Isolation and Protein Composition of Membranes of Neurosecretory Vesicles and Plasma Membranes from the Neural Lobe of the Bovine Pituitary Gland

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1. Homogenates of neural lobes of bovine pituitary glands were fractionated by differential ultracentrifugation. 2. Neurosecretory vesicles were isolated by sucrose-gradient ultracentrifugation and membranes were obtained after hypo-osmotic lysis of the particles. 3. A method is described for the isolation of a preparation of purified neuronal plasma membranes by using a fraction enriched in nerve endings as a starting material. 4. The purity of the subcellular fractions was estimated by enzyme assays and by examination with the electron microscope. 5. On the basis of the results it was estimated that neuronal plasma membranes constitute more than 30% of the protein of the nerve endings and neurosecretory vesicles more than 45% of the total amount of protein in the homogenate. 6. The proteins of membranes of neurosecretory vesicles and of plasma membranes were solubilized by means of sodium dodecyl sulphate. Polyacrylamide-gel electrophoresis of such preparations showed that both membranes contained a large number of proteins, including three glycoproteins.

In the neural lobe of the pituitary gland the hormones vasopressin and oxytocin are stored in vesicles limited by a trilaminar membrane (Weinstein *et al.*, 1961; Lederis, 1965; Bargmann, 1968). Besides hormones the vesicles contain soluble proteins, the neurophysins (Hollenberg & Hope, 1968; Dean & Hope, 1968) and ATP (Poisner & Douglas, 1968). So far no other soluble components have been demonstrated in the vesicles.

It is believed that secretion of hormone from the neural lobe occurs by exocytosis. The final stage of this process involves the fusion of the membrane of the neurosecretory vesicles with the plasma membrane. Electron-microscopic studies of the neural lobe of the hamster and rat have revealed pictures compatible with exocytosis (Douglas *et al.*, 1971; Santolaya *et al.*, 1972) and biochemically it has been demonstrated that the hormones are released from the neural lobe together with the neurophysins (Cheng & Friesen, 1970; Uttenthal *et al.*, 1971; Nordmann *et al.*, 1971; Vilhardt *et al.*, 1975) and ATP (Hong & Poisner, 1974). To elucidate the molecular mechanism involved in the membrane fusion a knowledge of the chemical composition of the two interacting membranes is necessary.

In recent years a series of investigations have been published describing the isolation of plasma membranes of nerve cells from the central nervous system (Morgan *et al.*, 1971; Cotman & Matthews, 1971; Henn *et al.*, 1972; Gurd *et al.*, 1974; Jones & Matus,

1974). The method usually adopted in these studies has been to isolate a preparation of synaptosomes from brain homogenates as described by Gray & Whittaker (1960) and by Arnaiz *et al.* (1967). After disruption of the synaptosomes, plasma-membrane fragments are isolated by density-gradient centrifugation. One of the disadvantages of this method is that the synaptosomal fraction may be contaminated with glial elements (see Morgan *et al.*, 1971). In addition it is difficult to estimate the contamination of the purified plasma membranes with membranes derived from synaptic vesicles. In most studies homogenates of whole brain have been used as starting material and the plasma membranes isolated have consequently originated from cells containing different transmitter substances and serving different functions. The use of the neural lobe of the posterior pituitary gland as starting material for isolation of plasma membranes of neuronal origin seems advantageous for several reasons. 1. The organ is less complex than the brain, containing mainly nerve endings and no nerve-cell bodies. The vast majority of the nerves are axons of the hypothalamo-neurohypophyseal tract engaged in transport and secretion of either vasopressin or oxytocin. 2. The buoyant density of the nerve endings is rather high (presumably owing to their content of neurosecretory vesicles), offering a possibility of separating them from less-dense pituitary elements. 3. Contamination of the plasma-membrane preparation by neurosecretory vesicles

seems unavoidable, but the extent of the contamination can be estimated by using vasopressin as a specific marker for the vesicles.

In the present work we describe a method for the isolation of a preparation of purified neuronal plasma membranes. The procedure involved is basically similar to that used in isolation of plasma membranes from brain tissue, i.e. lysis of a preparation of isolated nerve endings followed by fractionation of the lysate by density-gradient centrifugation. The protein composition of the preparation is studied by polyacrylamide-gel electrophoresis and compared with that of membranes from isolated neurosecretory vesicles.

Materials and Methods

Biological material

Pituitary glands were removed from adult cattle of both sexes within 15 min of the killing of the animals by shooting. The glands were transported to the laboratory in an ice-bucket. The neural lobes were freed of anterior- and intermediate-lobe tissue as described by Vilhardt & Hope (1974) and were placed in ice-cold 0.3 M-sucrose. Usually 12 glands were prepared at a time; the yield of neural-lobe tissue was 2–3 g wet wt.

Homogenization and differential centrifugation

The neural lobes (usually six at a time) were placed on an ice-cooled Perspex board and chopped to a fine mince with a stainless-steel knife. The mince was then homogenized in 12 ml of 0.3 M-sucrose in a Teflon-to-glass homogenizer with a radial clearance of approx. 0.17 mm (for details see Vilhardt & Hope, 1974).

The homogenate was then centrifuged by a modification of the method described by Vilhardt & Hope (1974). Fraction (I) was sedimented at 800 g_{av} for 15 min, fraction (II) at 3000 g_{av} for 15 min, fraction (III) at 5000 g_{av} for 15 min and fraction (IV) at 26000 g_{av} for 15 min. The final supernatant contained most of the microsomal material. All operations took place at 4°C in a MSE Mistral 6L refrigerated centrifuge (for fraction I) and an A40 and an FA65 rotor in a Spinco model L ultracentrifuge (for other fractions). The sediments were resuspended in 2 ml of 0.3 M-sucrose.

Preparation of membranes of neurosecretory vesicles

Portions (0.8 ml) of fraction (IV) were placed on discontinuous density gradients made up of 1 ml of 2 M-, 1 ml of 1.4 M-, 1 ml of 1.35 M- and 1 ml of 1.3 M-sucrose. The gradients had been stored for 18 h at 4°C before

use. Centrifugation was performed in a Spinco model L-2 65B ultracentrifuge in an SW 50.1 rotor at 110000 g_{av} for 60 min. Particles accumulating at the 1.4 M-/2 M-sucrose interface were recovered by cutting the gradient in a Schuster centrifuge-tube cutter. This fraction, consisting of highly purified neurosecretory vesicles, was diluted to 13.5 ml with 0.2 M-sucrose and sedimented at 100000 g_{av} for 45 min. The sediment was resuspended in 4 ml of 5 mM-Tris-acetic acid buffer, pH 7.5, and frozen for 60 min. After thawing, the suspension was sedimented, and the freezing and thawing procedure was repeated four more times. In some experiments the lysis of the vesicles was performed by resuspending the purified vesicles in 13.5 ml of 5 mM-Tris-HCl buffer at pH 8.4 and 0°C for 120 min.

Preparation of purified plasma membranes

The method outlined for isolation of plasma membranes is outlined in Scheme 1. Fraction (II) obtained by differential centrifugation was diluted with 13.5 ml of 0.3 M-sucrose and re-centrifuged at 3000 g_{av} for 15 min. The washed pellet, consisting mainly of nerve endings (R. V. Baker, H. Vilhardt & D. B. Hope, unpublished work) was resuspended in 0.3 M-sucrose (fraction II1) and placed on top of 2 ml of 25% (w/v) Ficoll in 0.3 M-sucrose. After centrifugation in an SW 50.1 rotor at 60000 g_{av} for 60 min the material accumulating on top of the Ficoll solution was recovered and diluted with 0.3 M-sucrose to 3.5 ml (fraction II2). Fraction (II2) was then placed on top of a solution of 8% (w/v) Ficoll in 0.3 M-sucrose and centrifuged in the SW 50.1 rotor at 110000 g_{av} for 30 min. The pellet at the bottom of the tube was resuspended in 0.3 M-sucrose (fraction II5) and centrifuged in the A40 rotor at 6500 g_{av} for 20 min. The sediment, consisting of purified nerve terminals, was resuspended in 13.5 ml of 5 mM-Tris-HCl buffer, pH 8.4, and left in the cold for 120 min to lyse the nerve endings (fraction II6). The lysate was centrifuged in an FA65 rotor at 100000 g_{av} for 45 min and the sediment was resuspended in 1.5 ml of 0.3 M-sucrose (fraction II7). The suspension was placed on top of a freshly prepared discontinuous sucrose density gradient of 1 ml of 1.2 M-, 1 ml of 1.0 M-, 1 ml of 0.95 M- and 1 ml of 0.6 M-sucrose and centrifuged in the SW 50.1 rotor at 60000 g_{av} for 90 min. The material accumulating at the 0.6 M-/0.95 M-sucrose interface was recovered by cutting the gradient (fraction II9). The fraction was diluted to 13.5 ml with water and sedimented in an FA65 rotor at 100000 g_{av} for 30 min. The pellet (fraction II12) was resuspended in 1 ml of water. This fraction consisted of purified plasma membranes. The time from the killing of the animals to the recovery of fraction (II12) was approx. 10 h.

Scheme 1. Fractionation procedure for the preparation of plasma membranes from nerve endings of bovine neural lobes

Analytical procedures

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Succinate dehydrogenase (succinate-tetrazolium oxidoreductase, EC 1.3.99.1) was measured by the method of Pennington (1961) as modified by Porteous & Clark (1965).

Monoamine oxidase [monoamine-oxygen oxidoreductase (deaminating), EC 1.4.3.4] was determined by the method described by Wurtman & Axelrod (1963), with [¹⁴C]tryptamine (New England Nuclear GmbH, Frankfurt am Main, Germany) as a substrate.

Lactate dehydrogenase (L-lactate-NAD⁺ oxidoreductase, EC 1.1.1.27) was assayed by following the

oxidation of NADH at 340 nm (Wróblewski & LaDue, 1955) as modified by Pickup & Hope (1971).

5'-Nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) was determined by the method of Ipata (1967) or by the method of Emmelot *et al.* (1964). P_i was determined by the method of Fiske & SubbaRow (1925).

Mg²⁺ + Na⁺ + K⁺-dependent ATPase* (ATP phosphohydrolase, EC 3.6.1.3) was measured as the difference in formation of P_i with and without 1 mM-ouabain present in the medium (Fiske & SubbaRow, 1925; Vilhardt & Hope, 1974).

Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) and non-specific cholinesterase (acetylcholine acyl-hydrolase, EC 3.1.1.8) activity were

* Abbreviation: ATPase, adenosine triphosphatase.

determined by the method described by Ellman *et al.* (1961) with acetylthiocholine as a substrate. Compound BW 284c51 [1,5-bis-(4-allyl dimethylammoniumphenyl)pentan-3-one di-iodide; final concentration 1.5 μ M; Burroughs Wellcome, Temple Hill, Dartford, Kent, U.K.] was used as inhibitor of the specific acetylcholinesterase and eserine sulphate (Sigma Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.; final concentration 10 μ M) as general inhibitor of cholinesterase activity.

Glucose 6-phosphatase (D-glucose 6-phosphate phosphohydrolase, EC 3.1.3.9) was measured by the method of de Duve *et al.* (1955), with an incubation period of 60 min.

RNA was measured by the method of Schmidt & Tannhauser (1945) as modified by Pickup & Hope (1971).

DNA was measured fluorimetrically after lipid extraction of the samples (Kissane & Robins, 1958). 3,5-Diaminobenzoic acid dihydrochloride (Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A.) at a concentration of 0.6 M was used to hydrolyse DNA and to form a fluorescent product with deoxyribose. Excitation took place at 405 nm and emission at 520 nm.

Acid ribonuclease [ribonuclease pyrimidine-nucleotide-2'-transferase (cyclizing), EC 3.1.4.22] was determined by the method of Roth & Milstein (1952) as modified by Smith & Winkler (1966), with ribonucleic acid from yeast (BDH Chemicals Ltd., Poole, Dorset, U.K.) as substrate.

Rotenone-insensitive NADH-cytochrome *c* oxidoreductase (EC 1.6.99.3) was assayed by the method of Duncan & Mackler (1966) by following the increase in absorbance at 550 nm; 10 μ l of 50% (w/v) rotenone in ethanol was added to the medium.

Rotenone-insensitive NADPH-cytochrome *c* oxidoreductase (EC 1.6.99.1) was measured by the method of Lu *et al.* (1969) by following the reduction of cytochrome *c* at 550 nm.

Phospholipid was determined as total P by the method described by Bartlett (1959).

Biological assays

Vasopressin was extracted by placing the acidified [acetic acid, final concn. 0.25%] samples in a boiling-water bath for 5 min. After centrifugation the supernatants were neutralized by NaOH and assayed for pressor activity by the procedure of Dekanski (1952) with the modifications described by Dean & Hope (1967). Standards of synthetic [8-arginine]vasopressin (Studer, 1963) were standardized against the Third International Standard (Bangham & Mussett, 1958).

The analytical results are expressed as relative specific activities, i.e. the specific activity of the fraction analysed divided by the specific activity of the homogenate.

Electron microscopy

The tissue (neural lobe) to be fixed was cut into small cubes (approx. 1 mm³) and placed in cooled fixative [1% (v/v) formaldehyde – 4% (v/v) glutaraldehyde – 100 mM-sodium phosphate, pH 7.2]. The fractions to be examined were sedimented by ultracentrifugation (100 000 *g*_{av.}, 60 min). The pellets were fixed for 2 h in 2% (v/v) glutaraldehyde – 2% (v/v) formaldehyde – 100 mM-sodium phosphate buffer, pH 7.4. After post-fixation with 2% OsO₄ the blocks were dehydrated through graded concentrations of ethanol and embedded in a mixture of Epon and Araldite. Sections were cut with a glass knife and stained with uranyl acetate and lead citrate. The stained sections were examined with a Siemens 1A electron microscope.

Polyacrylamide-gel electrophoresis

Plasma membranes (fraction II12) and membranes of neurosecretory vesicles were resuspended in 500 μ l of 1% (w/v) sodium dodecyl sulphate in 10 mM-sodium phosphate buffer (pH 6.5) and left for 3 h at room temperature (20°C) followed by 18 h at 4°C. The samples were then briefly heated under hot tap-water and centrifuged at 110 000 *g*_{av.} for 45 min at room temperature in a SW 50.1 rotor with adaptors for small volumes. The sediment constituted less than 3% of the total amount of protein in the samples. The supernatants were recovered and sucrose was added to a concentration of 8% (w/v).

The gels were made up by a modification of the method described by Weber & Osborn (1969). Glass tubes (7.5 cm $\times$ 0.5 cm internal diam.) were filled with 1.3 ml of 6% (w/v) Cyanogum 41 (95% acrylamide monomer and 5% *NN'*-methylenebisacrylamide; BDH Chemicals Ltd.) in 0.1 M-sodium phosphate buffer (pH 6.5) and 0.1% sodium dodecyl sulphate. Polymerization was catalysed by 0.07% ammonium persulphate in the presence of 0.035% *NNN'*-tetramethylethylenediamine. The tops of the gels were covered with 2-methylpropan-1-ol and left at room temperature overnight.

The gels were pre-run for 2 h at 5 mA/tube in a buffer of 0.1% sodium dodecyl sulphate in 0.1 M-sodium phosphate, pH 6.5. Then a 50 or a 100 μ l sample was placed on top of each gel with 10 μ l of 0.05% Bromophenol Blue and electrophoresis was carried out at 8 mA/tube in the same electrode buffer as for the pre-run. The electrophoresis was stopped when the Bromophenol Blue band had migrated to within 2 mm from the end of the gels. The gels were removed from the tubes and fixed overnight in 25% (w/v) trichloroacetic acid. Proteins were demonstrated by staining the gels for 60 min with saturated Amido Black in 25% (w/v) trichloroacetic acid, followed by destaining in 7.5% (v/v) acetic acid. Bands containing carbohydrates were

demonstrated by incubating the gels for 1 h in 1% periodic acid in 25% (w/v) trichloroacetic acid. After repeated washings in 25% (w/v) trichloroacetic acid the gels were immersed in commercial Schiff's reagent (BDH Chemicals Ltd.) and placed in the dark. Pink bands were developed in 30–60 min and the density of the bands was estimated by photometric scanning of the gels (Vitatron Universal Photometer, Dieren, Holland).

Results

Isolation of membranes of purified neurosecretory vesicles

The method used for preparing neurosecretory vesicles is essentially that described by Dean & Hope (1967). The fraction recovered from the density gradient consists almost entirely of neurosecretory vesicles. The specific activity of vasopressin in the preparation was 9.4 ± 1.5 i.u./mg of protein (mean $\pm$ s.d., eight preparations). Weak activities of succinate dehydrogenase and acid ribonuclease and small amounts of RNA could be demonstrated, indicating some contamination by mitochondria, lysosomes and probably free ribosomes (Table 1). The preparation exhibited no $Mg^{2+}+Na^{+}+K^{+}$ -dependent ATPase or glucose 6-phosphatase activities. Electron-microscopic examination of the fraction (Plate 1b) showed a population of tightly packed, uniform particles of varying electron density, closely resembling the neurosecretory vesicles seen in whole neural-lobe tissue (Plate 1a).

The vesicles seemed rather resistant to hypo-osmotic treatment. Resuspension of the neurosecretory vesicles in 4 ml of 5 mM-Tris-acetic acid buffer, pH 7.5, followed by freezing and thawing only released approx. 40% of their vasopressin content (Fig. 1). However, repeated freezing and thawing of the preparation followed by sedimentation and

resuspension released most of the vasopressin from the vesicles, leaving approx. 30% of the total protein in a sedimentable form. This treatment of the particles also caused some loss of phospholipid. Resuspension of the purified neurosecretory vesicles in 13.5 ml of 5 mM-Tris-HCl, pH 8.4, for 120 min caused the release of approx. 80% of the vasopressin in the preparation.

When resuspended vesicle membranes were placed on a discontinuous sucrose-density gradient (as used for purifying plasma membranes) and were centrifuged at $60000 g_{av.}$ for 90 min, 11% of the protein in the resuspension was recovered at the 0.6 M-/0.95 M-sucrose interface. Most of the protein of the membrane fraction was found in the pellet of the gradient tube.

Isolation of plasma membranes

Purification of nerve endings. Fraction (II) obtained by differential centrifugation is enriched in nerve endings (R. V. Baker, H. Vilhardt & D. B. Hope, unpublished work). The fraction contains other cellular elements, mostly free mitochondria and neurosecretory vesicles but also endoplasmic reticulum and ribosomes (Table 2). The contamination of the fraction by nuclei and whole cells (with DNA as a marker) is small.

Washing of fraction (II) caused a decrease in its content of microsomal material. Thus fraction (III) contained less than 30% of the rotenone-insensitive

Table 1. *Relative specific activities in a preparation of purified neurosecretory vesicles from bovine neural lobes*

Neurosecretory vesicles were isolated by differential and discontinuous sucrose-density-gradient centrifugation as described in the Materials and Methods section. The results are expressed as relative specific activities (specific activity in fraction/specific activity in homogenate). n.d., not detectable.

Vasopressin	2.13
Succinate dehydrogenase	0.09
Lactate dehydrogenase	0.02
Acid ribonuclease	0.10
Glucose 6-phosphatase	n.d.
$Mg^{2+}+Na^{+}+K^{+}$ -dependent ATPase	n.d.
RNA	0.13

Fig. 1. *Lysis of neurosecretory vesicles in dilute buffer*

Neurosecretory vesicles obtained by sucrose-density-gradient centrifugation were suspended in 5 mM-Tris-acetic acid, pH 7.5. After freezing and thawing the particles were sedimented at $100000 g_{av.}$ for 45 min, followed by resuspension in the buffer. This procedure was repeated a total of five times. Portions of the sedimented particles were assayed for protein (●), vasopressin (○) and phospholipid (Δ). The results are expressed as a percentage of the total amounts in the unlysed vesicles.

Table 2. *Relative specific activities in the fractions of a preparation of nerve endings from bovine neural lobes*

Fraction (II) obtained by differential centrifugation was washed and further fractionated by means of sucrose-Ficoll-density gradient centrifugation as described in the Materials and Methods section. Subfractions are numbered according to Scheme 1. Since it was not possible to assay all the substances in one experiment the Table is composed of data obtained from several individual experiments. The results are expressed as relative specific activities (specific activity in fraction/specific activity in homogenate). n.d., Not detectable.

Fraction ...	Relative sp. activities					
	(II)	(II1)	(II2)	(II3)	(II4)	(II5)
Vasopressin	1.38	1.19	0.88	1.34	0.17	1.07
Succinate dehydrogenase	2.48	3.87	1.24	4.75	0.31	1.40
Monoamine oxidase	2.71	3.16	—	—	—	1.17
5'-Nucleotidase	0.41	0.48	0.60	0.11	0.58	0.74
Mg ²⁺ +Na ⁺ +K ⁺ -dependent ATPase	1.74	2.98	3.25	1.57	1.68	3.34
NADH-cytochrome <i>c</i> oxidoreductase	1.68	1.47	1.85	1.03	2.03	1.05
NADPH-cytochrome <i>c</i> oxidoreductase	1.79	0.83	—	—	—	1.16
Glucose 6-phosphatase	1.99	0.84	—	—	3.45	1.09
Acid ribonuclease	2.54	1.73	1.13	2.29	0.98	1.51
Lactate dehydrogenase	0.52	0.67	0.81	0.26	0.14	0.83
DNA	0.14	0.31	n.d.	0.47	n.d.	n.d.
Acetylcholinesterase	0.92	1.16	2.31	0.81	1.09	1.76
Non-specific cholinesterase	1.02	1.29	2.02	0.24	2.30	0.78
RNA	0.45	0.22	0.36	0.14	0.41	0.19

NADPH-cytochrome *c* oxidoreductase and RNA present in fraction (II).

A further purification of the nerve endings was obtained by centrifugation of the fraction placed on top of a 25% (w/v) Ficoll solution. A considerable number of free neurosecretory vesicles and mitochondria, but also some nerve endings, were recovered at the bottom of the centrifugation tube (fraction II3). The majority of the nerve endings, however, were retained by the Ficoll solution (fraction II2). Subsequent centrifugation of fraction (II2) placed on top of 8% Ficoll sedimented most of the nerve endings (fraction II5), whereas microsomal material tended to remain on top of the Ficoll solution (fraction II4). This step in the fractionation also produced a partial separation of the specific acetylcholinesterase (mainly in fraction II5) from the non-specific cholinesterase activity (mainly in fraction II4).

The specific activities in fraction (II5) of vasopressin and of marker enzymes for mitochondria and microsomal elements were slightly higher than those found in the homogenate. This is probably due to the presence inside the nerve endings of neurosecretory vesicles and other subcellular particles.

Electron-microscopic examination of the fraction (Plate 2a) confirmed the presence of large numbers of nerve endings resembling those observed in micrographs of sections of whole neural-lobe tissue (Plate 1a). Some free neurosecretory vesicles were encountered, but practically no free mitochondria. Some of the nerve endings had lost their content of organelles and appeared as large, empty vesicles.

Purification of plasma membranes of the nerve endings. The nerve endings (fraction II6) were found to be rather resistant to mechanical disruption. Several procedures were tried and the final one adopted was that described by Cotman & Matthews (1971). The nerve endings were resuspended for 120 min in 5 mM-Tris-HCl, pH 8.4, after which only 11% of the lactate dehydrogenase was sedimentable. After sedimentation of the lysate the resuspended pellet was fractionated on a discontinuous density gradient. Four particulate fractions, including a pellet, were obtained. Fraction (II8) (Table 3) showed an enrichment of microsomal enzymes, whereas the markers for neurosecretory vesicles and mitochondria were found in the bottom fractions of the gradient (fractions II10 and II11). Electron-microscopic examination showed that fraction (II10) contained a considerable amount of apparently undisrupted nerve endings (Plate 2b), which is consistent with the finding of relatively high activities of Mg²⁺+Na⁺+K⁺-dependent ATPase in this fraction.

Fraction (II9) showed a considerable decrease in the activities of most enzymes assayed (the assays used for acetylcholinesterases were not sensitive enough to show the distribution of these enzymes on the gradient). The activity of Mg²⁺+Na⁺+K⁺-dependent ATPase, however, is considerably increased in fraction (II9), indicating a high content of plasma membranes. Electron microscopy of the sedimented fraction (II12) showed a preparation consisting almost exclusively of membranes, mostly forming large irregular vesicles (Plate 3). The size of the membrane elements made it likely that they were

(a)

(b)

EXPLANATION OF PLATE I

Whole neural-lobe tissue (a) and purified neurosecretory vesicles (b)

(a) Section through nerve endings in a bovine neural lobe; (b) purified neurosecretory vesicles obtained by density-gradient centrifugation of fraction (IV) of a homogenate of bovine neural lobes. The scales represent 1 μ m.

EXPLANATION OF PLATE 2

Purified nerve endings (a) and subfraction from lysate of nerve endings (b)

(a) Fraction (II5) containing nerve endings purified by centrifugation as described in the text. (b) Fraction (III10) obtained by centrifugation of a lysate of nerve endings on a discontinuous sucrose density gradient. The scales represent 1 μ m.

EXPLANATION OF PLATE 3

Isolated plasma membranes from bovine neural lobes

Plasma membranes (fraction II12) obtained by centrifugation of a lysate of nerve endings on a discontinuous sucrose density gradient. The scale represents 1 μ m.

Table 3. *Relative specific activities in subfractions from a preparation of lysed nerve endings from bovine neural lobes*
Purified nerve endings (fraction II5 in Scheme 1) were lysed as described in the Materials and Methods section and centrifuged on a discontinuous sucrose density gradient. Since it was not possible to assay all the substances in one experiment the Table is composed of data obtained from several individual experiments. The results are expressed as relative specific activities (specific activity in fraction/specific activity in homogenate). n.d., Not detectable.

Fraction ...	Relative sp. activities			
	(II8)	(II9)	(III0)	(III1)
Vasopressin	0.48	0.16	0.89	1.43
Succinate dehydrogenase	0.18	0.08	0.87	1.74
Monoamine oxidase	0.20	0.11	1.71	0.93
Glucose 6-phosphatase	2.31	0.27	0.48	0.96
Acid ribonuclease	0.63	0.07	0.24	0.71
Mg ²⁺ +Na ⁺ +K ⁺ -dependent ATPase	1.31	7.13	3.24	0.94
5'-Nucleotidase	1.63	1.02	0.81	n.d.
NADH-cytochrome <i>c</i> oxidoreductase	1.34	0.13	0.51	0.87
NADPH-cytochrome <i>c</i> oxidoreductase	1.51	n.d.	0.13	n.d.
Lactate dehydrogenase	0.26	n.d.	0.08	n.d.

derived from plasma membranes. A few electron-dense particles and some minor vesicles of doubtful origin were also observed. The yield of material in fraction (II9) was 200–400 µg of protein, or less than 0.2% of the protein in the homogenate.

Polyacrylamide-gel electrophoresis of solubilized membrane proteins

Preparations of purified plasma membranes and membranes of neurosecretory vesicles were solubilized by 1% sodium dodecyl sulphate and electrophoresed on polyacrylamide gels containing 0.1% sodium dodecyl sulphate. Staining of the gels for proteins revealed the presence of a large number of bands. Fig. 2 shows diagrammatically the appearance of the gels (plasma membranes to the right and membranes of neurosecretory vesicles to the left). A number of bands seemed to be common to both gels. Three fast-migrating proteins were only found in membranes of neurosecretory vesicles and could be neurophysins contaminating the preparation.

Staining of the gels for carbohydrates with the periodic acid-Schiff reagent showed the presence of three glycoproteins, identically located in the two gels. The exact location of the carbohydrate-containing proteins was determined by staining the gels with Amido Black followed by the periodic acid-Schiff reagent.

Discussion

The neurosecretory vesicles studied in the present experiments were prepared by the method described by Dean & Hope (1967). This preparation consists of highly purified vesicles with minimum contamination by mitochondria, lysosomes and microsomal material. It contained no plasma-membrane fragments. On the basis of the specific activity of vasopressin in the homogenate it could be calculated

Fig. 2. *Schematic drawing of polyacrylamide gels after electrophoresis of proteins from membranes of neurosecretory vesicles and plasma membranes*

Membranes of neurosecretory vesicles (a) and plasma membranes (b) were solubilized by 1% (w/v) sodium dodecyl sulphate and subjected to electrophoresis on 6% (w/v) polyacrylamide gels in the presence of 0.1% sodium dodecyl sulphate. The anode is at the bottom of the picture and the gels were stained with Amido Black. The intensity of the staining was determined by densitometry and in the diagram the solid bands are more heavily stained than the hatched bands. PAS indicates the bands giving a positive reaction with the periodic acid-Schiff reagent and BB the position of the electrophoretic front as determined with Bromophenol Blue.

that approx. 45% of the protein in the homogenate is associated with neurosecretory vesicles. This should be regarded as a minimum value, since it cannot be excluded that the vesicles may have lost some of their hormone content during the purification procedure. Of the vesicle protein approx. 30% was membrane-bound.

The starting material used for preparing plasma membranes is already enriched in nerve endings (fraction II). Incubation of fraction (II1) in suitable media has shown that this preparation can be stimulated to hormone secretion by high concentrations of K^+ (R. V. Baker, H. Vilhardt & D. B. Hope, unpublished work) and by cold exposure (Vilhardt *et al.*, 1975). Further fractionation by means of the Ficoll gradients rid the preparation of considerable amounts of free mitochondria, neurosecretory vesicles and microsomal material. A partial separation of the activities of the specific and non-specific acetylcholine esterases was achieved in fractions (II4) and (II5). This may reflect a separation of pituicyte elements from the nerve endings. From studies of brain tissue there are several reports (Bülbring *et al.*, 1953; Giacobini, 1959; Torack & Barnett, 1962; Tower & Young, 1973) that non-specific cholinesterase activity is primarily associated with glial cells. Pituicytes may resemble glial cells in this respect.

The relative specific activity of vasopressin in fraction (II5) is 1.07, whereas that of $Mg^{2+} + Na^+ + K^+$ -dependent ATPase is 3.43. This may indicate that the secretory vesicles in the nerve endings have lost some of their hormone content. Another explanation could be that whereas the neurosecretory vesicles are distributed throughout the axon the $Mg^{2+} + Na^+ + K^+$ -activated ATPase may be primarily localized to the plasma membrane of the nerve ending. Additional support for such a hypothesis may be found in the comparatively low activity of 5'-nucleotidase observed in fractions (II5) and (II9). The specificity of this enzyme as a plasma-membrane marker has, however, been questioned (Cotman & Matthews, 1971; Widnell, 1972). In the present experiments 5'-nucleotidase to some extent followed the distribution of the microsomal marker enzymes, which could reflect the presence in microsomal material of small fragments of plasma membrane.

The sucrose-density-gradient centrifugation of the lysed nerve endings led to a separation of the activity of $Mg^{2+} + Na^+ + K^+$ -dependent ATPase from mitochondria (succinate dehydrogenase, rotenone-insensitive NADH-cytochrome *c* oxidoreductase, monoamine oxidase), lysosomes (acid ribonuclease) and microsomal fraction [glucose 6-phosphatase, rotenone-insensitive NADPH (and NADH)-cytochrome *c* oxidoreductase]. Since there is no known specific marker substance for the membranes of neurosecretory vesicles, contamination of the plasma-

membrane fraction by such elements can only be estimated indirectly. From the specific activity of vasopressin in purified neurosecretory vesicles and the distribution of hormone and protein when such a preparation was lysed and centrifuged on a sucrose density gradient (in the same way as lysed nerve endings), it can be calculated that 18% of the protein in fraction (II9) might originate in neurosecretory vesicles. This is not surprising, considering that 12–15% of the total protein content of the homogenate is associated with membranes of neurosecretory vesicles. If $Mg^{2+} + Na^+ + K^+$ -dependent ATPase is accepted as a specific marker for plasma membranes it can be calculated on the basis of the present results that these membranes constitute 32% of the protein of the isolated nerve endings.

Polyacrylamide-gel electrophoresis of the water-insoluble proteins of plasma membranes and membranes of neurosecretory vesicles revealed the presence of a large number of proteins in both preparations. Comparison of the two gels is difficult because of the multitude of bands, but certain similarities are obvious and may indicate that some proteins, including the three glycoproteins, are common to the two membrane preparations. Electrophoresis of solubilized proteins from brain plasma membranes and synaptic-vesicle membranes has also demonstrated rather complex protein compositions of these structures (Cotman & Mahler, 1967; Mehl, 1968; Cotman *et al.*, 1968; Bosmann *et al.*, 1970; Waehneltdt *et al.*, 1971) often with distinct similarities (Morgan *et al.*, 1973).

Judging from the intensity of the stained bands (as measured by densitometric scanning) the similarities in the electrophoretic pattern of the two gels were not due to contamination of the plasma-membrane preparation with neurosecretory vesicle membranes. This is further supported by the absence of the three fast-migrating bands in the gel of the plasma membranes. Vilhardt & Højlmer (1972) examined the lipid composition of the membranes of the neurosecretory vesicles and of plasma membranes isolated from posterior pituitary glands. Their results showed that the two membranes are very similar to each other in their composition of lipid classes, with only minor differences in their fatty acid spectrum. From the present experiments it appears that the protein compositions of the two membranes also exhibit certain similarities.

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References

- Arnaiz, G. R. D. L., Alberici, M. & De Robertis, E. (1967) *J. Neurochem.* **14**, 215-225
- Bangham, D. R. & Mussett, M. V. (1958) *Bull. W. H. O.* **19**, 325-340
- Bargmann, W. (1968) *Handb. Exp. Pharmacol.* **23**, 1-39
- Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466-468
- Bosmann, H. B., Case, K. R. & Shea, M. B. (1970) *FEBS Lett.* **11**, 261-263
- Bülbring, E., Philpot, F. J. & Bosanquet, F. D. (1953) *Lancet* **i**, 865-866
- Cheng, K. W. & Friesen, H. G. (1970) *Metabolism* **19**, 876-890
- Cotman, C. W. & Mahler, H. R. (1967) *Arch. Biochem. Biophys.* **120**, 384-396
- Cotman, C. W. & Matthews, D. A. (1971) *Biochim. Biophys. Acta* **249**, 380-394
- Cotman, C. W., Mahler, H. R. & Hugli, T. E. (1968) *Arch. Biochem. Biophys.* **126**, 821-837
- Dean, C. R. & Hope, D. B. (1967) *Biochem. J.* **104**, 1082-1088
- Dean, C. R. & Hope, D. B. (1968) *Biochem. J.* **106**, 565-573
- de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955) *Biochem. J.* **60**, 604-617
- Dekanski, J. (1952) *Brit. J. Pharmacol. Chemother.* **7**, 567-572
- Douglas, W. W., Nagasawa, J. & Schultz, R. (1971) *Mem. Soc. Endocrinol.* **19**, 353-378
- Duncan, H. M. & Mackler, B. (1966) *J. Biol. Chem.* **241**, 1694-1697
- Ellman, G. L., Courtney, K. D., Andres, V. & Featherstone, R. M. (1961) *Biochem. Pharmacol.* **7**, 88-95
- Emmelot, P., Bos, C. J., Benedetti, E. L. & Rümke, P. (1964) *Biochim. Biophys. Acta* **90**, 126-145
- Fiske, C. H. & SubbaRow, Y. (1925) *J. Biol. Chem.* **66**, 375-400
- Giacobini, E. (1959) *Acta Physiol. Scand. Suppl.* **156**, 1-45
- Gray, E. G. & Whittaker, V. P. (1960) *J. Physiol. (London)* **163**, 35P-37P
- Gurd, J. W., Jones, L. R., Mahler, H. R. & Moore, W. J. (1974) *J. Neurochem.* **22**, 281-290
- Henn, F. A., Hansson, H.-A. & Hamberger, A. (1972) *J. Cell Biol.* **53**, 654-660
- Hollenberg, M. D. & Hope, D. B. (1968) *Biochem. J.* **106**, 557-564
- Hong, J. S. & Poisner, A. M. (1974) *Endocrinology* **94**, 234-240
- Ipata, P. L. (1967) *Anal. Biochem.* **20**, 30-36
- Jones, H. D. & Matus, A. J. (1974) *Biochim. Biophys. Acta* **356**, 276-287
- Kissane, J. M. & Robins, E. (1958) *J. Biol. Chem.* **233**, 184-188
- Lederis, K. (1965) *Z. Zellforsch. Mikrosk. Anat.* **65**, 847-868
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Lu, A. Y. H., Junk, K. W. & Coon, M. J. (1969) *J. Biol. Chem.* **244**, 3714-3721
- Mehl, E. (1968) in *Macromolecules and the Function of the Neuron* (Lodin, Z. & Rose, S. P. R., eds.), pp. 22-32, Excerpta Medica, Amsterdam
- Morgan, I. G., Wolfe, L. S., Mandel, P. & Gombos, G. (1971) *Biochim. Biophys. Acta* **241**, 737-751
- Morgan, I. G., Zanetta, J.-P., Breckenridge, W. C., Vincendon, G. & Gombos, G. (1973) *Brain Res.* **62**, 405-411
- Nordmann, J. J., Dreifuss, J. J. & Legros, J. J. (1971) *Experientia* **27**, 1344-1345
- Pennington, B. T. (1961) *Biochem. J.* **80**, 649-654
- Pickup, J. C. & Hope, D. B. (1971) *Biochem. J.* **123**, 153-162
- Poisner, A. M. & Douglas, W. W. (1968) *Mol. Pharmacol.* **4**, 531-540
- Porteous, J. W. & Clark, B. (1965) *Biochem. J.* **96**, 159-171
- Roth, J. S. & Milstein, S. W. (1952) *J. Biol. Chem.* **196**, 489-498
- Santolaya, R. C., Bridges, T. E. & Lederis, K. (1972) *Z. Zellforsch. Mikrosk. Anat.* **125**, 277-288
- Schmidt, G. & Tannhauser, S. J. (1945) *J. Biol. Chem.* **161**, 83-89
- Smith, A. D. & Winkler, H. (1966) *J. Physiol. (London)* **183**, 179-188
- Studer, R. O. (1963) *Helv. Chim. Acta* **46**, 421-425
- Torack, R. M. & Barnett, R. J. (1962) *Exp. Neurol.* **6**, 224-244
- Tower, D. B. & Young, O. M. (1973) *J. Neurochem.* **20**, 269-278
- Uttenthal, L. O., Livett, B. G. & Hope, D. B. (1971) *Phil. Trans. Roy. Soc. Ser. B* **261**, 379-380
- Vilhardt, H. & Højlmer, G. (1972) *Acta Endocrinol. (Copenhagen)* **71**, 638-648
- Vilhardt, H. & Hope, D. B. (1974) *Biochem. J.* **143**, 181-190
- Vilhardt, H., Baker, R. V. & Hope, D. B. (1975) *J. Neurochem.* in the press
- Waehneltdt, T. V., Morgan, I. G. & Gombos, G. (1971) *Brain Res.* **34**, 403-406
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406-4412
- Weinstein, H., Malamud, S. & Sachs, H. (1961) *Biochim. Biophys. Acta* **50**, 386-389
- Widnell, C. C. (1972) *J. Cell Biol.* **52**, 542-558
- Wróblewski, F. & LaDue, J. S. (1955) *Proc. Soc. Exp. Biol. Med.* **90**, 210-213
- Wurtman, R. J. & Axelrod, J. (1963) *Biochem. Pharmacol.* **12**, 1439-1441