbonic anhydrase; TI=trypsin inhibitor; LA= -lactoalbumin.
Fig. 1.8  IEF-PAGE (pH 4-6) of $^{35}$S-cysteine labeled proteins transported to the neural lobe 24 hours after injection of $^{35}$S-cysteine into the preoptic recess.
by IEF-PAGE at various times after the injection of \(^{35}\text{S}\)-cysteine into the preoptic recess. Fig. 1.9 shows that 30 minutes after the injection, the PON labeling profiles are dominated by a peak of radioactivity with pI=5.2. Much less label is present in the range of 4.3-4.9 and 5.8-6.0. The latter is not detectable after 1 hour. As can be seen from Fig. 1.9 B-E, the radioactivity associated with the pI=5.2 peak decreases gradually with time relative to that associated with the peaks having pI ranging from 4.3-4.9. By 6 hours (Fig. 1.9E), an approximately equal amount of label is present in peaks with pI=5.2, pI=4.9 and pI=4.6. In addition, the total radioactivity in the PON is also decreasing as a function of time and by 24 hours, only trace amounts of the pI=5.2 peak are present (Fig. 1.9F). In the infundibulum, at 30 minutes, the radioactivity is present in peaks with pI's similar to those in the PON as mentioned above (Fig. 1.10 A). The peaks with pI=5.8-6.0 however, are clearly present at 4 and 6 hours after the injection in this region (Fig. 1.10 C,D). With time, the relative amount of radioactivity present in peaks with pI ranging from 4.6-4.9 increases when compared to that in the pI=5.2 and pI=5.8-6.0 peaks (Fig. 1.10 D). In the neural lobe, no labeled peaks can be detected till 24 hours after the injection. Using an expanded pH gradient (pH 4-6), two peaks of radioactivity can be consistently resolved with pI=4.6 and pI=4.9 at day 1 and 3 (Fig. 1.11 A,B). No labeled proteins with pI=5.8-6.0 or pI=5.2 were detected at any time points examined in the neural lobe.
Fig. 1.9 IEF-PAGE (pH 3.5-10) of $^{35}$S-cysteine labeled proteins extracted from preoptic nucleus of frog at various times after injection of $^{35}$S-cysteine into the preoptic recess.

(A) 30 minutes post-injection.
(B) 1 hour post-injection.
(C) 2 hours post-injection.
(D) 4 hours post-injection.
(E) 6 hours post-injection.
(F) 24 hours post-injection.
PREOPTIC NUCLEUS

30 MIN

CPM x 10^(-1) GEL SLICE

SLICE NUMBER

1 HOUR

CPM x 10^(-1) GEL SLICE

SLICE NUMBER

5.8

5.2

4.9

4.6

1 HOUR
Fig. 1.9 (continued)
Fig. 1.9 (continued)
Fig. 1.10  IEF-PAGE (pH 3.5-10) of $^{35}$S-cysteine labeled proteins transported to the infundibulum at various times after the injection of $^{35}$S-cysteine into the preoptic recess.

(A) 30 minutes post-injection.

(B) 1 hour post-injection.

(C) 4 hours post-injection.

(D) 6 hours post-injection.
INFUNDIBULUM

30 MIN

5.8 5.2 4.9 4.6

0 2 4 6 8
CPM X 10^2/GE SLICE

5 10 15 20 25 30 35 40
SLICE NUMBER

1 HOUR

22

0 2 4 6 8
CPM X 10^2/GE SLICE

5 10 15 20 25 30 35 40
SLICE NUMBER
Fig. 1.10 (continued)
Fig. 1.11 Separation of $^{35}$S-cysteine labeled proteins extracted from the neural lobe on IEF-PAGE with a narrow pH range (4-6). Two peaks of radioactivity are resolved.

(A) Neural lobe extracts collected 24 hours after the injection.
(B) Neural lobe extracts collected 3 days after the injection.
DISCUSSION

The magnocellular neurosecretory cells of *Rana pipiens* may be specifically labelled by $^{35}$S-cysteine after a short pulse. This is the first direct evidence that these cells are capable of synthesizing cysteine-rich proteins. The distribution of these cells in the pre-optic nucleus (PON) is comparable to that illustrated in other amphibia by the immunocytochemical staining method, using antibody against porcine neurophysin II (Watkins 1975b) or bovine neurophysin I (Vandesande & Dierickx 1976b).

The initial evidence that newly synthesized and thus labeled proteins are transported from the perikarya in the PON to axon terminals in the neural lobe comes from the results of LM-autoradiography. The PON cells can be specifically labeled and clearly identified only after a short pulse, i.e. 2 hours, but not after a pulse longer than 6 hours. The neural lobe, on the contrary, is not significantly labeled till 24 hours after the isotope injection and the label persist in the neural lobe for as long as 7 days. Considering the half life, 1) of 13 days for vasopressin and oxytocin, 2) of 19.8 days for vasopressin-neurophysin and 3) of 13.3 days for oxytocin-neurophysin (Sachs et al. 1969, Pickering & Jones 1971, Jones & Pickering 1972, Burford & Pickering 1973, Pickering et al. 1975), it is unlikely that the diminishing labeling intensity in the PON cells is entirely due to turnover. Furthermore, the above results correlate with the time
course study in which labeled proteins extracted from PON, infundibulum and neural lobe were separated by PAGE. The resulting autoradiogram of the PON suggests that incorporation of $^{35}$S-cysteine into proteins including the Np band is rapid and approximates a pulse incubation, since no labeled proteins were detectable by 24 hours after the injection. In addition, the transient appearance of these labeled proteins, particularly the Np band, in the infundibulum and the persistence of this labeled protein band in the neural lobe from 4 hours through 5 days all imply that this Np protein is synthesized first in the PON and then transported intra-axonally to the neural lobe for storage via axons traversing the infundibulum. The fact that the neural lobe contains, in addition to the Np protein, other minor proteins which are not labeled, with the exception of the Rf-0.66 band, (Fig. 1.2E, 1.2F) and since no significant amount of labels can be detected in neural lobe of animals with their hypothalamo-neurohypophysial tract either transected (Table 1.1) or treated with vinblastine (Fig. 1.3A, 1.3B), also implies that the Np protein present in the neural lobe is coming from the cell bodies rather than from a de novo synthesis by the pituicytes. This is consistent with reports that synthesis of the neural lobe hormones and neurophysins occur only at the site of neuronal perikarya in the hypothalamus, whereas the neural lobe containing the axons and terminals of these same neurons is incapable of synthesizing these neurosecretory products (Sachs 1960, Sachs & Takabatake 1964, Takabatake & Sachs 1964, Sachs et al. 1969). Since Np protein is the only major protein present in the neural lobe that is rich in cysteine and originates from the PON, it is tentatively
identified as the neurophysin protein of *Rana pipiens*.

In addition, the estimated, minimum transport rate of 22 mm/day for the newly synthesized Np is similar to that reported for isotocin in the goldfish (Jones et al. 1973) and vasopressin, oxytocin and neurophysins in the rat (Norstrom & Sjostrand 1971, Pickering & Jones 1971, Jones & Pickering 1972, Burford & Pickering 1973). The results in the present study support the conclusion by Jones et al. (1973) that the transport rate of neurosecretory materials in the magnocellular neurosecretory neurons of cold-blooded vertebrates is similar to that in the warm-blooded vertebrates.

Further characterization of the labeled proteins transported to the neural lobe was carried out by three types of PAGE systems. When a higher concentration of 10% basic gel with presumably a better resolution than the 7% gel for low molecular weight proteins was used, a single peak of radioactivity was detected. Thus, analysis by the basic gel system is not able to resolve more than one peak of $^{35}$S-cysteine labeled proteins in the neural lobe. However, when IEF-PAGE and SDS-PAGE (10% or 12.5%) were used, two peaks of radioactivity were resolved by both systems. The results thus support the conclusion derived from the immunocytochemical studies of Vandesande & Dierickx (1976b) that two different neurophysins are present in the amphibian hypothalmo-neurohypophysial system. In another lower vertebrate, the cod, *Gadus morhua*, only one neurophysin has been isolated. However, Pickering (1968) did not exclude the possibility that there may be more than one neurophysin present. At least three neurophysin-like proteins have been identified in the chicken neural lobe by
Peek & Watkins (1977). In both the SDS and the IEF-PAGE, the amount of labels associated with the two peaks are apparently not equal; this may be due to difference in synthesis, or release, or the number of cells responsible for the production of these two proteins. Whether or not a constant ratio exists as found in the rat between vasopressin-neurophysin and oxytocin-neurophysin, (Burford et al. 1971) remains to be determined. Equally important is the identification of the respective peptide hormones, i.e. vasotocin and mesotocin with which these two Np proteins are associated in vivo.

The isoelectric points (pI) of 4.9±0.1 and 4.6±0.1 confirm that these two cysteine-rich proteins are acidic in nature. Also, the pI's are similar to those of vasopressin-associated (pI4.8) and oxytocin-associated neurophysin (pI=4.6) of the rat (Brownstein and Gainer 1977, Gainer et al. 1977a).

The determined molecular weights of Np I (23,000) and Np II (20,000) are both smaller than the 25,000 reported for neurophysin of Rana temporaria (Moens 1974). Yet, both are larger than all the known mammalian neurophysins (M.W. 10,000) (Seif and Robinson 1978). Molecular weights of the chicken neurophysins (fraction B and C) were determined by gel filtration to be about 50,000 and 31,500 respectively. Under similar conditions most mammalian neurophysins have a dimeric molecular weight of about 20,000 (Peek and Watkins 1977). Assuming that the chicken neurophysins are also in dimeric forms, then this means that the molecular weights for the monomers would be about 25,000 and 15,000 for B and C respectively. The significance of neurophysins with larger molecular weights is not clear at this time.
However, the study of their chemical structures by comparing their amino acid sequence would lend insight as to how neurophysins from such diverse sources throughout the vertebrate phyla share so many similar characteristics.

A pulse label paradigm has been used to study the biosynthesis of the frog neurophysin. One of the essential steps in the identification of a precursor protein is to be able to detect that during the pulse, a labeled protein with larger molecular weight (precursor) is first synthesized, and then decreases in radioactivity with time as the lower molecular weight polypeptide (product) is formed (Gainer et al. 1977c). IEF-PAGE with a broader pH gradient of 3.5-10.0 was used to detect if any $^{35}$S-cysteine labeled proteins exist preceding the appearance of the neural lobe peaks ($pI=4.9$ and $pI=4.6$). The results indicate that there are, in addition to peaks with $pI$ range similar to that of the two tentative neurophysins, two peaks with $pIs$: 5.8 and 5.2 in the PON shortly after the injection of isotope. The $pI=5.8$ peak is not detectable after 1 hour. However, the $pI=5.2$ peak remains a prominent peak and decreases in radioactivity with time relative to that in the $pI=4.9$ and $pI=4.6$ neural lobe peaks. The neural lobe peaks never attain the amounts of radioactivity expected from a simple conversion in the perikarya, since after 4 hours, the total radioactivity present in the PON also decreased with time, and by 24 hours, only trace amount of $pI=5.2$ can be detected. This suggests that an "intragranular maturation" process is taking place during axonal transport, as has been proposed for neurophysin biosynthesis in the mammalian systems (Sachs et al. 1969, Pickering et al.
1975, Gainer et al. 1977a). Evidence for axoplasmic transport is also revealed by the presence of pI 5.8 and 5.2 peaks in the infundibulum at 30 minutes and 1 hour. A similar decrease in radioactivity of the pI=5.2 peak and the concomitant increase in peaks with pI ranging from 4.6-4.9 is also seen from 1 through 6 hours. This implies the conversion process continued during axonal transport. The radioactivity associated with the pI=5.8 peak gradually increases from 1 to 6 hours. Although it decreases together with the pI=5.2 peak and the pI=4.7 peak by 24 hours, the pattern of change relative to that in peaks with pI ranging from 4.6-4.9 as a function of time, is not as obvious as for the pI=5.2 peak. Since no labeled proteins with pI=5.8 or 5.2 can be detected at any time point examined in the neural lobe, it is either a matter of sensitivity of the method or perhaps the processing of the putative precursor protein may have been completed by the time the NGVs arrive in the neural lobe. The above results are preliminary in the identification of precursor protein for the frog neurophysins, since it would be necessary to demonstrate that the pI=5.2 and pI=5.8 proteins have a higher molecular weight than the two neurophysins. Also, the unequivocal proof for the identification of the precursor proteins requires isolation of these molecules for further immunological, peptide mapping and amino acid sequencing studies to confirm that sequence homology does exist between precursor and product.
In conclusion, two $^{35}$S-cysteine labeled proteins, Np I and Np II found in the acid extracts of frog neural lobe are synthesized in the perikaryon of PON cells. They arrive at the neural lobe between 2 and 4 hours after the injection of $^{35}$S-cysteine into the preoptic recess, indicating an axoplasmic transport rate of 22 mm/day. Their molecular weights have been determined to be about 23,000 and 20,100, with pIs of 4.9±0.1 and 4.6±0.1. It is believed that Np I and Np II are neurophysins of Rana pipiens, and proposed that one is associated with vasotocin and the other with mesotocin. Two $^{35}$S-cysteine labeled proteins (pI=5.8±0.1 and pI=5.2±0.1) are found to be present in the PON within one hour of isotope injection. Preliminary evidence indicates that at least one (pI=5.2) of them may be the putative precursor protein for the neurophysins.
LITERATURE CITED


PART II. LIGHT AND ELECTRON MICROSCOPIC OBSERVATIONS IN THE HYPOTHALAMO-NEUROHYPOPHYSIAL SYSTEM OF THE FROG RANA PIPIENS AFTER MICROIONTOPHORESIS OF VINBLASTINE INTO THE MEDIAN EMINENCE
One of the remarkable structural features of the hypothalamic, magnocellular peptidergic neurons is the presence of large axon dilatations referred to as Herring bodies. They occur along the axon between its perikaryon and its terminals in the neural lobe (NL), and are particularly numerous in the neural lobe (Dellmann & Rodriguez 1970, Dellmann 1973, Dellmann 1974). These Herring bodies have been classified into three main types by Dellmann and Rodriguez (1970): Type I Herring bodies are characterized by numerous neurosecretory granulated vesicles (NGVs); type II Herring bodies contain many autophagic vacuoles, dense bodies, varying numbers of NGVs and empty vesicles; type III Herring bodies contain an extensive network of axonal endoplasmic reticulum (PER), mitochondria and a few NGVs.

Based on these morphological characteristics, Dellmann and Rodriguez (1970) hypothesized that type I Herring bodies are storage sites for the NGVs, and may be caused by a temporary, localized arrest of the axonal transport. The accumulation of NGVs, thus represents the consequence of a constant anterograde axoplasmic transport of NGVs from the perikaryon. After short disturbances, resumption of the axonal transport may occur; however, after long disturbances, local disposal of NGVs may take place and cause the autophagic events seen in the type II Herring bodies. Type III Herring bodies, on the other hand, are considered to represent the beginning of the state of restitution. Consequently, the functional significance of the Herring bodies may be that they provide sites for
a regulatory mechanism by which excess of neurosecretory products, namely vasopressin (vasotocin), oxytocin (mesotocin) and their neurophysins are stored and metabolized.

Consistent, in part, with the above hypothesis, is an electron microscopic autoradiographic study concerning the movement of neurosecretory products through the anatomic compartments of the neural lobe by Heap et al. (1975) who demonstrated that newly synthesized, labeled NGVs first appeared in axon terminals and then in progressively larger and larger profiles of "swellings" (Herring bodies). Therefore, the NGVs found within Herring bodies appear to be "older". Furthermore, the presence of two distinct populations of NGVs isolated from the rat neural lobe has led Nordmann and co-workers (1979) to propose that one population representing the newly arrived NGVs is derived from axon terminals whereas the other population, representing the "aged" NGVs is derived from the axon swelling (Herring bodies).

In order to further test the hypothesis of Dellmann and Rodriguez (1970), a temporary, localized arrest of axoplasmic transport in the neurosecretory axons of the hypothalamo-neurohypophysial system was attempted. Since antimitotic drugs (e.g. colchicine and vinblastine) are capable of blocking the axoplasmic transport of neurosecretory cells, either in the perikaryon (Norstrom et al. 1971, Flament-Durand et al. 1972, 1975, Dustin et al. 1975, Hindelang-Gertner et al. 1976) or in the hypothalamo-neurohypophysial tract (Rodriguez et al. 1977), vinblastine was microiontophoretically ejected into the median eminence of the grass frog (Rana pipiens). The sequence of fine structural changes in
areas adjacent to the ejection site and in the neural lobe are described in the present paper.
MATERIALS AND METHODS

Animals and operative procedures

Groups of 3-4 frogs (Rana pipiens) were anesthetized by immersion in 0.1% Finquel solution. The infundibulum and median eminence were exposed paraorally (Dellmann and Owsley 1968), and the meninges were removed to facilitate the insertion of a micropipette prior to microiontophoresis.

Microiontophoresis

Microelectrodes with a tip diameter of about 4-7 um were filled with 18 mM vinblastine sulfate solution, mounted onto a microdrive and positioned 10-20 um deep into the center of the median eminence. A positive current of 10-20 nA was passed for 5 minutes. The animals recovered from anesthesia within 30 minutes, and were killed 1, 3, 8, 15, and 30 days after the ejection. Animals ejected with 18 mM Na₂SO₄ were killed 3 days after to serve as controls.

Preparation of tissues for light and electron microscopy

Animals were perfused through the heart with, or the whole brain was immersion fixed in 5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.4). The preoptic area, infundibulum, median eminence and neural lobe were dissected and remained in the same fixative for an additional 2-5 hours. Tissue samples were then rinsed in 0.2 M phosphate buffer with 4% sucrose, post-fixed in 1% OsO₄ in 0.03 M veronal acetate buffer (pH 7.4) with 4% sucrose (VA buffer) for 1 hour, rinsed with VA buffer
and stained en bloc with 2% uranyl acetate in VA buffer for 2 hours. Following dehydration with graded alcohol and acetone the tissues were embedded in an Epon-Aradlite mixture. Sagittal sections (3 um) were cut and stained with the aldehyde fuchsin stain of Gomori as modified by Coates and Teh (1978) for plastic sections. The site of blocked axoplasmic transport was localized at the light microscopic level. Subsequently, thin sections were obtained, stained with 2% aqueous uranyl acetate and 10% lead citrate and examined in a Hitachi U-12A electron microscope at 50 KV.

**Light microscopic autoradiography**

\(^{35}\)S-cysteine (5-6 uCi/frog) was injected into the preoptic recess of control and vinblastine treated animals. Three days later, the animals were fixed and the tissues processed as above. Serial sections 3 um thick were collected and processed as previously described for LM-autoradiography (See PART I. Materials and Methods).
RESULTS

Light microscopy

Infundibulum and median eminence. In control animals, the hypothalamo-neuropophysial tract is difficult to trace using the Gomori stain in sagittal sections of the infundibulum, since the axons only occasionally contain NGVs for any distance (Dellmann and Owsley 1968). However, in the median eminence, axons containing clusters of Gomori-positive material are seen more readily in perivascular regions. Both the infundibulum and the median eminence are lined by a simple columnar ependyma towards the infundibular recess. Also, tanycytes and pituicytes are frequently seen in the median eminence (Fig. 2.1A). No alterations are noted in these two areas 24 hours after the ejection of vinblastine. However, at day 3, Gomori-positive Herring bodies of varying sizes are seen proximal to the ejection site in the infundibulum of vinblastine-treated animals (Fig. 2.1B). Herring bodies are present through 15 days after the ejection, but are no longer detectable at 30 days.

Neural lobe. The neural lobe of control animals contains numerous Gomori-positive axons and perivascular axon terminals and some pituicytes (Fig. 2.1C). The same pattern of organization remains in the neural lobe of experimental animals at day 1 but is subsequently lost at day 3, when many large Gomori-negative, spherical structures are present among the remaining Gomori-positive terminals (Fig. 2.1D).
Fig. 2.1. Light micrographs of 3 um semithin plastic sections stained with Goromi's aldehyde fuchsin stain.

(A) Infundibulum of a control animal.
   IR: infundibular recess, E: ependyma cells (x 100).

(B) Three days after the ejection of vinblastine, proximal to the ejection site in the infundibulum, Gomori-positive Herring bodies (arrows) are present (x 256).

(C) Neural lobe of a control animal, the Gomori stain reveals the presence of many neurosecretory axons and terminals (x 256).

(D) Neural lobe of a vinblastine-treated animal 3 days after the ejection. Large clusters of Gomori-negative material are present among normal axons and terminas (x 256).
The neural lobes of the vinblastine-treated animals have reacquired normal appearance at 15 days after the ejection.

**Electron microscopy**

**Infundibulum and median eminence** In control animals, the thin neurosecretory axons (300-600 nm diameter) passing through the infundibulum and the median eminence contain many neurofilaments, microtubules, mitochondria, smooth axonal endoplasmic reticulum (AER) and a few NGVs (100-300 nm diameter) (Fig. 2.2).

At day 1 after the ejection, many neurosecretory axons in the inner zone of the median eminence adjacent to the ejection site, are filled with paracrystalline structures (Fig. 2.3). Similar paracrystals are found in tanycytes and some glial cells in the median eminence as well (Fig. 2.3, Fig. 2.4C). In longitudinal sections, these paracrystalline structures appear as parallel arrays of dense lines (Fig. 2.4A, B, Fig. 2.5) and in cross sections as honeycomb-like patches (Fig. 2.4C). NGVs and other organelles, including AER and mitochondria are often located in the periphery of axons filled with these paracrystals (Fig. 2.3, Fig. 2.4A, Fig. 2.5). In addition, numerous whirl-like or parallel arrays of neurofilaments and networks of AER are found in other axons in the same area (Fig. 2.5).

At day 3, in the median eminence distal to the ejection site, axons filled with paracrystals (Fig. 2.6A) or containing clusters of mitochondria (Fig. 2.6C) and apparently degenerated axons are present (Fig. 2.6D). Proximal to the ejection site in the infundibulum, many
Fig. 2.2 Neurosecretory axons in the infundibulum of a control animal. Notice that many parallel neurosecretory axons contain a few NGVs (N), microtubules (MT), axoplasmic reticulum (A), neurofilaments (Nf) and mitochondria (m) (x 24,000).
Fig. 2.3 One day after the ejection of vinblastine, the photomontage reveals that in addition to a few Herring bodies (arrow head), many neurosecretory axons passing through the inner zone (IZ) of median eminence are filled with paracrystalline structure (arrows), which is also found in the ependymal cells (E).

IR: infundibular recess, P: pituicyte (x 6,050).
Fig. 2.4 One day after the ejection of vinblastine, adjacent to the site in the median eminence.

(A) Paracrystalline structures (arrows) appear as parallelly arranged dense lines in neurosecretory axons sectioned longitudinally (x 28,800).

(B) These paracrystalline structures acquire a honeycomb-like (arrow) appearance in axons sectioned transversely (x 20,000).

(C) Patches of paracrystals (arrow) are also found in the neighboring glial cell. L: multilamellar body, mv: multivesicular body, G: Golgi apparatus (x 21,900).
Fig. 2.5  One day after the ejection of vinblastine, adjacent to the ejection site in the median eminence, neurosecretory axons containing numerous neurofilaments (Nf) and networks of axonal smooth endoplasmic reticulum (A) are also found in addition to the paracrystal (arrow) filled axon. H: Herring bodies (x 28,800).
Fig. 2.6 Three days after the ejection of vinblastine, adjacent to the ejection site in the median eminence.

(A) Axon profiles containing paracrystalline structures (arrows) are still present. G: glial processes (x 24,000).

(B) Cluster of mitochondria is present in a glial process (x 24,000).

(C) Clusters of mitochondria (m) are also present in some neurosecretory axons (x 24,000).

(D) Degenerated axons (D) engulfed by glial cells (G) are found in the area distal to the site of ejection (x 24,000).
Herring bodies are present, that are characterized by large numbers of NGVs and vesicles in addition to dense networks of AER (Fig. 2.7A,B). Also three types of NGVs of different size and electron density were observed. While many of the vesicles have the same electron density and diameter as the AER, others are non-granulated and their size approaches that of the NGVs. The profiles of AER within these Herring bodies are numerous; their tubules are not of uniform diameter; some have a blind end, while others are without a membrane delineated ending. The cisternae are dilated and filled with moderately electron-dense material.

At day 8, in the infundibulum, accumulations of NGVs are found in many of the dilated axons, whereas the larger Herring bodies (2-4 um) are filled with NGVs and AER (Fig. 2.9). Some of the large Herring bodies (4 um), in addition to NGVs, are packed with various organelles, the most prominent of which are the numerous profiles of AER (Fig. 2.10). Their cisternae are long and branching, and at higher magnification they are found to be filled with electron dense material (Fig. 2.11). Also present are secondary lysosomes, autophagic vacuoles, dense lamellar bodies and centrally located bundles of neurofilaments (Fig. 2.12). In addition, in the lateral infundibulum, many empty spaces with some organelle remnants framed by glial processes are also present (Fig. 2.8).

At day 15, Herring bodies congested with NGVs are still seen in the infundibular region (Fig. 2.13, Fig. 2.14, Fig. 2.15). However, the networks of AER are no longer as conspicuous (Fig. 2.14, Fig. 2.15) as those seen in Herring bodies in the same area at day 3 and 8.
Fig. 2.7  Three days after the ejection, proximal to the ejection site in the infundibulum.

(A) Large Herring bodies (3 um) containing NGVs, vesicles, neurofilaments and tubules of AER.
The cisternae of the AER are filled with electron dense material (x 36,000).

(B) Other Herring bodies in the same area contain NGVs, vesicles, mitochondria and tubules of AER, and fusion between NGVs with lighter electron density are also seen (arrows) (x 27,000).
Fig. 2.8  Eight days after the ejection of vinblastine, proximal to the ejection site in the lateral infundibulum, the photomontage shows that in addition to many Herring bodies (arrows), empty spaces (T) framed by processes of pituicytes are also present (x 5,000).
Fig. 2.9 Eight days after the ejection of vinblastine, proximal to the ejection site, many dilated neurosecretory axons and Herring bodies are found in mid-sagittal section of the infundibulum containing the hypothalamo-neuro-hypophysial tract. L: lipid inclusion (x 6,900).
Eight days after the ejection of vinblastine, proximal to the ejection site in the infundibulum, giant Herring bodies (4 μm) congested with NGVs, secondary lysosomes (arrows), extensive AER (A) and bundles of neurofilaments (Nf) are present (x 12,000).
Eight days after the ejection of vinblastine proximal to the ejection site in the infundibulum.

(A) Extensive network of AER are present throughout the entire Herring body (x 20,000).

(B) Higher magnification of an area in (A). The tubules of the AER are branching and interconnected (x 40,000).

(C) Long and curved tubules of AER are also seen in Herring bodies together with dense bodies, NGVs and agranular vesicles (x 28,000).
Fig. 2.12 Eight days after the ejection of vinblastine, proximal to the ejection site in the infundibulum.

(A) and (B) A Herring body and a dilated neurosecretory axon contain centrally located bundles of neurofilaments (Nf); other organelles are located at the periphery (x 18,000).

(C) In addition to NGVs and AER, secondary lysosomes and autophagic vacuoles (arrows) are present within Herring bodies (x 27,000).
Fig. 2.13 Fifteen days after the ejection of vinblastine, proximal to the ejection site, the photomontage reveals the presence of many Herring bodies with numerous NGVs as the predominant feature in the lateral infundibulum (x 5,140).
Fig. 2.14 Fifteen days after the ejection of vinblastine, proximal to the ejection site in the infundibulum, the photomontage shows that the large Herring bodies are filled with many NGVs, neurofilaments (Nf), autophagic vacuoles (large arrow), and agranular vesicles (small arrow) (x 15,400).
Fig. 2.15 Fifteen days after the ejection of vinblastine, proximal to the ejection site in the infundibulum.

(A) Photomontage of a longitudinally sectioned neurosecretory axon having three dilatations where NGVs are preferentially located (x 8,590).

(B), (C), and (D) Higher magnification of the dilated portions of the axon in (A), containing NGVs, mitochondria, AER and neurofilaments. Microtubules (arrows) are however, scarce (x 24,000).
Autophagic vacuoles and neurofilaments are still present in some Herring bodies proximal to the ejection site (Fig. 2.14, Fig. 2.16B). In the longitudinal sections of neurosecretory axons with several dilatations, clusters of NGVs are preferentially located in the dilated portions (Fig. 2.14, Fig. 2.15). At this stage, neurosecretory axons of normal morphology (i.e. thin fibers with a few NGVs) are seen in the mid-sagittal sections of the infundibulum (Fig. 2.17). They are also seen transversing the inner zone of the median eminence (Fig. 2.16A,B).

At day 30, both the infundibulum (Fig. 2.18) and the median eminence (Fig. 2.19) have reacquired their normal morphologic characteristics.

**Neural lobe** The neural lobe of control animals contains numerous neurosecretory axons and terminals together with many pituicytes. The NGVs within these axons and terminals are of uniform electron density and range in size from 100-300 nm. The pituicytes contain a relatively large nucleus surrounded by a thin layer of cytoplasm (Fig. 2.20).

At day 1, NGVs of varying electron density and size are present in many of the axons. Fusion between the larger NSVs of less electron density are also seen. In addition, clusters of small electron-lucent vesicles are seen in terminals abutting the perivascular region (Fig. 2.21).

At day 3, axons and terminals with heterogeneous contents are observed. Some contain NGVs of darkened granular matrix, others contain NGVs that have lost part of their limiting membranes and acquired different electron densities, and finally some contain just an amorphous homogeneous inclusion of light electron density (Fig. 2.22). Many of
Fig. 2.16 Fifteen days after the ejection of vinblastine, in the inner zone of median eminence.

(A) Low magnification of an area showing the presence of neurosecretory axons with normal appearance. E: ependymal cell, IR: infundibular recess (x 4,700).

(B) Bundles of neurosecretory axons enclosed by processes of ependymal cells are present. Herring bodies with autophagic vacuoles (arrows) and neurofilaments (Nf) in addition to NGVs may still be found at this stage (x 10,800).
Fig. 2.17 Fifteen days after the ejection of vinblastine, the montage shows that only a few axons are dilated (arrows) and filled with NGVs among many long, parallel neurosecretory axons found in the mid-sagittal section of the infundibulum (x 6,200).
Fig. 2.18 Thirty days after the ejection of vinblastine, in the infundibular tract, many neurosecretory axons that have reacquired normal morphology and glial process (arrows) are found (x 14,400).
Fig. 2.19 Thirty days after the ejection of vinblastine, in the median eminence, neurosecretory axons of normal appearance prevail in this area (x 14,400).
Fig. 2.20 An area of neural lobe of a control animal with numerous neurosecretory axons, terminals and a few pituicytes (P). C; capillary (x 6,000).
Fig. 2.21 Neural lobe, 1 day after the ejection of vinblastine. (A) and (B) Large Herring bodies containing NGVs of varying electron density are present. Axon terminals abutting the perivascular space are characterized by the presence of many electron-lucent vesicles (arrows) (x 12,000).
Fig. 2.22 Neural lobe, 3 days after the ejection of vinblastine. Among normal neurosecretory axons and terminals, many degenerating axons (D) have been engulfed by processes of pituicytes. Autophagic vacuoles (arrows) are also present in some Herring bodies (x 12,500).
these neurosecretory axons have been engulfed by the thin processes of the pituicytes (Fig. 2.23). By day 8, the cytoplasm of the pituicytes is essentially packed with axons and terminals in different stages of degeneration, including polymorphous inclusions of different sizes, multilamellar dense bodies and lipid inclusions in addition to those described above (Fig. 2.24A, B).

At day 15 and 30, neurosecretory axons and terminals in the neural lobe containing NGVs of homogeneous granular content prevail (Fig. 2.25, Fig. 2.26, Fig. 2.27). Only a few pituicytes contain some degenerated axons (Fig. 2.25, Fig. 2.26A) at day 15, while others resume a less extended shape with dense cytoplasm (Fig. 2.26B). At day 30, pituicytes contain extensive networks of rough endoplasmic reticulum (RER) and well developed Golgi apparatus (Fig. 2.27B, Fig. 2.28A,B). Residual bodies, the end product of degenerated axons are also present (Fig. 2.28A).

**Light microscopic autoradiography**

Autoradiographs show heavy labeling in the perivascular regions of the neural lobe of control animals 3 days after the injection of $^{35}$S-cysteine into the preoptic recess (Fig. 1.3A). In contrast, no appreciable labeling can be found in the neural lobes of vinblastine-treated animals. Instead, clusters of labeled materials are present proximal to the site of vinblastine ejection (Fig. 1.3B).
Fig. 2.23 A photomontage of neural lobe 3 days after the ejection of vinblastine.
At the perivascular region, a long process of a pituicyte has engulfed many neurosecretory axons or terminals in different stages of degeneration (D). C: capillary (x 10,800).
Fig. 2.24  Neural lobe, 8 days after the ejection of vinblastine.

(A) The pituicyte is involved in the digestion of the degenerating neurosecretory axons and terminals (D).
P: pituicyte, N: normal axons (x 9,000).

(B) Many multilamellar bodies (L), dense bodies (d) and lipid inclusions (arrows) are degenerated neurosecretory axons and terminals (D) at late stages of digestion by the pituicytes (P) (x 9,000).
Fig. 2.25  Neural lobe 15 days after the ejection of vinblastine.

Neurosecretory axons and terminals of normal appearance are present, while a few degenerated axons (D) may still be found in processes of pituicytes (P) (x 12,000).
Fig. 2.26  Neural lobe 15 days after the ejection of vinblastine.

(A) An area showing the presence of many normal axons and terminals, and also a large residual body (R) within the process of a pituicyte (x 10,800).

(B) Many pituicytes at this stage have resumed normal appearance as shown (x 9,000).
Fig. 2.27  Neural lobe 30 days after the ejection of vinblastine.

(A) The neural lobe at this stage is packed with neurosecretory axons and terminals. One pre-terminal axon (A) contains many microtubules and a few NGVs (x 13,500).

(B) Higher magnification of an area with typical characteristics of neurosecretory axons and terminals. A pituicyte (P) contains within its cytoplasm an extensive network of rough endoplasmic reticulum (x 16,000).
Fig. 2.28 Neural lobe 30 days after the ejection of vinblastine.

(A) Cytoplasm of a pituicyte containing large dense bodies presumably resulting from undigested neurosecretory axons (x 15,000).

(B) A process of pituicyte is in contact with a neighboring pituicyte by a desmosome (arrow). The cytoplasm of both cells contain polyribosomes, rough endoplasmic reticulum, and mitochondria (x 15,200).
DISCUSSION

The results presented in this study demonstrate that a localized, temporary block of the axoplasmic transport in the hypothalamo-neurohypophysial system of the frog has been achieved by microiontophoretic ejection of vinblastine into the median eminence. Consequently, Herring bodies are induced proximal to the ejection site, while the events observed distal to it are compatible with the notion that degeneration and subsequent regeneration of many of the neurosecretory axons take place.

Infundibulum and median eminence

No obvious structural alteration can be detected with light microscopy 24 hours after the ejection of vinblastine, however, observation with the electron microscope reveal many neurosecretory axons filled with paracrystalline structures at the site of ejection. These paracrystalline structures are presumably formed by the binding of vinblastine to the microtubule protein tubulin. Similar paracrystalline lattices have been found in cultured fibroblasts (Bensch and Malawista 1969), rat neural lobe (Rufner et al. 1972), autonomic nerves (Tomlinson et al. 1976), and many other cell types when treated with low concentrations ($10^{-5}-10^{-4}M$) of vinblastine and vincristine (Dustin 1978). Moreover, there is both biochemical (Marantz et al. (1969) and immunological (Nagayama and Dales 1970) evidence for the tubulin nature of these paracrystals. The general consensus is that in any cell containing a pool of tubulin, paracrystalline
structures can be observed after treatment with vinblastine (Dustin 1978). Therefore, it is not surprising that patches of paracrystals are also present in the glial and ependymal cells in the vicinity of these neurosecretory axons.

Three days after the ejection, paracrystalline structures are still present in neurosecretory axons of the median eminence. Either they represent a physical obstruction to axoplasmic transport or vinblastine could have depleted locally all the microtubules and tubulins that are considered to be essential for axonal transport by Schmitt (1968) and Ochs (1971). This alteration of microtubules in the neurosecretory axons of the hypothalamo-neurohypophysial tract (1 day) after the ejection of vinblastine into the median eminence was not observed by Rodriguez et al. (1977) in the rat neurosecretory axons, in which a vinblastine "resistant" type of microtubules is presumed to be present. However, these authors based their conclusions on observations made at 3 days after application of vinblastine into the median eminence, whereas the response of microtubules toward the vinblastine occurs apparently earlier than that.

The vinblastine-induced blockade of the axoplasmic transport is evident 3 days after the ejection, as seen by light and electron microscopy. Proximal to the ejection site, in addition to the accumulation of Gomori-positive material, light microscopic autoradiography, 3 days after the simultaneous injection of $^{35}$S-cysteine into the preoptic recess and ejection of vinblastine into the median eminence, reveals accumulation of labeled material. The neural lobe, on the other hand, is devoid of any label, indicating that the newly synthesized peptide
hormones and neurophysins can no longer be transported to the neural lobe. Proximal to the ejection site, many experimentally induced Herring bodies are present which are characterized by numerous NGVs and an extensive axonal smooth endoplasmic reticulum (AER). The fact that these organelles are the first ones to accumulate proximal to the ejection site is consistent with the findings that both NGVs and AER are transported via the fast anterograde axoplasmic transport (Droz et al. 1975, Grafstein 1977, Mason and Bern 1977).

Eight days after vinblastine application, the number of Herring bodies in the infundibulum is still very pronounced, which suggests that the blockade is still effective. Some striking features are found in the giant Herring bodies. The large size as compared to that of the undilated neurosecretory axons reflects plasticity of the neurosecretory system. The extensive networks of AER and the agranular vesicles of variable sizes are likely to be the source of membrane for the enlarged axolemma (Droz et al. 1975, Grafstein 1977). Similar accumulation of AER agranular vesicles has been reported in the growth cone of growing nerves (Yamada et al. 1971) and in regenerating neurosecretory axons (Rodriguez and Dellmann 1970, Dellmann and Rodriguez 1971, Dellmann 1973). In both cases, AER has been implicated as the source of the new axolemma. In addition, the presence of many autophagic vacuoles and secondary lysosomes, indicative of catabolic activity (Whitaker et al. 1970, Boudier et al. 1979), is similar to that reported in type II Herring bodies of the neural lobe (Dellmann 1973). It thus appears that in these experimentally induced Herring bodies the prolonged storage of NGVs (8 days) leads to their local disposal. This is in agreement
with the hypothesis of Dellmann and Rodriguez (1970) that Herring bodies represent areas where unreleased NGVs are stored and broken down. These Herring bodies should not be considered as undergoing degeneration since they are in continuity with the perikaryon and there is no glial reaction as observed around degenerating neurosecretory axons after stalk transection (Dellmann 1973). These observations further support the concept that the lytic activity exemplifies a normal regulatory mechanism for the turnover of the peptide hormones and neurophysins within these neurons (Dellmann and Rodriguez 1970, Rufner 1973, Picard et al. 1977, Boudier et al. 1979). In the lateral infundibulum, many empty spaces with some organelle remnants framed by glial processes might be tracts once occupied by axons that have degenerated by this time. These tracts may play a role in orienting axonal growth during development or during regeneration as recently proposed for ependymal channels (Gottlieb 1980).

The bundles of neurofilaments seen within these large Herring bodies at 8 days may be organelles that are transported via the slow component, since they were not observed before. This correlates well with the findings that axonal cytoskeleton proteins, comprising the neurofilaments and microtubules, are transported in the slow component of the axoplasmic transport (Lasek and Hoffman 1976). These cytoskeletons are not degraded as they are conveyed within the axons but turn over rapidly upon entering the presynaptic terminals (Lasek and Black 1977). However, microtubules do not accumulate in these Herring bodies. There may be some intracellular regulating factors (e.g. local calcium concentration, covalent modification of tubulin)
that cause the disassembly of microtubules (Haga et al. 1974, Solomon 1976). The significance of the proliferation of neurofilaments at this stage is not clear.

By day 15, Herring bodies are still present in the infundibulum with NGVs as the predominant feature rather than the network of AER. It is possible that the AER has already been disposed of, or transported away, a sign that the axonal transport has probably been resumed between day 8 and 15, since there is a 7 day gap between the selected observation periods in the present study. It is unlikely that the AER moves down as a unit through a densely packed fibrillar framework in the fast component of the axonal transport. Based on the observation that "vesiculotubular structures" are frequently seen in continuation with the AER, Tsukita and Ishikawa (1980) proposed that part of the AER network may be pinched off into vesiculotubular structures and move down the axon to fuse again with AER at any moment.

In the longitudinal sectioned axons, clusters of NGVs are located in the dilated portion of the axons, where neurofilaments are present while microtubules are scarce. Similarities (i.e. the storage of NGVs, the spherical dilated shape and the scarcity of microtubules) shared by axon dilatations and terminals may indicate that the former represent localized areas where disassembly of microtubules occurs in response to a local increase of Ca\(^{++}\) concentration as has been proposed by Lasek and Hoffman (1976) in a model for the selective depolymerization of the axonal cytoskeletons in the axon terminals. These authors postulate that the Ca\(^{++}\) concentrations in the non-growing axon terminals is higher than that in the axon. The elevated level of Ca\(^{++}\)
in the axon terminals may come from AER, mitochondria or the extracellular space and may cause the disassembly of microtubules (Haga et al. 1974). The absence of neurofilaments in the axon terminals may be due to the presence of an endogenous Ca\(^{++}\)-activated protease, which specifically degrades neurofilaments (Gilbert et al. 1975). The presence of neurofilaments but not microtubules in the experimentally induced Herring bodies could be attributed to the local Ca\(^{++}\) concentration, i.e. sufficient to cause microtubules disassembly but not to activate the protease for neurofilaments, since microtubules disassembly in vitro can be brought about by concentrations of Ca\(^{++}\) as low as 1-30 μM (Haga et al. 1974) while the axoplasmic neurofilament protease requires Ca\(^{++}\) at a higher concentration than 0.5 mM for activation in vitro (Pant et al. 1979, Pant and Gainer 1980).

By 30 days after the ejection, both the infundibulum and the median eminence appear normal. The reestablishment of the normal fine structure also implies a functional recovery, that is the resumption of the axoplasmic transport. Thus the newly arrived AGVs and other organelles are considered to have been transported toward the neural lobe. Further autoradiography studies are needed to provide evidence that axoplasmic transport has resumed.

**Neural lobe**

Changes in the neural lobe include the degeneration of some of the neurosecretory axons and terminal distal to the ejection site at day 3 and 8, and the eventual recovery of the fine structure of the entire neural lobe between day 15 and 30 after vinblastine administration.
At day 1, many Herring bodies containing the NGVs with less electron-dense cores than normal are present. These NGVs are larger and many of them are fused. This is the first indication of the beginning of degeneration (Dellmann 1973). This is in agreement with the postulate of Nordmännen et al. (1979) that prior to their destruction and/or lysosomal digestion, NGVs in the Herring bodies are going through an aging process that renders the core osmotically active, they thus become larger and less electron dense than those in the axon terminals. In addition, many axon terminals abutting the perivascular space contain numerous electron-lucent vesicles indicative of release (Douglas et al. 1971, Morris et al. 1978).

The morphological changes in some of the neurosecretory axons and terminals in the neural lobe at days 3 and 8 are further evidence that they undergo degeneration. Autophagy is evident in those axons and terminals that are already engulfed by the processes of the pituicytes. This phagocytic activity of pituicytes has been reported to accompany invariably the degeneration of transected neurosecretory axons (Dellmann 1973). Although degeneration of some fibers due to mechanical damage at the ejection site cannot be ruled out, degenerating fibers are more abundant in vinblastine-treated animals than in control animals. Therefore it is likely that the degeneration may result from a blockade that is long enough to prevent communication via axoplasmic transport between the distant perikaryon and the axon terminal thus simulating conditions in a severed neurosecretory axon. However, not all neurosecretory axons and terminals in the neural lobe degenerate as is the case after transection (Dellmann 1973). Furthermore, light microscopic
autoradiography results (Fig. 1.3A,B) show the neural lobe of vinblastine treated animals to be devoid of any labeled material. This finding indicates that the blockade of axonal transport is complete. The non-degenerating axons in neural lobe at day 3 and 8 thus represent those axons whose axoplasmic transport have also been blocked by vinblastine. Whether the difference in response is due to lower concentration of vinblastine in areas at some distance from the ejection site requires further studies.

By day 15, the phagocytic activity of pituicytes apparently has subsided; pituicytes with engulfed neurosecretory axons and terminals are rarely seen, which is in agreement with the findings of Dellmann (1973). Neurosecretory axons and terminals appear normal. Regeneration in some neurosecretory axons and the re-establishment of axonal transport in others may be taking place, since transected neurosecretory axons have been reported to regenerate at the cut end and neurosecretory neurons are thought to have self-reparative properties (Bern 1967, Dellmann 1973, Mason and Bern 1977). Definitive proof for the regeneration requires a clear demonstration of new fibers growing into the neural lobe between day 8 and 15. On the other hand, it is also possible that some neurosecretory neurons may have been eliminated due to retrograde degeneration. Current studies of the preoptic nucleus cells at the corresponding stages should provide the necessary information.

The fine structure of the neural lobes at 30 days is essentially the same as that of control neural lobes except for a few pituicytes
which contain residual bodies of the degenerated axons and terminals. Whether the recovery of the neural lobe from the effect of vinblastine is completed earlier than 30 days, remains to be determined by observations at shorter post-operative time intervals.

In summary, microiontophoretic ejection of vinblastine into the median eminence effectively blocks the axoplasmic transport in the hypothalamo-neurohypophysial system of the frog. The blockade apparently induces the formation of Herring bodies proximal to the ejection site at day 3, and local disposal of NGVs by lysosomes is seen at day 8. The changes within and the subsequent disappearance of these axon dilatations suggests that the blockade is temporary. The early response (day 3-8) of some of the neurosecretory axons in the distal neural lobe to vinblastine resembles that after axonal transection and indicates that some of the axons degenerate, while others remain apparently intact. Regeneration of the degenerated axons that have been disposed of by the phagocytic activity of the pituicytes is presumed to have taken place at a later stage (day 15-30).


PART III. PREPARATION OF ANTIBODY AGAINST PURIFIED BRAIN TUBULIN AND THE VISUALIZATION OF IN VITRO ASSEMBLED MICROTUBULES BY THE UNLABELED PEROXIDASE ANTI-PEROXIDASE METHOD
INTRODUCTION

Microtubules are ubiquitous cellular organelles present in all eucaryotic cells with the single exception of the anucleated red blood cells of most mammals. They are involved in a wide variety of cell functions, e.g. maintenance of cell shape, motility, secretion, mitosis and axoplasmic transport (for review see Soifer 1975, Snyder and McIntosh 1976, Kirschner 1978, Dustin 1978).

The major structural protein of microtubules is tubulin, a heterodimer composed of two closely related polypeptides, alpha and beta-tubulins (Bryan and Wilson 1971, Feit et al. 1971, Luduena and Woodward 1973, Eipper 1974). The relative mobility of alpha and beta-tubulins in the SDS or SDS-urea gel systems is dependent on the state of the sulfhydryl groups, and the separability of the two subunits is thought to depend on charge rather than size difference (Eipper 1974, Luduena and Woodward 1975). The conservative nature of the tubulins has been demonstrated by partial amino acid sequencing studies (Luduena and Woodward 1973), amino acid composition analysis (Bryan and Wilson 1971, Luduena and Woodward 1973, Eipper 1974, Cleveland et al. 1977a). In addition, antibodies raised against tubulins show a high degree of cross-reactivity with tubulins from various sources (Fulton et al. 1971, Bibring and Baxandall 1971, Dales 1972, Brinkley et al. 1975b).

Much progress has been made on the mechanism of assembly since the first description of a reversible in vitro assembly-disassembly system for microtubules by Weisenberg (1972) and Borisy and Olmsted
(1972). The system allows the identification of three possible mechanisms by which cells may regulate assembly. The first is the efficient removal of Ca$^{++}$, the second, the presence of GTP or ATP, and the third, the requirement for an oligomeric nucleation center (Kirschner 1978). Other aspects including the effect of temperature, sulfhydryl agents, divalent cations, and the role of microtubule-associated proteins (Olmsted 1976, Gaskin and Gethner 1976, Mellon and Rebhun 1976, Sloboda et al. 1976) have also contributed substantially to the understanding of regulating microtubules assembly in vitro.

Nevertheless, many details concerning the distribution of tubulin in vivo, and its possible interaction with other organelles remain to be worked out. In view of the fact that a dynamic equilibrium exists between the soluble tubulins and the assembled microtubules in the cells (Inoue and Sato 1967, Inoue 1976). The assembly-disassembly may be one of the mechanisms by which microtubules exert their functions. We are particularly interested in studying the role of tubulins and microtubules in axoplasmic transport and axonal regeneration, antibody against tubulin should provide a powerful tool for this type of study. There are reports for the preparation of antibody against tubulin, and it has often been used in the studying the mitotic spindle, and the cytoplasmic organization of microtubules in cultured cells by immunofluorescence microscopy (Fuller et al. 1975, Weber et al. 1975, Wiche and Cole 1976, Marchisio et al. 1978, Ecket and Snyder 1978). Although invaluable in presenting an overview of the organization and localization of cytoskeletons in the whole cell, the limited resolution of the light microscope, i.e. 200nm (Weber 1976), allows no direct
identification of individual stained microtubules, nor could accurate localization of tubulin be possible. Therefore, it is necessary to develop an immunocytochemical method which would allow examination at the electron microscopic level. The unlabeled peroxidase-antiperoxidase labeling method of Sternberger et al. (1970) and Sternberger (1973) for the localization of tissue antigens would be the most adequate, since it has been employed at the light microscopic level, and the results correspond well with those obtained by the immunofluorescence in describing the overall organization and localization of microtubules in the cells (De Mey et al. 1976, De Brabander et al. 1977, Karsenti et al. 1978). More recently, Henderson and Weber (1979) have also combined the immunoperoxidase labeling with the stereo-electron microscopy to show the three-dimensional organization of microfilaments and microtubules in detergent-extracted cells.

The present study describes the preparation of antibody against purified tubulin and the visualization of in vitro assembled microtubules by the unlabeled peroxidase-antiperoxidase method.
MATERIALS AND METHODS

Purification of microtubule protein tubulin

Cycles of assembly and disassembly. Porcine brains of about 90-100 grams (wet weight) were used for the purification of tubulin by repeated cycles of assembly and disassembly according to the method of Shelanski et al. (1973), with slight modifications as follows:
1) The brain tissues were first minced with a razor blade in Dis buffer (0.1M Mes, 1 mM EGTA, 1 mM MgSO$_4$, pH 6.4) with a volume ratio of 100 gm. tissue/100 ml. buffer, and then homogenized at 4°C in a Waring blender.
2) The resulting crude fraction was centrifuged in a Sorvall SS-34 rotor at 14,000 rpm for 20 minutes. 3) The supernatant (Sup 0) fraction was further centrifuged in a Beckman rotor 65 at 40,000 rpm for 1 hour at 4°C. 4) The resultant supernatant (Sup I) fraction was diluted 1:1 with RA buffer (0.1M Mes, 1 mM EGTA, 0.1 mM GTP and 8 M glycerol, pH 6.4) and warmed to 37°C for 1-2 hours to allow microtubule assembly (Sup IA). 5) Sup IA was then centrifuged at 40,000 rpm for 1 hour at 25°C, and the resulting pellet (Ppt II) was redissolved in ice-cold Dis buffer with protein concentration adjusted to 15 mg/ml. and stirred at 4°C overnight to allow disassembly of the microtubules. 6) Ppt II was then centrifuged at 37,000 rpm for 30 minutes at 4°C to remove particulates. 7) The supernatant (Sup III) fraction was diluted again 1:1 with RA buffer and allowed another cycle of assembly. 8) The two times assembled microtubules (2xMT) were either used for further purification on a phosphocellulose column or added with an equal
volume of glycerol for storage at \(-20^\circ\text{C}\).

**Phosphocellulose column chromatography** A modified procedure of Weingarten et al. (1975) was used. The 2xMT fraction was dialysed against Mes-EDTA buffer (25 mM Mes, 0.5 mM MgCl\(_2\), 1 mM 2-mercaptoethanol, and 0.1 mM EDTA, pH 6.4), centrifuged at 16,000 rpm at \(4^\circ\text{C}\) for 20 minutes to remove aggregates before loaded onto a phosphocellulose column (K9, with a bed volume of 2.5 ml.), which was equilibrated with at least 4 bed volumes of Mes-EDTA buffer. The flow rate was maintained at 5 ml./hour. After the sample was loaded, the column was then washed with 2 bed volumes of Mes-EDTA buffer before eluting with Mes-EDTA buffer containing 0.8 M NaCl. \(A_{280}\) of each fraction was measured to locate the protein peaks.

**SDS-polyacrylamide gel electrophoresis (SDS-PAGE)**

The method described by Weber and Osborn (1969) or Laemmli (1970) was used to check the purity of the purified tubulin and determine the molecular weight. Separating gel (7.5%), electrophoresis buffer, and sample buffer of the system of Weber and Osborn (1969) were prepared according to Table A.3.1-3.3. Samples were dissolved in sample buffer and heat denatured in presence of 2-mercaptoethanol before loading onto gels. Electrophoresis was carried out at 4 mA/gel, stopped when tracking dye (bromophenol blue) had moved 1 cm from the bottom of the gel. The gels were then removed from the tubes, fixed in 12.5% TCA, stained with 0.25% coomassie B blue R-250 in solution containing 9% acetic acid and 45% methanol for 2 hours and then diffusion-destained in 7% acetic acid. Separating gel (8% or 12.5%), stacking gel,
electrophoresis buffer and sample buffer of the system of Laemmli (1970) were prepared according to Table A.3.4-3.6. Conditions for electrophoresis and the subsequent staining and destaining procedures were carried out the same as described in PART I Materials and Methods.

**Visualization of microtubules assembled in vitro**

**Negative staining preparation**  Purified tubulin fraction or 2xMT were used to allow assembly for 30 minutes. The in vitro assembled microtubules may be visualized by the modified procedures of De Mey et al. (1976). One drop of sample was placed on a nickel grid coated with formvar for 1 minute. The grid was then rinsed with a few drops of 1 M diethyleneglycol and fixed with 2.5% glutaraldehyde in Dis buffer for 1 minute. After rinsing with Dis buffer, the grid was placed on a drop of 0.5% aqueous uranyl acetate for 30 seconds, rinsed with distilled water, drained and dried.

**Thin-sectioned preparation**  Assembled microtubules were pelleted in 65 motor (40,000 rpm, 1 hour) at 25°C, casted in 2% Noble agar, the soft blocks were then fixed in 5% glutaraldehyde in Dis buffer, post-fixed in 1% OsO₄, dehydrated and embedded in Epon-Araldite. Thin sections were prepared and stained with uranyl acetate and lead citrate before examination in the electron microscope (Hitachi U-12A).

**Preparation of antiserum against tubulin**

Antigen for immunization was prepared as follows: PC-tubulin (10 mg/ml.) was cross-linked by an equal volume of 2% glutaraldehyde in Dis buffer at 0°C for 10 minutes (Fuller et al. 1975). The
precipitated tubulin was collected by centrifugation (Beckman, JA-21 rotor, 16,000 rpm, 15 minutes) at 4°C, washed 3 times with 3 ml. of phosphate buffer saline I (PBS-I, 0.01 M phosphate buffer, 0.85% NaCl, pH 7.4) and homogenized in 7 ml. of PBS-I. Complete Freund's adjuvant (7 ml.) was added and the mixture was homogenized. Pre-immune serum was obtained from all rabbits. Each rabbit was given an intradermal injection of 3 mg proteins at multiple sites on the back. Booster injections were given at monthly intervals with approximately 2 mg protein. One week to 10 days after the last injection, the rabbits were bled via cardiac puncture. All sera were heat inactivated at 56°C for 30 minutes, added with 0.01% Na-azide (final concentration), and stored in aliquots at -20°C.

Alternatively, tubulin was separated from the high molecular weight associated proteins present in the 2xMT fraction by SDS-PAGE. From each gel, the stained band corresponding to tubulin was sliced out, pooled and homogenized. Equal volume of incomplete Freund's adjuvant was added and emulsified before immunization (Wiche and Cole 1976).

**Tests for the determination of antigenic specificity**

**Passive hemagglutination test** The method of Garvey et al. (1977) was carried out with modifications as follows: Freshly drawn sheep blood in Alsever's solution (3 ml.) was washed three times with 5 ml. cold PBS-II (0.15 M phosphate buffer, 0.85% NaCl, pH 7.2). The final pelleted sheep red blood cells (SRBC) were suspended in PBS-II. The cell density was standardized by adding distilled water to an
aliquot of cell suspension (9:1, v/v). The optical density of the cell lysate was read at 520 nm. The properly diluted cell suspensions read 0.58-0.60. The washed SRBC (3 ml.) was then added with an equal volume of 0.005% tannic acid in PBS-II, stirred till thoroughly mixed and incubated at 37°C for 10 minutes. The tanned SRBC were then pelleted (500g, 5 minutes), washed for 3 times and resuspended in PBS-II. Tubulin (10 mg/ml, 1 ml) and 4 ml. PBS-II were added to the tanned SRBC and kept at room temperature for 10 minutes. Control cells were prepared by adding either 1 ml. of PBS-II or 1 ml. bovine serum albumin (10 mg/ml.) in place of tubulin. The antigen coated, tanned SRBC were then centrifuged at 500 g for 5 minutes at 4°C, washed and resuspended in 1 ml. of 2% gelatin in PBS-II for use. All sera were absorbed with an equal volume of SRBC to remove non-specific binding. The control pre-immune serum and the antiserum were first diluted 20 fold and serial two-fold dilution of each was then prepared in a microtiter plate. Antigen coated cells or control cells were added to each well, covered and mixed well. Incubation was carried at room temperature for at least 3 hours before reading the result.

**Two-dimensional immunoelectrophoresis** The method of Laurell (1965) was carried out with modifications as follows: Agarose gel matrix was prepared by dissolving 1.2 gm. agarose, 1.2 gm. Noble agar, 70 mg. Na-azide, 70 mg. EDTA and 20 gm. sucrose in 200 ml. 0.05 M barbitol buffer, pH 8.6. Antigen (2xMT) was electrophoresed in the first dimension for 1 hour with 0.1% SDS (final concentration) included in the antigen well. The gel slab was cut into strips containing the separated antigen, transferred to a separate plate where
agarose containing the antiserum was casted. The second electrophoresis was carried out at right angles to the first direction for 3 hours (40 volts). The slabs were then washed in 2% NaCl for 10 hours to remove unreacted serum proteins, dried and stained with 6% amino black in 45% methanol-10% acetic acid.

**Immunocytochemical staining of microtubules**

The unlabeled antibody-enzyme method of Sternberger et al. (1970), modified by De Mey et al. (1976) was used. In vitro assembled microtubules collected on formvar coated nickel or gold grids were rinsed with 1 M diethyleneglycol, fixed with 2.5% glutaraldehyde, rinsed with Dis buffer and put on a drop of normal goat serum (NGS) in Tris-buffered saline (TBS, 0.05 M Tris-NC1, 0.85% NaCl, pH 7.4, 1:30 v/v) for 10 minutes, put on a drop of anti-tubulin antiserum (1:200 or 1:400 v/v in TBS) with 1% NGS for 30 minutes, rinsed with TBS for 20 minutes, put on a drop of goat-anti-rabbit IgG (GAR) (1:20 in TBS for 30 minutes, washed with TBS, put in a drop of peroxidase-anti-peroxidase (PAP) (1:50 in TBS) with 1% NGS for 30 minutes, washed with TBS and reacted with 3.-3' diaminobenzidine-H₂O₂ solution. The working solution of DAB-H₂O₂ was prepared freshly by the method of Moriarty et al. (1973). During the reaction time, the DAB-H₂O₂ solution was kept in the dark and stirring slowing for 2-7 minutes. The grids were then stained with 2% OsO₄ in 0.05 M veronal acetate buffer (pH 7.5) for 10 minutes, rinsed with the same buffer and air-dried before examination in the electron microscope.
RESULTS

Purification of Microtubule Protein Tubulin

Tubulin was purified from procine brain via the repeated cycles of assembly and disassembly. Table 3.1 is the summary of yield for each purification step of a typical run. From 90 grams (wet weight) of porcine brain, 65 mg of 2xMT, representing approximately 2% of the soluble proteins may be recovered. The 2xMT fraction was further purified by passing through a phosphocellulose (PC) column (Fig. 3.1). The tubulin fraction did not adsorb to the column, therefore it was eluted as a large peak (Peak I) in the initial wash fraction. The proteins retained by the column were subsequently eluted as a single peak (II). Fractions in peak I and II were pooled separately and designated as PC-tubulin and MAP (microtubule-associated proteins) respectively.

The purity of PC-tubulin, and the components of the MAP fraction were examined by SDS polyacrylamide gel electrophoresis (Weber and Osborn 1969). Fig. 3.2 shows that the gel loaded with 2xMT contains a major band migrated at the position with a 52,000 molecular weight and some minor bands predominantly in the high molecular weight range. The gel overloaded with PC-tubulin (100 ug) is free of any detectable contaminating proteins. The gel loaded with the MAP fraction still reveals the presence of protein with mobility similar to tubulin and an enrichment of the high molecular weight proteins.
Table 3.1. Purification of Microtubules Protein Tubulin.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol. (ml.)</th>
<th>Protein Concentration a (mg./ml.)</th>
<th>Total Protein (mg.)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude b</td>
<td>200 ml.</td>
<td>-----</td>
<td>-----</td>
<td>100</td>
</tr>
<tr>
<td>Sup 0</td>
<td>68</td>
<td>43.2</td>
<td>2938</td>
<td>77</td>
</tr>
<tr>
<td>Sup I</td>
<td>67</td>
<td>34.4</td>
<td>2305</td>
<td>64</td>
</tr>
<tr>
<td>Sup IA</td>
<td>127</td>
<td>14.8</td>
<td>1880</td>
<td>64</td>
</tr>
<tr>
<td>PPT II</td>
<td>10</td>
<td>20.6</td>
<td>206</td>
<td>7</td>
</tr>
<tr>
<td>Sup III</td>
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<td>10.2</td>
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<td>3</td>
</tr>
<tr>
<td>Sup IIIA</td>
<td>18</td>
<td>5.0</td>
<td>90</td>
<td>3</td>
</tr>
<tr>
<td>PPT IV</td>
<td>3.8</td>
<td>17.0</td>
<td>64.6</td>
<td>2</td>
</tr>
</tbody>
</table>

aProtein concentration was determined by the method of Lowry (1951).

bPorcine brain of wet weight 90 gm was used as starting material.
Fig. 3.1  Separation of tubulin from microtubule-associated proteins (MAP) on a phosphocellulose (PC) column. Two-times polymerized microtubules (2xMT) was resuspended to a final concentration of 15 mg/ml in cold Mes-EDTA buffer, and a total volume of 1 ml was loaded onto a PC column with a bed volume of 2.5 ml, and a flow rate of 5 ml/hr. Peak I contains the unbound tubulin, representing about 78% of the proteins recovered, whereas peak II contains the MAP fraction which may be eluted by Mes-EDTA buffer containing 0.8 M NaCl.
O.BM NaCl

PROTEIN CONCENTRATION (mg/ml)

FRACTION NUMBER

I

II
SDS-polyacrylamide gel electrophoresis (Weber and Osborn 1969) of microtubule protein at different stages of purification: (1) MAP fraction; (2) 2xMT fraction; (3) PC-tubulin; and (4) protein markers of known molecular weights; BSA: bovine serum albumin (M.W. 68,000), OVA: ovalbumin (M.W. 43,000), TRY: trypsin (M.W. 23,000), LYS: lysozyme (M.W. 14,400). The first arrow indicates the area where MAP of higher molecular weights are present, the second arrow points at the position where tubulin is located.
PORCINE BRAIN MICROTUBULE SDS-PAGE

BSA

OVA

TRY

LYS

1 2 3 4
The molecular weight of the PC-tubulin was determined to be about 52,000 (Fig. 3.3). The standard marker proteins of known molecular weights used were bovine serum albumin (BSA: 68,000), ovalbumin (OVA: 43,000), trypsin (TRY: 23,000) and lysozyme (LYS: 14,400). When SDS-PAGE system of Laemmli (1970) was used, the single band of tubulin in the system of Weber and Osborn (1969) resolved into alpha- and beta-tubulins (Fig. 3.4A) with molecular weight of 60,000 and 54,000 respectively (Fig. 3.4B).

The ability of the purified microtubule protein tubulin to assemble in vitro indicative of its biological function was checked by negative staining and thin-section preparations examined in the electron microscope. Under optimum conditions for assembly, the purified PC-tubulin by itself was not able to assemble into microtubules. However, if MAP was added to the PC-tubulin, or 2xMT was used, assembly into microtubules resembling those found in vivo was overwhelming. Fig. 3.5 (A-D) and Fig. 3.6 (A-C) are some representative micrographs of these in vitro assembled microtubules. The average diameter of these microtubules was determined to be about 21 nm, the length could be 490 nm or longer. Side-arms projecting from the wall of the microtubules can be better seen in cross-sectioned material (Fig. 3.6C). In addition to the normal tubules, abnormal assembly forms are seen occasionally. They are composed of protofilaments identical in appearance to those of the microtubules, but appear to be open in a sheet (Fig. 3.6 D-G) rather than rolled into tubules.
Fig. 3.3  Molecular weight of tubulin was derived from the standard curve constructed by plotting the known molecular weights of marker proteins vs. their respective Rf values on a semi-log scale.
BSA: bovine serum albumin, OVA: ovalbumin,
TRY: trypsin, LYS: lysozyme.
Fig. 3.4A Separation of 2xMT by SDS-PAGE system of Laemmili (1970). Alpha (a) and beta (b) tubulins may be resolved.

3.4B Molecular weights of alpha (a) and beta (b) tubulins were determined as described before to be about 60,000 and 54,000 respectively.
Fig. 3.5  Negative staining preparation of the *in vitro* assembled microtubules as seen in the electron microscope.

(A) Low magnification of microtubules obtained after 2xMT fraction in Dis buffer was incubated in presence of 4M glycerol, and 0.1M GTP at 37°C for one hour (x 33,000).

(B) At higher magnification (x 68,000), protofilaments (arrows) may be clearly seen.

(C) Under the same condition as described in (A), essentially identical microtubules may be obtained after MAP fraction was added back to PC-tubulin (x 42,000).

(D) Higher magnification of (C), reveal the presence of protofilaments (arrows). (x 100,000).
Fig. 3.6  Electron micrographs of in vitrō assembled microtubules in thin sections.

(A) Longitudinal section of microtubules (x 40,000).

(B) At higher magnification, side-arm projections (arrows) may be seen along the wall of microtubules (x 70,000).

(C) Transverse section of microtubules, each has a dense wall and a light center, side-arm projections may also be seen (x 70,000).

(D) Sheet-like (arrow) microtubules are occasionally seen in thin sectioned material (x 70,000). Similar structures may also be found in negative staining preparations, (E) x 34,000, (F) x 60,000, and (G) x 60,000.
Preparation of antiserum against tubulin

The specificity of antisera obtained was determined by two methods. Using the passive hemagglutination test, Fig. 3.7 shows an end point in well 2 (titer of 1:40), when 2xMT was used as antigen, an end point in well 5 (titer of 1:320) is shown when PC-tubulin was used as antigen, and an end point in well 1 (titer of 1:20) is shown when MAP was used as antigen. Parallel tests using pre-immune sera gave negative readings for all three antigens tested (Fig. 3.7B). When two-dimensional immunoelectrophoresis was used, a single precipitation line was revealed (Fig. 3.8). The results thus indicate the presence of monospecific antibody against tubulin.

Visualization of in vitro assembled microtubules by the peroxidase-antiperoxidase method

Individual microtubules assembled in vitro and fixed with glutaraldehyde may be stained by the peroxidase-antiperoxidase (PAP) method (Fig. 3.9 A-C). The reaction product is found to deposit along the long and curved microtubules, which appear as heavily stained array of PAP complexes with an average diameter of 53 nm. Short segments of thin tubules are seen occasionally with a diameter of about 21 nm, similar to that of the unlabeled microtubules. These thin tubules are seen more frequently in preparations in which antiserum was diluted 400 fold instead of 200 fold.
Fig. 3.7 Passive hemagglutination test for titer determination of antiserum against tubulin. Immune (A) or pre-immune (B) serum was first diluted 20 fold and added to well 1, 11, and 12. Serial two fold dilutions of the 1:20 serum stock were then made and added into well 2-9. No serum was added to well 10. Tanned SRBC and BSA coated tanned SRBC were added in place of the antigen-coated tanned SRBC in well 11 and 12 respectively in all tests to serve as controls.
Fig. 3.8  Two dimensional immunoelectrophoresis. 2xMT was added to the antigen well (*) and separated in the first dimension then, the second electrophoresis at right angle into the antiserum against tubulin containing gel was carried out. F-3 is the antiserum obtained from a rabbit immunized with SDS-PAGE purified tubulin.
Fig. 3.9  

Microtubules assembled \textit{in vitro} are seen decorated with PAP complex with an average diameter of 53 nm after staining with the unlabeled peroxidase anti-peroxidase method.

(A) \( x \ 94,500 \), arrows indicate stretches of microtubules that are devoid of the PAP complex.

(B) \( x \ 101,500 \).

(C) \( x \ 94,500 \).
DISCUSSION

Since tubulin is present in virtually all eucaryotic cells, and the molecular structure of this protein dimer has been shown to be highly conserved (Snyder and McIntosh 1976, Kirschner 1978), it is thus expected to be a weak immunogen. The preparation of a specific antibody against tubulin would demand that the antigen be free of any contaminating proteins.

Microtubule protein, tubulin, has been partially purified from procine brain using the temperature-dependent cycles of assembly and disassembly originally developed by Shelanski et al. (1973). Further purification to homogeneity as judged by SDS-PAGE was achieved by column chromatography on phosphocellulose (PC) as described by Weingarten et al. (1975). The presence of 2-mercaptoethanol in the PC column buffer was found to be necessary for successful dissociation of the two peaks (Fig. 3.1). The resulted PC-tubulin moves as a single band in the SDS-PAGE system of Weber and Osborn (1969) and has a molecular weight of approximately 52,000. Alternatively it is resolved into two subunits (alpha- and beta-tubulin) with molecular weights of 60,000 and 54,000 respectively in the SDS-PAGE system of Laemmli (1970). These results are consistent with the molecular weights determined for tubulin in comparable gel systems by others (Fine 1971, Bryan 1974, Cleveland et al. 1979). The MAP fraction, in addition to proteins with high molecular weights known to affect both the initiation and elongation
of microtubule assembly in vitro (Dentler et al. 1975, Murphy and Borisy 1975, Bloodgood and Rosenbaum 1976, Sloboda et al. 1976, Kim et al. 1979), contains also proteins with similar electrophoretic mobility as tubulin. It may be that separation of tubulin from the MAP on phosphocellular column was not complete, or it may represent the Tau protein, also reported to have the ability to facilitate microtubule assembly in vitro with molecular weight ranging from 53,000 to 62,000 (Weingarten et al. 1975, Witman et al. 1976, Cleveland et al. 1977b, 1979, Penningroth et al. 1976).

In addition to the criterion of molecular size, the ability of PC-tubulin to assemble into microtubules has also been checked after incubation under conditions favorable for in vitro assembly. Pc-tubulin by itself, is unable to assemble unless the MAP fraction was added, a process essentially the same as to reconstitute the 2xMT fraction. The assembled microtubules and side-arms projecting from the wall of microtubules, presumably formed by the high molecular weight proteins resemble in all aspects those reported in the negative-staining preparation (Witman et al. 1976, Weingarten et al. 1975) or in thin-sectioned materials (Murphy and Borisy 1975, Dentler et al. 1975).

The antibody elicited against the PC-tubulin was found to be monospecific as judged by two-dimensional immunoelectrophoresis (Fig. 3.8). Single specificity is concluded despite of the trailing peak since the two peaks are fused. The trailing is likely to be due to non-specific aggregation of the tubulins during separation in the first dimension, since 0.1% SDS (final concentration) has to be
included in the 2xMT for proteins to migrate out from the antigen well toward the anode. The titers of the different batches of antisera obtained were determined by the passive hemagglutination test. The highest titer ever obtained was 1:5120. In general, they are relatively low, ranging from 1:240 to 1:320. Monospecific, but low titer antisera were also obtained from rabbits immunized with SDS-PAGE purified tubulin. Due to the relative low titers of the antisera, no attempt was made to fractionate the antisera, which were subsequently used to stain the in vitro assembled microtubules by the peroxidase-antiperoxidase (PAP) method.

Microtubules assembled in vitro may indeed be stained by the PAP method using the antisera specific for tubulin (Fig. 3.9). The PAP reaction product decorated microtubules acquire an average diameter of 53-7 nm. Considering the size of the PAP complex, i.e. 20 nm (Sternberger 1973), of the neurotubules, i.e. 20-26 nm (Peters et al. 1970) and of the immunoglobulin molecule, i.e. 10 nm (Valentine and Green 1967), the size of the PAP-microtubules is consistent with the notion that a uniform circumferential labeling of a fiber with double layer antibody should increase its diameter by 30-40 nm, provided that no shrinkage of the microtubules and the double antibody layer due to fixation and labeling conditions has occurred (Henderson and Weber 1979). A larger diameter of 100 nm has been reported for the PAP-labeled microtubules by De Mey et al. (1976). However, the antibody used was elicited against the 2xMT fraction, which is known to contain microtubule-associated proteins. Hence it is not clear as to the specificity of
their antisera. If antibody against these MAPs is also present in the antisera, the staining of the side-arm projections should considerably increase the size of the labeled fibers. The establishment of the dimensions of peroxidase-labeled microtubules and tubulins apparently requires more rigorous studies under standardized conditions for both the sectioned material and the negative staining preparation before it is applicable to quantitative morphometric study. Whereas with the specific antibody against tubulin available, the PAP method should have the adequate resolving power to localize tubulin not only in the microtubules, but also in other cellular configurations that one has not been able to demonstrate yet. Work is currently underway to apply this method for histological, and thin-sectioned materials, and the distribution of tubulin and microtubules in neurosecretory axons with blocked axoplasmic transport will be studied.
LITERATURE CITED


SUMMARY AND CONCLUSIONS

Biosynthesis, axonal transport and storage of neurophysin in the amphibian (Rana pipiens) magnocellular peptidergic neurosecretory system was studied, and the results were compared with the same process reported in mammals.

Evidence provided by light microscopic autoradiography indicates that neurons of the preoptic nucleus (PON) are capable of synthesizing cysteine-rich proteins. The time course of appearance of these $^{35}$S-cysteine labeled proteins in different regions of the hypothalamo-neurohypophysial system was studied by slab gel autoradiograph. $^{35}$S-cysteine labeled proteins extracted from the preoptic nucleus, the infundibulum and the neural at 1, 2, 4, 6 and 12 hours, and 1, 3 and 5 days were separated by basic polyacrylamide gel electrophoresis. The resulted autoradiograms reveal the presence of $^{35}$S-cysteine labeled proteins in the PON from 1 hour post-injection on. However, in the infundibulum and neural lobe, a major labeled protein Np band first appeared 4 hours after the injection. This labeled Np band corresponds to the only major coomassie blue-stained band found in the neural lobe. After 1 day, labeled proteins were not observed in the PON and infundibulum, whereas in the neural lobe, the labeled Np band persisted throughout the entire observation period of 5 days. In view of the fact that this Np protein is the only major protein present in the neural lobe, rich in cysteine, and synthesized by neurons of the PON rather than pituicytes of the neural lobe, it is
tentatively identified as the neurophysin protein of *Rana pipiens*. The minimum rate of transport for neurophysin is then calculated as 0.9 mm/hr (22 mm/day), which is similar to that reported for the goldfish (1 mm/hr) (Jones et al. 1973) and the rat (1 mm/hr) (Pickering and Jones 1971, Nörstrom and Sjöstrand 1971, Jones and Pickering 1972, Burford and Pickering 1973). The results in the present study thus support the conclusion by Jones et al. (1973) that the axoplasmic transport rate in the magnocellular neurosecretory system of poikilotherms is similar to that of homeotherms.

The number of species of neurophysin in the frog neural lobe was determined by injecting $^{35}$S-cysteine into the preoptic recess. Three days after the injection, proteins extracted with 0.1N HCl and precipitated with 10% TCA were analyzed by isoelectric focusing in polyacrylamide gel (IEF-PAGE). Two peaks of radioactivity with pI's of 4.9±0.1 and 4.6±0.1 were resolved with molecular weights of 23,000 (Np I) and 20,100 (Np II) as determined by SDS-PAGE (12.5%). These results indicate there may be two separate neurophysins for Arg-vasotocin and mesotocin. Further work is needed for the identification of the respective peptide hormones with which these two neurophysins are associated in vivo.

Also, two peaks with pI's of 5.8±0.1 and 5.2±0.1 in addition to the neurophysin peaks found in the neural lobe were observed by IEF-PAGE of radioactive proteins extracted from the preoptic nucleus and infundibulum 30 minutes and 1 hours after the injection on $^{35}$S-cysteine. The pI=5.2 peak gradually decreased in radioactivity as the two neurophysin peaks increased as a function of time, thus suggesting a
putative precursor role for the pI 5.2 protein species. An unambiguous proof for the identification of this protein as precursor for neurophysins requires the demonstration that the pI=5.2 protein is larger in molecular weight then the two neurophysins and that sequence homology exists between the putative precursor and product.

Axoplasmic transport in the hypothalamo-neurophysial system of the frog has been effectively blocked by microiophoretic ejection of vinblastine into the median eminence. The blockade apparently induces the formation of Herring bodies, i.e. axon dilatations, since accumulation of Gomori-positive material proximal to the ejection site is readily detectable at the light microscopic level 3 days after the ejection. The sequence of fine structural changes in areas adjacent to the ejection site in the infundibulum and in the distal neural lobe were studied at days 1, 3, 8, 15 and 30 days after the ejection.

At the electron microscopic level, paracrystalline structures presumably formed by the binding of vinblastine to tubulin, the subunit protein of microtubules, fill many of the neurosecretory axons in the median eminence at day 1. These paracrystals apparently have caused neurosecretory granulated vesicles (NGVs) to accumulate proximal to them. At day 3, the axon dilatations in the infundibulum contain an increased number of NGVs, an extensive smooth endoplasmic reticulum and neurofilaments. In addition, autophagic vacuoles and secondary lysosomes indicative of catabolic activity become pronounce in Herring bodies at day 8. By day 15, the extensive smooth endoplasmic reticulum and bundles of neurofilaments are no longer present, whereas NGVs and lysosomal activity to a less extent are still present. Both infundibulum
and median eminence appear normal by day 30. The above morphological observations suggest that the blockade is temporary. Changes at the corresponding stages in the neural lobe include the degeneration of some of the neurosecretory fibers and terminals distal to the ejection site at day 3 and 8, and the eventual recovery of the fine structures of the entire neural lobe between 15 and 30 days. The degeneration may be the result of the blockade that is long enough to prevent communication via axoplasmic transport between the distant perikaryon and terminal, thus simulating conditions in a transected neurosecretory axon. The fact that not all axons and terminals have degenerated and the results of the light microscopic autoradiography suggest that the blockade caused by vinblastine can be achieved without necessarily causing degeneration.

The recuperation of the fine structure both in the hypothalamic-neurohypophysial tract and in the neural lobe implies a functional recovery of the system, that is the resumption of axoplasmic transport. However, further work is required to demonstrate that 15 days after the vinblastine treatment, newly synthesized neurosecretory material, i.e. peptide hormones and neurophysins within the NGVs may arrive in the neural lobe and that new neurosecretory axons are growing into the neural lobe between day 8 and 15.

The microtubule protein tubulin was purified to apparent homogeneity from porcine brain by temperature dependent cycles of assembly-disassembly and ion-exchange column chromatography on phosphocellulose (PC). The purity of the PC-tubulin was checked by SDS-PAGE (Weber and Osborn 1969) to be free of high molecular weight
microtubule-associated proteins (MAP) and the molecular weight of PC-tubulin was determined to be about 52,000. The single band of PC-tubulin in the SDS-PAGE system of Weber and Osborn (1969) may be resolved into alpha and beta tubulins by the system of Laemmli (1970) with molecular weights of about 60,000 and 54,000 respectively. PC-tubulin was unable to assemble into microtubules under conditions where in vitro assembly from 2xMT is readily accomplished. However, the addition of the MAP fraction to the PC-tubulin greatly facilitate in vitro assembly of the latter into microtubules with an average diameter of 21 nm.

Glutaraldehyde cross-linked PC-tubulin or SDS-PAGE purified tubulin was used as antigen to elicit antibody against tubulin in rabbits. As expected antisera with low titers were obtained. The results of two-dimensional immunoelectrophoresis indicate the antibody to be monospecific against tubulin. Hence antisera against tubulin were used to stain the in vitro assembled microtubules by the unlabeled peroxidase-antiperoxidase (PAP) method of Sternberger (1973). The PAP complex-decorated microtubules attain an average diameter of 53 nm, which may be accounted for by the size of the PAP complex (20 nm), and the double-layer antibody (20 nm). This method has the advantage over the widely employed immunofluorescence technique to be applicable to glutaraldehyde-fixed tissue sections both at the light and electron microscopic level. It will thus permit the study of the distribution within neurons of microtubules or tubulin-containing
structures, the localization of soluble tubulins and the interaction of both with other organelles. This information will be of great value in understanding the role of tubulin and microtubules in axonal transport and axonal regeneration.
LITERATURE CITED


"Teach me Thy way, O Lord; I will walk in Thy truth;  
Unite my heart to fear Thy name.  
I will give thanks to Thee, O Lord my God, with all my heart,  
And will glorify Thy name forever.  
For Thy lovingkindness toward me is great,  
And Thou hast delivered my soul from the depth of Sheol." Amen!  


I thank my Lord Jesus for His saving grace, love and provisions  
that have made my stay in Ames the most fulfilled years of my life.

My sincere gratitude is due to Dr. Dieter Dellmann for his guidance  
and support during the course of this study and the preparation of this  
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I also wish to thank Dr. J. Viles and Dr. J. A. Thomas for serving  
on the graduate committee.

Special appreciation is extended to Mrs. Susan Jennifer Woroch Stahl  
for being such a wonderful friend and a great help in times of need,  
especially for her excellent photographic assistance.
Finally, I dedicate this and my love to my parents, family members and especially to my husband, who shared many burdens as well as joys of mine both at home and in school. It is with his love and understanding that I am able to persist and complete this work.
<table>
<thead>
<tr>
<th>Stock A (pH 8.8-9.0)</th>
<th>Stock D</th>
</tr>
</thead>
<tbody>
<tr>
<td>IN HCl</td>
<td>48 ml.</td>
</tr>
<tr>
<td>Tris</td>
<td>36.3 gm.</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.23 ml.</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>to 100.0 ml.</td>
</tr>
<tr>
<td>Stock B (pH 6.6-6.8)</td>
<td>Stock E</td>
</tr>
<tr>
<td>IN HCl</td>
<td>48 ml.</td>
</tr>
<tr>
<td>Tris</td>
<td>5.98 gm.</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.46 ml.</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>to 100.0 ml.</td>
</tr>
<tr>
<td>Stock C</td>
<td>H$_2$O</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>28 gm.</td>
</tr>
<tr>
<td>Bis</td>
<td>0.735 gm.</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>to 100.0 ml.</td>
</tr>
<tr>
<td>Stock C'</td>
<td></td>
</tr>
<tr>
<td>Acrylamide</td>
<td>60 gm.</td>
</tr>
<tr>
<td>Bis</td>
<td>0.4 gm.</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>to 100.0 ml.</td>
</tr>
</tbody>
</table>

To prepare:


b) 10% gel, mix stock A, C, H$_2$O and G in volume ration of 1:1.3:1.7:4.

c) Stacking gel, mix stocks B, D, E, and F in volume ratio of 1:2:1:4, and expose to fluorescent light for 10-15 minutes.
Table A.1.2 Preparation of 10% and 12.5% SDS-Polyacrylamide gels

<table>
<thead>
<tr>
<th>Stock solutions</th>
<th>Final conc. of separating gel</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10%</td>
<td>12.5%</td>
<td>Stacking gel</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>10.0 ml</td>
<td>12.5 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>1% bis-acrylamide</td>
<td>3.9 ml</td>
<td>3.1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>1.5M Tris-HCl (pH 8.7)</td>
<td>7.5 ml</td>
<td>7.5 ml</td>
<td>--</td>
</tr>
<tr>
<td>0.5M Tris-HCl (pH 6.8)</td>
<td>--</td>
<td>--</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>distilled water</td>
<td>8.2 ml</td>
<td>6.5 ml</td>
<td>5.35 ml</td>
</tr>
<tr>
<td>10% Ammonium sulfate</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.3 ml</td>
<td>0.3 ml</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>TEMED (1:1)</td>
<td>0.02 ml</td>
<td>0.02 ml</td>
<td>0.01 ml</td>
</tr>
</tbody>
</table>

Total volume

30.0 ml  30.0 ml  10.0 ml

\(^a\)This reagent should be prepared freshly before use.
Table A.1.3 Preparation of isoelectric focusing in polyacrylamide gels

<table>
<thead>
<tr>
<th>Stock solutions</th>
<th>Vol. (ml.)</th>
<th>Vol. (ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(pH 3.5-10)</td>
<td>(pH 4-6)</td>
</tr>
<tr>
<td>Monomer stock</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>6.25</td>
<td>6.25</td>
</tr>
<tr>
<td>0.8% Bis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50% Glycerol</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>8.75</td>
<td>8.75</td>
</tr>
<tr>
<td>40% Ampholine (LKB)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.5-10.0</td>
<td>1.25</td>
<td>0.5</td>
</tr>
<tr>
<td>4-6</td>
<td>--</td>
<td>0.75</td>
</tr>
<tr>
<td>0.2% Riboflavin</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>5.75</td>
<td>5.75</td>
</tr>
<tr>
<td>Total volume</td>
<td>25 ml</td>
<td>25 ml</td>
</tr>
</tbody>
</table>

Table A.1.4 Preparation of Epon-Araldite embedding medium

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Weight (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDSA</td>
<td>15.85</td>
</tr>
<tr>
<td>Araldite</td>
<td>1.4</td>
</tr>
<tr>
<td>Epon 812</td>
<td>9.85</td>
</tr>
<tr>
<td>DMP-30</td>
<td>0.65</td>
</tr>
</tbody>
</table>
### Table A.3.1 Preparation of 7.5% SDS-polyacrylamide gels (Weber and Osborn 1969)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Monomer stock</strong></td>
<td></td>
</tr>
<tr>
<td>16.59% Acrylamide-0.446% Bis</td>
<td>13.5</td>
</tr>
<tr>
<td>Gel buffer(^a) (1x)</td>
<td>10.5</td>
</tr>
<tr>
<td>0.14% Ammonium persulfate</td>
<td>1.5</td>
</tr>
<tr>
<td>1% TEMED in gel buffer</td>
<td>4.5</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td>30.0 ml</td>
</tr>
<tr>
<td>(^a)See Table A.3.2</td>
<td></td>
</tr>
</tbody>
</table>

### Table A.3.2 Preparation of electrophoresis gel buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Weight (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{Na}_2\text{HPO}_4\cdot\text{H}_2\text{O})</td>
<td>3.9</td>
</tr>
<tr>
<td>(\text{Na}_2\text{HPO}_4)</td>
<td>10.22</td>
</tr>
<tr>
<td>SDS</td>
<td>1.0</td>
</tr>
<tr>
<td>(\text{H}_2\text{O}) to 1000 ml</td>
<td></td>
</tr>
</tbody>
</table>

### Table A.3.3 Preparation of sample buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01 M Phosphate buffer (pH 7.0)</td>
<td>89 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>10 ml</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>1 ml</td>
</tr>
</tbody>
</table>
Table A.3.4 Preparation of 8% SDS-polyacrylamide gels (Laemmli 1970)

<table>
<thead>
<tr>
<th>Reagent stock</th>
<th>Separating Vol. (ml)</th>
<th>Stacking Vol. (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acrylamide-0.8% Bis</td>
<td>5.3</td>
<td>0.5</td>
</tr>
<tr>
<td>1M Tris-Cl (pH 8.8)</td>
<td>7.5</td>
<td>---</td>
</tr>
<tr>
<td>1M Tris-Cl (pH 6.8)</td>
<td>---</td>
<td>0.625</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.2</td>
<td>0.05</td>
</tr>
<tr>
<td>5% TEMED</td>
<td>0.1</td>
<td>0.025</td>
</tr>
<tr>
<td>2.5% Ammonium persulfate&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.2</td>
<td>0.05</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>6.7</td>
<td>3.75</td>
</tr>
</tbody>
</table>

Total volume: 20 ml, 5 ml

<sup>a</sup>Prepare freshly before use.

Table A.3.5 Preparation of 5x<sup>a</sup> electrophoresis gel buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Weight (gm)</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-Cl (pH 8.3)</td>
<td>15.15</td>
<td>0.125 M</td>
</tr>
<tr>
<td>Glycine</td>
<td>72.0</td>
<td>0.96 M</td>
</tr>
<tr>
<td>SDS</td>
<td>5.0</td>
<td>0.5%</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>to 1000.0 ml</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Dilute 5x before use.

Table A.3.6 Preparation of sample buffer

<table>
<thead>
<tr>
<th>Reagent stock</th>
<th>Volume (ml)</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Tris-Cl (pH 6.8)</td>
<td>1.25</td>
<td>0.0625 M</td>
</tr>
<tr>
<td>10% SDS</td>
<td>4.0</td>
<td>2%</td>
</tr>
<tr>
<td>110% 2-mercaptoethanol</td>
<td>0.91</td>
<td>5%</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>13.84</td>
<td>--</td>
</tr>
</tbody>
</table>

Total volume: 20.0 ml