A SIMPLIFIED METHOD FOR THE PREPARATION OF PLASMA MEMBRANES FROM OVINE ANTERIOR PITUITARY GLANDS

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1. Introduction

The plasma membranes of many tissues are considered as the site at which the hormone initiates its action. It has thus been suggested that the anterior pituitary plasma membranes contain the receptor sites for the hypothalamic releasing hormones [1–3].

Several methods for the isolation of plasma membranes from intact tissues have been described [4, 5]. Most of them concern rat liver [6–10]. These methods, when applied to the isolation of the plasma membranes from anterior pituitary tissue, gave poor results because the density of the pituitary plasma membranes appears to be different from that of rat liver. To our knowledge, no specific method has been proposed for the purification of the pituitary plasma membranes.

Besides morphological studies, many authors consider the determination of 'marker enzyme' activity as very important for the characterization of plasma membrane preparations. In order to follow progress in the purification of our material, we assessed the specific activity of the three most characteristic enzyme markers of the plasma membranes: 5′-nucleotidase, (Na^+- K^+- Mg^{2+})-ATPase and adenylate-3′,5′-cyclase [8, 11]. In the present paper, a simple and rapid method is described for the preparation of a fraction enriched in plasma membranes from ovine anterior pituitary glands.

2. Materials and methods

The pituitary glands from adult ewes were collected in the slaughterhouse (SORGA, Paris) in ice-cold Hanks' medium [12]. With minimum loss of time, the glands were delivered to the laboratory and the posterior lobes were removed. All subsequent procedures were carried out at 4°C. The anterior lobes were weighed, minced and homogenized in 7 vol of 0.25 M sucrose, 0.5 mM $CaCl_2$ and 5 mM Tris buffer. pH 7.4 [10]. Homogenization was performed in a loose-fitting glass homogenizer with a Teflon rod, by means of 10 gentle up and down strokes. The homogenate was then filtered through a fine cheese-cloth. The filtrate was centrifuged at 120 *g* for 10 min. The pellet was discarded. The supernatant was centrifuged at 5900 *g* for 35 min. Fractionation of the 5900 *g* supernatant produced microsomal and mitochondrial fractions. The 5900 *g* pellet was used to prepare a fraction enriched in plasma membranes. The pellet was resuspended in the initial buffer (1 ml/*g*). This suspension was layered over a discontinuous sucrose gradient (1 ml/layer) of densities 1.20, 1.18, 1.16, 1.14 prepared in a Spinco rotor SW 65 tube. The sucrose solutions all contained 0.5 mM $CaCl_2$ and 5 mM Tris buffer (pH 7.4). The tubes were filled to the top with buffer solution and centrifuged for 25 min at 300 000 *g*. Five fractions, including the pellet, were collected with a Pasteur pipette at interfaces between the layers of the following densities: buffer/1.14, 1.14/1.16, 1.16/1.18, 1.18/1.20 and the pellet. They were resuspended in 0.5 mM $CaCl_2$, 5 mM Tris buffer, pH 7.4 (3 vol), twice washed free of sucrose (15 min centrifugation at 75 000 *g*) and stored at −196°C. Fig. 1 is a flow chart of the fractionation procedure.

Protein concentration of various subcellular fractions was determined by the method of Lowry et al.

Table 1
Activity of 'plasma membrane marker enzymes' in sheep pituitary subcellular fraction. [1]

Fraction	5'-Nucleotidase [2]		(Na^+, K^+, Mg^+)ATPase [2]		Adenyl cyclase [3]			
					Basal activity		$NaF(10^{-2}M)$	
	Activity	RSA [4]	Activity	RSA [4]	Activity	RSA [4]	Activity	RSA [4]
Homogenate	2.8 (7)		5.9 (7)		0.6 (7)		5.0 (7)	
Supernatant [1]	3.1 (7)	1.1	6.7 (7)	1.1	0.6 (6)	1.0	5.6 (6)	1.1
Pellet [1]	2.2 (7)	0.8	5.6 (7)	0.9	0.7 (6)	1.3	6.1 (6)	1.2
Supernatant 2	5.5 (7)	2.0	6.1 (7)	1.0	0.5 (4)	0.9	5.6 (4)	1.1
Pellet 4	10.1 (7)	3.6	7.4 (7)	1.2	1.4 (3)	2.5	8.1 (3)	1.6
Pellet 5	11.5 (2)	4.1	7.9 (2)	1.3	1.1 (2)	1.8	11.3 (2)	2.3
Gradient *d.* < 1.14	48.3 (7)	17.2	29.1 (7)	4.9	6.7 (7)	11.1	32.4 (7)	6.4
d. 1.14/d.1.16	10.8 (7)	3.8	21.9 (7)	3.7	3.0 (7)	5.0	16.4 (7)	3.3
d. 1.16/d.1.18	5.2 (7)	1.9	19.4 (7)	3.3	1.9 (7)	3.1	11.4 (7)	2.3
d. 1.18/d.1.20	1.9 (4)	0.7	10.4 (4)	1.8	0.4 (2)	0.7	2.6 (2)	0.5
d. < 1.20 (pellet)	0.7 (4)	0.2	3.7 (4)	0.6	0.1 (3)	0.2	0.9 (3)	0.2

[1] Numbers in parenthesis indicate number of preparations tested.
[2] Activities are expressed as Po_4^{---} μmoles per mg proteins per hr at 37°C.
[3] Activities are expressed as nmoles cAMP per mg protein per hr at 37°C.
[4] Relative specific activity = fraction activity/homogenate activity.

[13], using above serum albumin as the standard. The activities of three plasma membrane enzyme markers were assayed at each step of the fractionation procedure and in the five fractions of the sucrose gradient. The 5'-nucleotidase and $(Na^+ - K^+ - Mg^{2+})$-ATPase activities were measured by incubation of samples for 30 min at 37°C in the following solutions: 100 mM KCl; 5 mM $MgCl_2$; 50 mM Tris, pH 7.2 containing 5 mM 5' AMP for 5' nucleotidase (according to Emmelot et al. [8]) and 5 mM KCl; 2 mM $MgCl_2$; 60 mM NaCl; 100 mM Tris, pH 7.5 containing 7 mM ATP for ATPase (according to Emmelot et al. [8] with slight modifications). After centrifugation, the released inorganic phosphate was measured in the supernatant [14].

The adenylate cyclase activity was assayed using Krishna's method [15] modified by Ramachandran and Lee [16]. In accordance with this modification, cAMP was separated from ATP and other nucleotides by filtration on an aluminium oxide column. Assays were performed by incubating samples for 30 min at 37°C in a buffer containing 1.6 mM [α^{32}P]ATP, 10 mM theophylline, 5 mM $MgCl_2$, 0.1% bovine serum albumin, 17 mM Tris, pH 7.4 and an ATP regenerating system with 5 mM phosphoenolpyruvate and 80 μg per ml of pyruvate kinase. The basal activity of adenylate cyclase and the activity of this enzyme in the presence of NaF were determined.

3. Results and discussion

Table 1 records the enzymatic activities determined in the various subcellular fractions. The specific activity of the three characteristic plasma membrane enzymes is highest in the fraction collected on 1.14 density layer. The relative specific enzymatic activities of this fraction with respect to the whole homogenate are approximately 17, 5 and 11 times for 5'-nucleotidase, $(Na^+ - K^+ - Mg^{2+})$-ATPase and adenylate cyclase.

The degree of purification of the sheep pituitary plasma membranes as assessed by the three marker enzyme activities is of the same order or better than that observed for the same enzymes for liver plasma membranes [8, 10, 17] and for fat cell plasma membranes [18].

Our data demonstrate a recovery of roughly 0.5 mg protein per gram wet weight of pituitary. Lauter et al. [17] indicated that the theoretical yield should be 1.98 mg membrane protein per gram wet weight of liver. They noted that most of the procedures give a yield of about 15%. If this estimation is applicable to the anterior pituitary tissue, our data indicate a recovery of 25%.

The plasma membrane fraction obtained by Emmelot et al. [8] and by Berman et al. [10] from rat liver cells was located at the sucrose density

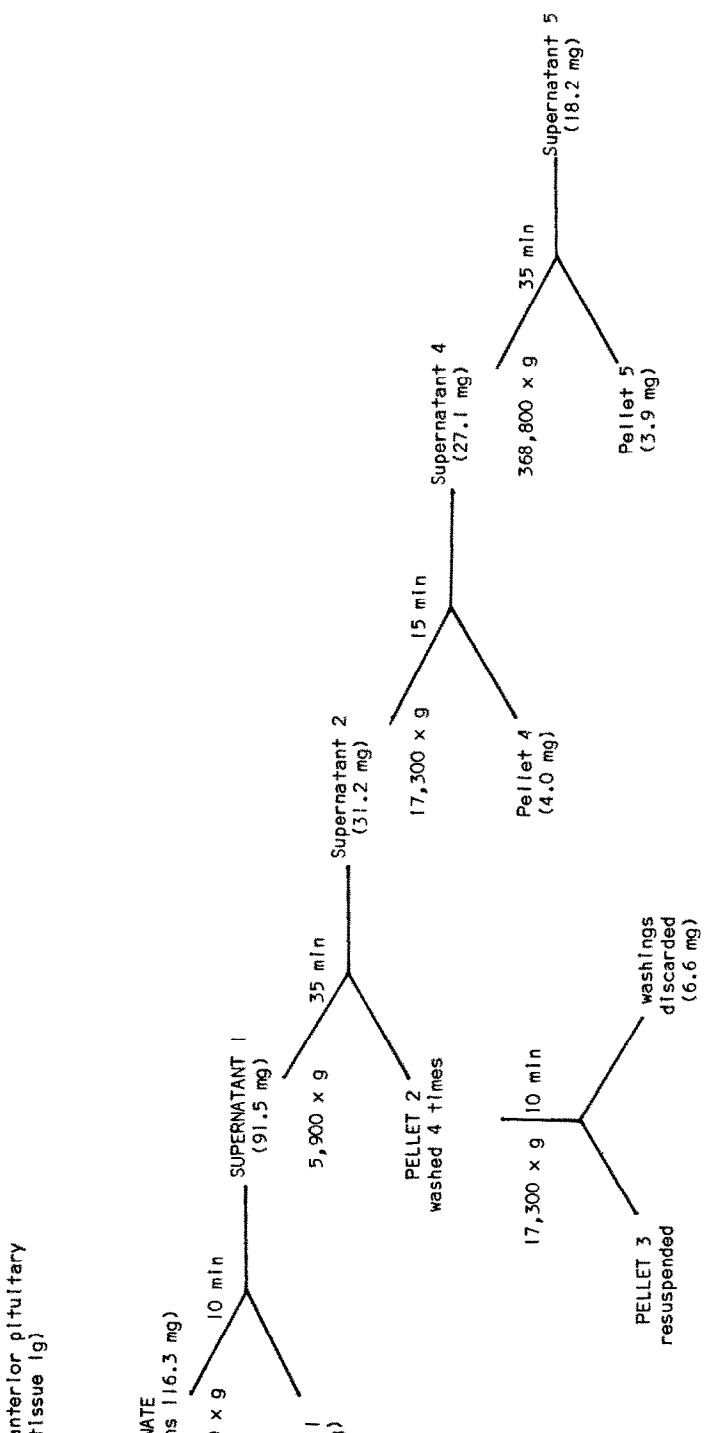

Fig. 1. Schematic representation of the procedure for subcellular fractionation of sheep pituitary.

1.16/1.18 interface. Labrie et al. [2] reported that the plasma membranes from bovine anterior pituitary glands sedimented in a step-wise sucrose gradient at interfaces 1.14/1.16 and 1.16/1.18. In our procedure, bands containing plasma membranes appear in the lower density region (less than 1.14). A similar location was found for plasma membranes from isolated fat cells [18]. As noted by Lauter et al. [17] 'unlike most isolation procedures for other subcellular, fractions, the method for the isolation of plasma membrane fragments may have to be worked out individually for different species, tissues and/or physiological and pathological conditions'. It is possible therefore that the density of the bovine pituitary plasma membranes is different from that of the ovine glands.

Two out of the eight preparations of the pituitary plasma membrane fractions were examined by electron microscopy. The fractions were found to be rich in plasma membranes and virtually free of secretory granules. However, this does not rule out the possible presence of contamination from other cell constituents. Such would require a detailed examination of other specific enzyme markers.

Our purpose in this work was to develop a simple and rapid method for the preparation of pituitary plasma membranes which could be used in the study of the binding of hypothalamic hormones.

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