Total lipid and membrane lipid analysis of normal animal and human lenses

J. Stevens Andrews and Thomas Leonard-Martin

Comparisons of lens fiber cell membrane isolation methods were made. Although membrane lipid yield from stirring overnight in hypotonic medium followed by low-speed centrifugation was comparable to that from homogenization and high-speed centrifugation, losses in phosphatidyl ethanolamine were observed with the former method. Lyophilization with vacuum replacement by nitrogen did not exert any deleterious effects. A significant difference in total lens lipid content was observed between human and the three species of animals investigated.

Key words: lipids, lens, animal, human, membrane, matrix

Isolation of a water-soluble and a water-insoluble fraction (membrane fraction) from human lenses, both normal and cataractous, is an active area in the investigation of cataractogenesis.[1–5] Most methods involve cell disruption in buffer by homogenization followed by a high-speed centrifugation in order to isolate the water-insoluble fraction. Frequently this fraction is then further purified by urea extraction or by sucrose density centrifugation. In many cases the supernatant fractions are discarded because the primary goal is to analyze the water-insoluble fraction.

These techniques do not permit comparative examination of alternative methodologies for obtaining the water-insoluble fraction, nor do they permit accumulation of reliable data on the total composition of the human lenses. These limitations are particularly true when applied to lipid analysis because vesicle formation by homogenization occurs and a fraction of the vesicles may remain in the water-soluble fraction and be discarded. Thus concomitant analysis of both fractions after centrifugation permits methodology evaluation and accumulation of total composition data for each lens analyzed.

Although the tissue lipid extraction solvent of Folch[6] remains the solvent of choice, another method of subsequent purification is available that has the advantage of yield quantification by means of dry weight.[7] Thus, at each step of the lipid purification and fractionation, the question of losses may be evaluated. Furthermore, summation of corresponding data obtained from the supernatant and insoluble fractions allows conclusions concerning the total composition of the lens.

The data presented in this manuscript represents the application of these methods to normal animal and human lenses on a microscale—in most cases to single human lenses. The data include a comparison of homogeni-

From the Department of Ophthalmology, Vanderbilt University School of Medicine, Nashville, Tenn.
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Reprint requests: J. S. Andrews, Ph.D., Department of Ophthalmology, Vanderbilt University School of Medicine, Nashville, Tenn. 37232.

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zation and stirring in a hypotonic buffer, the effects of lyophilization, and the speed of centrifugation as each affects the lipid distribution between the supernatant and precipitate fractions. Total lipid concentrations of animal and human lenses are also presented.

Methods

Normal human lenses were obtained from the local eye bank within 24 hr after death, isolated, and stored in sealed containers at −20°. Beef and rabbit lenses were obtained locally and were either used fresh or stored as above. Mouse lenses were supplied frozen by the Eye Research Laboratories of the National Institutes of Health. Chloroform and methanol were redistilled in glass. All other reagents were American Chemical Society grade or better.

Homogenization was done by hand with a ground-glass tube and Teflon pestle. The tissue was suspended in 5 ml of 0.001M ammonium bicarbonate buffer, pH 7.2, by seven passes of the pestle. Unless otherwise noted, the suspension was diluted to approximately 280 ml with buffer and stirred for 1 hr before centrifugation. Centrifugation and all the above operations were conducted at 4°. Some lenses were disrupted by gently stirring overnight at 4° in 280 ml of 0.001M ammonium bicarbonate buffer prior to centrifugation. Centrifugation speeds varied between 1000 and 30,000 × g for 15 to 20 min.

After pooling of the supernatant fractions, the precipitates were collected in a small volume of buffer and both fractions were lyophilized. The dry residues were suspended in a small volume of water, 6.6 vol of methanol were added, and an aliquot was taken for dry weight estimation. Both total and lipid dry weight estimates were obtained on a Cahn electrobalance. Preliminary investigation demonstrated that use of a 30 μl aliquot in an aluminum pan (approximately 7 mg) exposed for 30 min to 37° followed by 30 min at 105° would yield a reproducible weight after 5 min of equilibration. Consecutive exposures to 105° did not significantly alter sample weights. Periodic checks during the investigation demonstrated the validity of this method of obtaining dry weights.

After the dry weight sample was obtained, 13.4 vol (based on the added water volume) of chloroform were added and the mixture was briefly stirred.[6] The Folch extracts of each fraction were collected by vacuum filtration through a sintered glass disc of medium porosity. The extracts were then cycled between −20° and room temperature three times in order to dissociate any proteolipid complexes. Each extract was then taken to dryness under water-pump vacuum with a nitrogen bleed over the sample. The dried residue was then taken up in a small volume (approximately 1 ml) of chloroform:methanol (2:1), transferred to a small sample vial, and re-evaporated to dryness under a stream of nitrogen. The dried residue was dissolved in 0.5 ml of chloroform:methanol (19:1), which had been equilibrated with water, and an aliquot was taken for dry weight (extract dry weight). The remainder of the sample was applied to a dextran gel column (0.5 by 15 m), and fractions were eluted.[7] The first elution solvent, containing the purified lipid, was immediately taken to dryness under vacuum with a nitrogen bleed. The subsequent three fractions containing gangliosides and contaminants were pooled and also taken to dryness. Each residue was transferred from the collection flask to a sample vial and evaporated to dryness under a stream of nitrogen. The residues were dissolved in 0.5 ml of an appropriate solvent, and an aliquot of both the lipid and contaminant fractions was taken for dry weight. These weights were then used to calculate the yield of dry weight applied to the column. Whenever storage of samples was necessary, they were stored at −20° under nitrogen.

The purified lipid was further fractionated on a small, silicic acid column into neutral lipid and phospholipid.[8] Aliquots for dry weight measurements were also taken from each of these fractions as described above, and the total was compared to the purified lipid dry weight. Total cholesterol in each neutral lipid fraction was chemically assayed.[9] In some experiments, between 100 and 120 μg of phospholipid dry weight were applied to a 10 by 10 cm high-performance thin-layer liquid chromatography plate, (E. Merck, Darmstadt, West Germany) and two-dimensional chromatography was conducted.[10] After solvent migration in the second direction, the plates were air-dried for at least 15 min and were then sprayed with a triketohydrinene hydrate (Ninhydrin; City Chemical Corp., New York) reagent. Color was developed at 105° and the positive spots were carefully outlined. The plate was then sprayed with concentrated sulfuric acid and heated to 180°. The developed spots were scraped off and individually collected in Pyrex microtubes. The tube contents were oxidized, and the inorganic phosphate was assayed after removal of silicic acid by centrifugation.[11] An aliquot of the original phospholipid fraction was also oxidized and assayed.

All glassware used in the lipid purification pro-

Table I. Human lens (56 year) fraction dry weights and chemical assay estimates

Fraction or constituent	*Homogenized-extracted (mg)*	*Homogenized-lyophilized (mg)*	*Stirred-lyophilized (mg)*
Lens total dry weight	70.67	64.00	68.50
Extract total dry weight	4.71	4.70	3.94
Total column fractions	4.52	4.18	3.82
Purified lipid	2.75	2.54	2.27
Neutral lipid	1.33	1.25	1.08
Cholesterol	1.20	1.20	1.11
Phospholipid	2.25	2.04	1.63
Estimated*	0.930	0.390	0.983

*Total μg P × 25 = estimated phospholipid.

cedures was acid-washed, and no detergent solutions were allowed to contact the glassware used for inorganic phosphate assay.

Results

The first experiment attempted to examine the effects of stirring overnight as compared to homogenization and the possibility of phospholipid loss during lyophilization. To this end, one of a pair of lenses was stirred overnight, the other was homogenized in 5 ml of buffer; two 2 ml aliquots were removed. One aliquot of the homogenate was extracted immediately and the other was lyophilized. The stirred lens was also lyophilized. The dried residues from both the homogenized and stirred lenses were suspended in water and extracted. All extracts were then subjected to the procedures described in Methods, and the results were calculated as total lens lipid.

Table I presents the dry weight data obtained at each step of the procedure calculated on a per lens basis. Several minor problems were apparent from an examination of the data. Variations in lens total dry weight were primarily sampling problems; that is, particulate matter tended to remain on the sides of the stirring vessel and the lyophilization flasks. This observation is probably responsible for the low values for extract weight and purified lipid weight, since these figures remained a constant percent of the corresponding figures for the homogenized-extracted sample. Comparison of homogenized-extracted and homogenized-lyophilized samples revealed only small differences directly attributable to lyophilization. Dry weight yields from the silicic acid column showed that more material was coming off the column than had been applied. Because the amount of cholesterol chemically assayed was very close to the weight of neutral lipid, it seemed probable that the methanol fraction was contaminated with significant fines from the column, which were included in the phospholipid fraction weight. However, very significant losses apparently occurred in the homogenized-lyophilized sample. Therefore the experiment was repeated with more emphasis on the effects of lyophilization.

In this experiment, a pair of 71-year-old normal human lenses were homogenized in 5 ml of 0.001M ammonium bicarbonate buffer. One homogenate was transferred to a 250 ml flask with two 1 ml rinses of buffer, and the other was transferred in the same manner and then diluted with 135 ml of buffer. The former lens, after methanol addition and removal of an aliquot for dry weight determination, was extracted immediately. The latter homogenate was divided among several bottles, rinsed, and lyophilized. After suspension in water and pooling, the residues were sampled for dry weight and were extracted. In this experiment, nitrogen entered the lyophilization apparatus instead of air, and methanol-washed silicic acid was used for chromatography.

The dry weight results are presented in Table II. In this experiment, there are significant differences only in those parameters where insoluble materials may, and in this case did, render sampling difficult, i.e., lens dry weight and extract dry weight. It is interesting that there was only a 1% difference

Table II. Human lens (71 year) fraction dry weights and chemical assay estimates

Fraction or constituent	*Homogenized-extracted (mg)*	*Homogenized-lyophilized (mg)*
Lens total dry weight	88.2	81.0
Extract total dry weight	3.30	4.57
Total column fractions	4.90	4.95
Purified lipid	3.20	3.18
Neutral lipid	1.55	1.37
Cholesterol	1.58	1.50
Phospholipid	1.87	1.62
Estimated*	1.31	1.47

*Total μg P × 25 = estimated phospholipid.

Table III. Human lens (64 year) fraction dry weights and chemical assay estimates

Fraction or constituent	*Stirred, low-speed centrifugation (mg)*		*Homogenized, high-speed centrifugation (mg)*	
	Precipitate	*Supernatant*	*Precipitate*	*Supernatant*
Lens total dry weight	18.667	35.267	22.400	34.400
Extract total dry weight	2.517	0.817	2.750	0.883
Total column fractions	2.734	1.517	2.716	1.367
Purified lipid	2.167	0.667	2.133	0.567
Neutral lipid	0.917	0.333	1.017	0.133
Cholesterol	0.790	0.155	0.856	0.113
Phospholipid	1.017	0.467	1.317	0.467
Estimated*	0.558	0.105	0.784	0.100

*Total μg P × 25 = estimated phospholipid.

between the two lenses in total yield from the dextran gel columns. Although the dry weights still exhibited significant variability between the two samples, the chemical assay figures between lenses were close enough (approximately 10%) to conclude that lyophilization does not result in significant loss of phospholipid or cholesterol.

Preliminary experiments with a pair of normal human lenses in which stirring and homogenization followed by dilution were investigated suggested that significantly more total lipid (79% vs. 62%) was isolated in the precipitate fraction of the stirred lens by a relatively low-speed centrifugation (1000 × g) than in the precipitate fraction of the homogenized lens centrifuged at the same speed. To further investigate this significant difference in membrane isolation, a pair of normal human lenses were stirred, and the resulting suspension was equally divided. One half was centrifuged at 1000 × g for 15 min and the other half was centrifuged at 3600 × g. Surprisingly, there was no difference (88% vs. 87%) in the amount of purified lipid extracted from the precipitate fractions when expressed as a percentage of the total lipid in each extract.

None of the experiments performed thus far are comparable to the usual homogenization followed by high-speed centrifugation. Therefore one further experiment was conducted with a pair of normal human lenses. In this case, one lens was homogenized and then centrifuged at 30,000 × g for 20 min; the other was stirred overnight and centrifuged at 1000 × g for 15 min. All fractions, both precipitate and supernatant, were lyophilized before extraction. The dry weight results are presented in Table III along with the chemical assay results for cholesterol and phospholipid. Although there are some variations between comparable samples, it is apparent that the precipitate sample from the stirred lens represents a similar membrane fraction that is obtained with much less effort and time.

However, an analysis of the precipitate phospholipid fractions from each lens dem-

Table IV. Human lens (64 year) phospholipid composition (μg) by chromatography

Constituent	*Stirred*		*Homogenized*	
	Precipitate	*Supernatant*	*Precipitate*	*Supernatant*
Origin*	7.1	1.8	7.9	3.9
Lysophosphatidyl choline	12.5	1.8	5.3	2.1
Lysophosphatidyl serine	28.1	2.1	5.3	1.5
Sphingomyelin	325	28.3	336	59.2
Lysophosphatidyl ethanolamine	37.1	6.0	97.6	12.0
Phosphatidyl choline	7.1	2.7	6.3	0
Phosphatidyl serine	5.5	1.2	14.4	.2
Phosphatidyl inositol	6.3	0	6.3	0
Phosphatidyl ethanolamine	12.3	4.5	53.4	5.1
Sum	441.0	48.4	532.5	84.0
Total phospholipid yield†	558 (79%)	105 (46%)	784 (68%)	100 (84%)

*Sample application point.
†μg P × 25.

Table V. Percent lipid in human and animal lens (mg lipid/mg dry weight)

Animal lenses	*Human lenses*		*Statistical significance*
	Homogenized	*Stirred*	
Rabbit 0.90	3.89	3.31	
Beef 0.87	3.97	5.25	
Mouse 1.10	3.63	3.80	
	3.93	4.30	
	3.93	3.80	
	4.75	4.20	
	3.46	4.20	
	3.90		
	3.48		
	$\bar{x} = \overline{3.88} \pm 0.17$	$\bar{x} = \overline{4.12} \pm 0.36$	N.S.
$\bar{x} = 0.96 \pm 0.12$	(Overall human lens $\bar{x} = 3.99 \pm 0.25$)		H.S.

N.S. = not significant; H.S. = highly significant.

onstrated significant losses in the stirred lens of phosphatidyl ethanolamine and phosphatidyl serine (Table IV). Furthermore, although the lysophosphatidyl serine was increased, the lysophosphatidyl ethanolamine fraction was also lower in the stirred lens precipitate. The yield of individual phosphatides from the stirred lens was higher, however, than that from the homogenized lens (precipitate fractions). Finally, because of the method used, it is possible to corroborate the results of an earlier publication[12] describing an assay of the lipid concentration of the human lens. The lipid concentration was calculated as the milligram of purified lipid per milligram dry weight. The human lens data were from an accumulated 15 experiments with single or pairs of lenses, both stirred and homogenized. In cases where the supernatant and precipitate were analyzed separately, the purified lipid values and fraction dry weights were added to obtain the total lens values. Animal lens values were obtained from one stirred beef lens, eight homogenized rabbit lenses, and a pool of 140 homogenized mouse lenses. These results and statistical evaluations of the results are presented in Table V.

Discussion

Cell disruption by stirring in a hypotonic medium is a valuable technique for the isolation of all membrane species, since the method is not as prone to vesicle formation as homogenization. Thus much lower centrifugation speeds may be used to isolate a comparable membrane fraction from a stirred lens. Stirring overnight, however, even at 4°

under nitrogen, introduces the possibility that compositional changes may occur. Evidence supporting a selective loss of phosphatidyl ethanolamine during stirring appears in Table IV, since loss of both the lyso- and complete forms were observed without a concomitant increase in estimated total lipid phosphorus. This observation strongly suggests that the phosphorus originally present in both structures was converted to a water-soluble form before extraction occurred, i.e., during overnight stirring. On the other hand, the small differences in total phospholipid introduced by this method would not seem significant unless individual phospholipids were specifically investigated, since the data in Tables I, II, and III for cholesterol and phospholipid are in general agreement with the values appearing in the literature.

Large-scale dilution has been proposed as a means of reducing contamination of lens membrane preparations by soluble proteins.[13] Although this step may introduce error into lens dry weight estimates because of adsorption losses of insoluble protein and associated lipid, the amount of error amounts to approximately 5% and affects experimental results only when absolute amounts are measured. Curiously, although confounded (in the statistical sense) with the effects of dilution, lyophilization of either homogenized or stirred lenses appears to reduce dry weight estimates of the lens but does not affect the absolute amounts of total lipid found (Table III). This observation suggests the presence of a volatile component in the buffered lens suspension, since the amount of buffer salts that are present in the homogenized, non-lyophilized lens preparations (79 μg) is not enough to account for the milligram-range differences observed.

The optimal method for human lens cell membrane preparation and subsequent lipid analysis thus appears to be homogenization in a small volume of aqueous medium (particularly with frozen, partially dehydrated lenses) followed by a high-speed centrifugation. Isolation of the precipitate and supernatant fractions for analysis results in a complete analysis of the lens lipids. Dissociation of fiber cell membrane and matrix may then be accomplished by urea treatment.

Finally, the significant difference in lipid concentration between those animal lenses investigated and human lenses may be, at least in part, a function of the size of the fiber cell. If human lens fiber cells are smaller in diameter than rabbit lens fiber cells, then more cells would be necessary to fill a comparable volume. However, in order to account for a fourfold increase in cell surface area for a given volume, the cell diameter must be reduced by a factor of 8. It seems unlikely that such a large disparity in cell size would be ignored by electron microscopists. Therefore it seems equally possible that the cytoplasmic matrix associated with the membrane may have a significant lipid content in the human fiber cell.

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