OBTAINING THE OUTER SEGMENTS FROM THE PHOTORECEPTOR CELLS OF THE RETINA AND DETERMINATION OF RHODOPSIN IN A SUSPENSION OF THE SEGMENTS

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The outer segments are specialized membrane structures of the photoreceptor cells of the retina, containing photosensitive pigments and responsible for the primary act of reception of light. The isolation of the outer segments as a homogeneous fraction is an essential condition for the biochemical study of certain problems in reception. A number of methods of isolating the fraction of the outer segments have been described in the literature, but they have the disadvantage of yielding a fraction with a low level of purity, and during the process of isolation of the segments, moreover, they are subjected to prolonged treatment with hypertonic solutions [4,5,7].

The object of the present investigation was to improve the method of obtaining the fraction of outer segments of a high level of purity and integrity with minimal length of hypertonic treatment, and also to determine the content of rhodopsin directly in the suspension of these segments and to study the possibility of using this index as a criterion of the purity of the fraction obtained.

EXPERIMENTAL METHOD

The experiments were carried out on the eyes of cattle obtained from the slaughter house in a cold state and kept in darkness. Dissection of the eyes and all subsequent operations were carried out in red light in a cold room at 2-5°. The fraction of the outer segments was obtained by a combined method – by differential centrifugation and centrifugation in a sucrose density gradient [3-5,7] – with certain modifications. The rhodopsin content was judged from the optical density (ΔE_{500}), measured spectrophotometrically, and equal to the difference between the optical densities of the dark and illuminated suspension. This determination is based on the property of the pigment rhodopsin that it has a maximum of absorption at a wavelength of 500 mμ. Protein was determined colorimetrically [6]. The fractions were isolated, washed, and suspended in 0.25 M sucrose solution containing 0.02 M tris buffer (pH 7.3).

EXPERIMENTAL RESULTS

After a series of experiments, the following conditions were adopted as optimal for obtaining the outer segments.

1. The homogenate (30-40 retinas to 30-40 ml sucrose) was obtained by gentle disintegration of the tissue by vertical movement of the pestle of the glass homogenizer.

2. The homogenate was centrifuged at 800g for 5 min. The liquid was removed, the residue was washed twice, and the liquids were combined.

3. The homogenate was centrifuged at 1600 g for 10 min. The liquid was removed and the residue washed twice at the same speed and suspended in sucrose in a dose equal to 10-12 ml for the residue of outer segments obtained from 30-40 retinas (Fraction 1). Fraction 1 is the final fraction of outer segments obtained by Futterman's method [4].

4. For centrifugation in a density gradient, the following were poured successively into test tubes: 2 ml of 2 M sucrose solution;* 5 ml of 0.93 M sucrose solution, and 1 ml of Fraction 1 (the proportions between the layers are given for tubes with a capacity of 10 ml). To obtain clearly demarcated layers, the filling must begin with the lower layer of 2 M sucrose solution, and the remaining solutions are added carefully down the wall of the tube. The

*To simplify the method, the layer of 2 M sucrose solution was sometimes omitted. In these circumstances the cell structures heavier than the outer segments passed through the layer of 0.93 M sucrose solution and formed a residue directly on the bottom of the tube.

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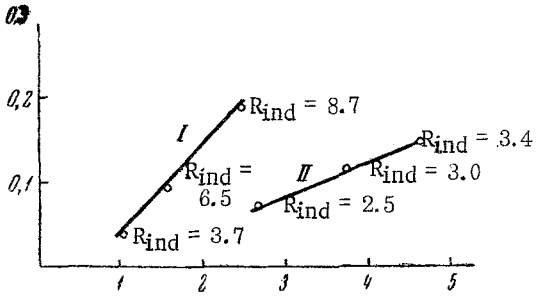

Fig. 1. Rhodopsin curves of Fractions 1 and 2 and R_{ind} calculated from the formula:

$$R_{ind} = \frac{-\Delta E_{500} \cdot 100.}{C}$$

I) Fraction 2; II) Fraction 1. Along the axis of abscissas, protein content of fraction (C in mg); along the axis of ordinates, difference of optical densities ($-\Delta E_{500}$).

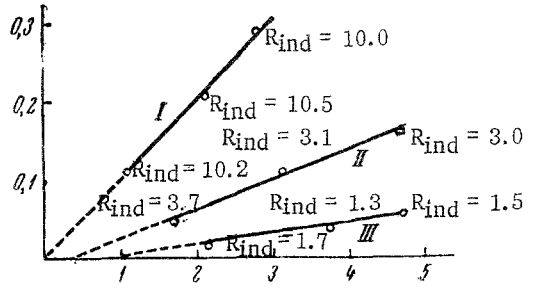

Fig. 2. Rhodopsin curves of homogenate and Fractions 1 and 2, and R_{ind} calculated from the formula:

$$R_{ind} = \frac{-\Delta E_{500} \cdot 100}{C - C_0}.$$

III) Homogenate. Remainder of legend as in Fig. 1.

Relative Content of Rhodopsin in Separate Fractions

Experiment no.	Name of fraction	Protein content in mg ($C - C_0$)	R_{ind}	Ratio between R_{ind} of fraction 2 and R_{ind} of fraction 1	Experiment no.	Name of fraction	Protein content in mg ($C - C_0$)	R_{ind}	Ratio between R_{ind} of fraction 2 and R_{ind} of fraction 1
1	Homogenate	2,270	1,7	2,2*	3	Fraction 1	1,872	6,6	2,2
	"	3,672	1,3			"	1,472	8,5	
			1,5			"	2,200	7,7	
	Fraction 1	1,200	3,7			"	4,400	5,0	
	"	2,982	3,0			"	8,800	4,7	
			3,3					6,9±1,0	
	Fraction 2	1,072	10,2	6,8*		Fraction 2	0,860	16,6	2,2
	"	2,040	10,5			"	1,636	14,9	
			10,3			"	2,312	16,0	
						"	3,360	14,7	
								15,5±0,7	
2	Fraction 1	0,124	4,0	2,7	4	Fraction 1	0,280	8,8	2,1
	"	1,770	3,8			"	1,840	9,6	
	"	3,700	4,2			"	3,600	9,3	
			4,0±0,4					9,2±0,1	
	Fraction 2	0,373	9,9			Fraction 2	0,080	18,7	
	"	0,917	10,7			"	0,640	20,0	
	"	1,617	11,8			"	1,192	20,2	
			10,8±0,8					19,6±0,9	

*In this experiment, R_{ind} of Fractions 1 and 2 was compared with R_{ind} of the homogenate.

tubes were centrifuged in a horizontal rotor for 10 min at 5000 g, after which the layer of outer segments, contained in 0.93 M sucrose solution, was carefully withdrawn by a pipet with a pointed end.

5. The suspension was diluted in three volumes of cold bidistilled water to give a roughly isotonic concentration of sucrose.

6. The suspension was centrifuged at 2500 g for 5 min. The supernatant fluid was removed, and the residue was suspended and washed twice at the same speed (Fraction 2).

To compare the purity of the fraction of segments at the various stages of purification, Fraction 1, as usually used by other authors [4], and Fraction 2 were used. Studies of these fractions in the optical microscope showed that the second is much purer than the first.

As an objective criterion for comparing the purity of the fractions, the content of rhodopsin, the specific protein of the outer segments, was determined. To avoid loss of rhodopsin during extractions, it was determined directly in the suspension of outer segments [1]. Since a linear relationship exists between the amount of rhodopsin and the total protein content of the fraction, during purification of the fraction, the amount of rhodopsin in relation to the total protein content in the suspension of segments must increase. To determine the value of ΔE_{500}, 0.2 ml of a 0.1 M solution of hydroxylamine (pH 7.0) and 2 ml of the sucrose suspension of the fractions (homogenate, Fraction 1 or 2) were poured into two dishes. The contents of the dishes were mixed with a thin glass rod, and then one of them was placed in darkness, while the suspension in the other was illuminated through a water filter in an intensity of 1500 lx. After illumination, the optical density of the dark suspension was measured and compared with that of the illuminated suspension at a wavelength of 500 mμ. The optical density determined was a different value due to the contained rhodopsin.

It is clear from Fig. 1 that a linear relationship exists between the rhodopsin content (value of $-\Delta E_{500}$) in the suspension and its total protein content. The ratio between $-\Delta E_{500}$ and the total protein content of the fraction (see Fig. 1) is the relative quantitative characteristic of rhodopsin, or the rhodopsin index (R_{ind}), expressed on a conventional scale of optical density:

$$R_{ind} = \frac{-\Delta E_{500} \cdot 100}{C}, \qquad (A)$$

where C is the total protein content in the dish, and $-\Delta E_{500}$ is the difference in optical densities at a wavelength of 500 mμ.

If the value of R_{ind} is determined by the formula A for different protein contents of Fractions 1 and 2 (see Fig. 1), a linear series of increasing values is obtained, although theoretically the values of R_{ind} should be the same. The reason for this is that the so-called rhodopsin curves, expressing the relationship between $-\Delta E_{500}$ and the protein content in the suspension in this case do not pass through the zero of the system of coordinates. This may be because the total protein content in the suspension is much higher than the content of rhodopsin protein. The value of R_{ind}, when the protein content in the suspension is different, can be equal only if the content of other proteins, whose optical density does not vary with illumination, is zero or very small, as is the case, for example, when the rhodopsin is determined in digitonin extracts, when mainly rhodopsin is extracted [2]. During determination of R_{ind} in the suspension of outer segments, a case is possible when $-\Delta E_{500}$, due to the rhodopsin content, is equal to zero, although the dish contains a large quantity of protein. It is this protein content, conventionally described as C_0, which must be subtracted from the total protein content in the suspension in order to bring the rhodopsin curves near to the zero point.

Hence, the value of C_0 characterizes a certain quantity of other proteins in the suspension of segments, evidently protein impurities with no maximum of absorption at 500 mμ, when the value of $-\Delta E_{500}$, i.e., the rhodopsin content, is equal to zero. So that the values of R_{ind} could be compared for any protein content in the suspension, the final formula must be expressed as follows:

$$R_{ind} = \frac{-\Delta E_{500} \cdot 100}{C - C_0}. \qquad (B)$$

The value of C_0 may be found graphically by prolonging the rhodopsin curves to their intersection with the axis of abscissas or by solving a system of equations about two points relative to C_0 (Fig. 2). It is clear from Fig. 2 that R_{ind} for Fractions 1 and 2, calculated by formula (B), were equal for different protein contents of the fractions, and this formula may therefore be used for comparing the purity of various fractions with a different protein content. Analysis of the results obtained (see Fig. 2) shows that the higher the purity of the fraction, the lower the value of C_0 and the higher the value of R_{ind}. With a sufficiently high level of purity, the rhodopsin curves practically pass through the zero point. One of the experiments in which this was obtained is illustrated in Fig. 2. In different experiments the values of R_{ind} of Fractions 1 and 2 varied, but their ratio in all the experiments described was approximately the same, about 2.5. Hence, Fraction 2 obtained by the method described above was approximately 2.5 times purer than Fraction 1 obtained by the method described by other authors [4] and approximately 7 times purer (as rhodopsin content) than the original homogenate of the retina (see table).

The variation of R_{ind} in the individual experiments was evidently due to differences in the level of dark adaptation of the animals before slaughter, and also to possible individual variation.

The results obtained demonstrate that the method described can be used to obtain a comparatively pure fraction of the outer segments. The value of R_{ind} may be used as a criterion of the purity of the fractions obtained. This value may be determined directly in the suspension of outer segments, without extracting the rhodopsin with digitonin. In this case, for determination of R_{ind}, the parameter C_0 must be taken into account. This represents the protein content of the suspension when $-\Delta E_{500}$, i.e., the rhodopsin content, is equal to zero.

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