

THE ISOLATION OF RETINAL OUTER SEGMENT FRAGMENTS

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ABSTRACT

Bovine retinal outer segment fragments were isolated by density gradient centrifugation in a high centrifugal field. Assays of the final preparation for enzymes of the mitochondrial respiratory chain indicated mitochondrial contamination not in excess of 1 per cent. Glucose-6-phosphatase and TPNH-cytochrome *c* reductase activities, presumably diagnostic for microsomes, were also absent. Electron micrographs did not disclose the presence of significant numbers of particles other than fragments of the outer segment discs. The red fragments were characterized by an ascorbate-oxidizing system and a high lipid content.

Electron microscopic observations by numerous investigators (1-6) have established fundamental similarities between the ultrastructure of the outer segment of the retinal receptor and that of other membranous structures such as the mitochondrion and chloroplast. Wald (7-9), MacNichol (10, 11), and others have presented unequivocal evidence that the outer segment houses the carotenoid visual pigments required for vision in bright or dim light. Futterman (12-14) has undertaken to elaborate the mechanism which governs carotenoid maintenance and metabolism. Nonetheless, investigation of the device by which the energy of light absorbed by the carotenoid is ultimately transduced into an electrical impulse has been very meagre. The starting assumption of our studies has been that this device must have certain enzymic principles in common with energy-conserving mechanisms found in organelles possessing similar membranous ultrastructure.

While all investigators may not share this assumption, all must agree that the serious study of the enzymology of this unique organelle demands its physical isolation from contaminating particles. It now appears inescapable that such isolation has not been achieved in the past. The standard su-

crose suspensions of outer segments, from which extracts of the visual pigments are classically derived (8, 9, 15-18), are almost certainly contaminated by mitochondria. Our own observation of the light sensitivity of the ATPase found in digitonin extracts of rhodopsin (19) must in due course be reexamined on this account. The purpose of the present communication is to describe the methodology we have used to isolate the outer segments, and to present the criteria used in evaluation of the completeness of the separation.

Many investigators have used one or more modifications of the method of Saito (20), which entails breaking off large numbers of outer segments, or their distal fragments, by shaking the retina in dilute sucrose and then filtering the suspension through gauze. Despite claims made for this method, we have found that the outer segment does not invariably break free of the mitochondria-laden inner segment. This appendage, complete with connecting cilium and enclosed mitochondria, can be seen with the electron microscope in suspensions of outer segments prepared in this way. The inner segments are not removed by gauze filtration, and are not easily removed by sedimentation.

Some investigators prefer homogenizing the whole retina with subsequent elimination of contaminating particles by sedimentation at low g , sometimes on a density gradient. This method, in our hands, results in the retention of substantial mitochondrial contamination, evidently for two reasons. First, homogenizing tends to make the elongated outer segments form balls, sometimes with several segments enmeshed together. Mitochondrial fragments, present in any starting preparation, appear to become trapped inside the balls and are subsequently eliminated only after vigorous homogenizing. This treatment separates the individual outer segment discs from the enclosing plasma membrane within which they were stacked. Second, even prolonged sedimentation at low g (typically 1100 to 1800 g , see reference 18) will not ordinarily eliminate mitochondria from 30 per cent sucrose suspensions of outer segments (which are normally isopyknotic in 30 per cent sucrose). The fact that they can be eliminated by thorough grinding followed by high speed centrifugation is demonstrated in the present report.

Biochemical and electron microscopic criteria were used to determine the success of the methods. Much of the analysis and electron microscopy were performed on material sedimented on a layered sucrose density gradient which permitted a simple, approximate estimation of the nature of the particles sedimenting to each layer. However, a continuous linear density gradient was ultimately selected for routine daily isolations.

MATERIALS AND METHODS

RETINAL PREPARATIONS: Adult cattle eyes were enucleated within a few minutes following decapitation and were immediately placed on ice in darkness. All subsequent handling of material was carried out at 0 to 5°C and under dim red light. After 1 to 6 hours, retinas were excised and dropped into either a 45 or 30 per cent sucrose solution 0.01 M in Tris-acetate (pH 7.5) at a ratio of 1 retina per ml of solution. In either solution retinas were very briefly homogenized with the Thomas glass and Teflon mortar and pestle before centrifugation for 15 minutes at 1100 g . The supernatant suspension of crude retinal outer segments (ROS) was retained (this procedure was repeated with the supernatant suspension from the run in 30 per cent sucrose until no further sediment was observed). Both 45 and 30 per cent suspensions were diluted to a sucrose concentration of 15 per cent with 0.01 M Tris-acetate and sedimented for 15 minutes at 2500 g to ensure packing of the pellet. The supernatant fluid was removed and the

pellet was rinsed and suspended in 0.25 M sucrose-Tris-acetate, pH 7.5. This suspension was vigorously homogenized, centrifuged, and the sediment taken up in a minimal volume of 0.25 M sucrose-Tris. This suspension usually containing about 30 mg of protein in a volume of 3 ml, was split into 3 volumes of 1 ml, each of which was pipetted gently onto the top of a sucrose density gradient consisting of either (a) four discrete layers of densities 1.12, 1.14, 1.16, and 1.18 occupying a total volume of 4 ml; or (b) a continuous gradient varying linearly in density from 1.11 to 1.13 and occupying the same volume. These gradients were then centrifuged in the Spinco SW-39L (Swinging Bucket) rotor at maximum speed ($R_{av} = 131,000 g$) for periods of 15 minutes to 1.5 hours. The bands were removed by either of two methods. One involved inserting a hypodermic needle through the side of the tube at the base of the band and permitting the contents to drain out slowly. Because of the wide separation of the bands, in the case of the layered gradient, it was as reliable and much easier to remove all bands from top to bottom by careful syringing with a disposable pipette.

RECOMMENDED METHOD: The results to follow have led to adoption of the following routine method for isolating the ROS discs. A brief homogenization of retinas in 45 per cent sucrose is followed by sedimentation at 1100 g . The supernatant suspension is then diluted with 0.01 M Tris-acetate to 15 per cent with respect to sucrose and is centrifuged again at 1100 g . The pellet is rinsed and resuspended in a minimal volume of density 1.10 sucrose-0.01 M Tris-acetate and is vigorously homogenized for two minutes at 0°C. The suspension is gently pipetted onto the top of a linear gradient with density continuous from 1.11 to 1.13 in each of 3 5-ml tubes. The tubes are then centrifuged at 39,400 rpm in the SW-39L rotor of the Spinco Model L centrifuge for 30 minutes. Only the thick red band at the top of the gradient is removed. A spectrophotometric assay for cytochrome oxidase activity is then run. If mitochondrial contamination is detected, homogenization and density gradient centrifugation can be repeated until an arbitrary criterion has been satisfied. The resulting suspension can be frozen till used. The method yields a total of 20 to 25 mg of protein from 80 adult cattle eyes.

CONTROL PARTICLES: Heavy beef heart mitochondria (HBHM) were prepared by the method of Hatefi and Lester (21); submitochondrial electron transport particles (ETP_H) by the method of Smith and Hansen (22), and liver microsomes by the method of MacLennan *et al.* (23). Mitochondrial phospholipid was prepared by the method of Fleischer *et al.* (24).

ENZYMIC ASSAYS: DPNH oxidase and succinoxidase were assayed polarographically using the

methods of Hatefi *et al.* (25). The net respiration, expressed as $m\mu$ atoms of oxygen taken up in 1 minute, was computed by subtracting the endogenous rate from the rate observed in the presence of substrate. Specific respiration was computed by dividing the net rate by the mg of protein introduced in the particle sample.

The oxidation of ascorbate was measured polarographically at 30°C in a final volume of 2 ml. The particle suspension (0.2 ml) was added to 1.7 ml of a solution which was 0.68 M in sucrose, 2.7 mM in ADP, 1.8 mM in ATP, 13.6 mM in $MgCl_2$, 54.5 mM in glucose, and 22.7 mM in Tris-acetate, pH 7.5. The particle was allowed to equilibrate until its endogenous rate had been established. Potassium ascorbate (0.1 ml of 0.1 M) was added and the new rate was recorded. Control measurements were made in the absence of particle, and spontaneous ascorbate oxidation, if any, was subtracted before calculation of the net rate of the particle. Specific respiration was computed as in the preceding assays.

Cytochrome oxidase was assayed at 38°C in the Beckman DU Spectrophotometer according to the method of Wharton and Griffiths (26). First order rate constants were estimated as recommended by Smith (27).

Succinate-cytochrome *c* reductase was assayed at 38°C in the DU spectrophotometer by the method of Hatefi *et al.* (28). TPNH-cytochrome *c* reductase was assayed by the same method, TPNH being substituted for succinate.

ATPase was assayed in the light at 37°C in the Dubnoff shaker, the medium containing 0.05 ml of M Tris-acetate, pH 7.5, 0.03 ml of 0.1 M $MgCl_2$, 0.1 ml of 0.1 M ATP, 0.1 ml of particle, and 0.72 ml of H_2O . After a 5 minute preincubation of other components in the shaker, the reaction was initiated by addition of either particle or ATP (both at 0°C), carried out for 10 minutes, and terminated by the addition of 1.0 ml of 1.5 M $HClO_4$. Following removal of protein by centrifugation, an aliquot of 0.5 ml of the supernatant was taken for the determination of phosphate. Control reactions with substrate and no particle, and *vice versa*, were similarly treated. In one series of experiments, oligomycin (Wisconsin Alumni Research Foundation, Madison, Wisconsin) in 50 per cent ethanol was added to the assay in the ratio of 2 μg per mg protein. Control assays contained 50 per cent ethanol only.

Glucose-6-phosphatase was assayed at 37°C in a medium (final volume 1.0 ml) containing 0.10 ml of 10 per cent glucose-6-phosphate in 0.01 M Tris-acetate, pH 6.5 (29), 0.80 ml of H_2O , and 0.10 ml of the particle suspension. This mixture was incubated for 15 to 30 minutes, depending on the particle, and the reaction was terminated by addition of $HClO_4$. Subsequent procedures and controls were the same as for the ATPase assay.

CHEMICAL METHODS: Inorganic phosphate was determined by the method of Lindberg and Ernster (30). Protein was determined either by the biuret method of Gornall *et al.* (31) or by the method of Lowry *et al.* (32). Lipid phosphorus was determined after the extraction of particles in chloroform:methanol by the method of Fleischer *et al.* (24).

By the use of a method similar to that of Wharton and Tzagoloff (33), copper was determined in samples of the particle suspensions after these had been digested over a flame in 0.2 ml of a 50:50 mixture of reagent grade sulfuric and nitric acids. The charred black solution was further oxidized and decolorized by the addition of 0.1 ml of 71 per cent $HClO_4$, and heating was continued until clear. With thick suspensions, additional perchloric acid was necessary. To the colorless solution were added, with shaking, 0.1 ml of 5 per cent thioglycolate, 0.5 ml of 0.1 per cent 2,2-biquinoline in glacial acetic acid, and 1.0 ml of saturated sodium acetate. Finally, 1.0 ml of isoamyl alcohol was added. After vigorous shaking, the reaction mixture was centrifuged and a 1.0 ml sample was pipetted from the alcohol layer. A positive reaction was indicated by a pink color ($\lambda_{max} = 535 m\mu$). The blank and copper standard contained, respectively, water and 0.01 mg per ml of copper, equal in volume to the particle aliquot. Copper was also determined by the electron paramagnetic resonance (ESR) signal, with the collaboration of Professor Helmut Beinert of the Enzyme Institute.

Iron was determined by a method precisely analogous to the spectrophotometric copper method, except that the color-developing reagent was bathophenanthroline (4,7-diphenyl-1,10-phenanthroline) instead of biquinoline.

Visual pigment was determined in the Beckman DK-2 recording spectrophotometer, in a medium containing 0.05 ml of the particle suspension, 0.03 ml of 10 per cent deoxycholate, pH 8.2, and 0.92 ml of 0.01 M Tris-acetate, pH 7.5. After the initial spectrum was taken, 0.01 ml of M $NH_2OH \cdot HCl$ (neutralized with M Tris) was added to the medium and a second spectrum taken. The contents of the cuvette were then bleached by a 1 minute exposure, at a distance of 20 cm, to a 150-watt tungsten spotlight, after which the final spectrum was taken. The reference cuvette in each case contained all the components except the particle. In some cases, sodium thiosulfite (dithionite) or potassium ferricyanide was added in an attempt to demonstrate reduction or oxidation of hemes through their difference spectra.

Carotenoids were estimated in aliquots of the particle suspensions which had been extracted and saponified by boiling, until clear, in a medium containing 2.0 ml of 95 per cent ethanol, 0.2 ml of 12 N KOH and 2.0 ml of reagent grade $CHCl_3$. Ten volumes of water were added and the mixture, after boiling until free of chloroform, was acidified with

10 N H₂SO₄. It was subsequently extracted with anhydrous ethyl ether, the aqueous phase was removed, and the ethereal extract was dried with anhydrous sodium sulfate after several water washes. The ether was removed by evaporation. The residue was taken up in 1.0 ml of CHCl₃ and was observed in the DK-2 spectrophotometer against a CHCl₃ blank. The contents of the cuvette were next emptied, at room temperature, into a test tube containing 2.0 ml of saturated SbCl₃ in CHCl₃ (Carr-Price reagent, reference 34). The spectrum of this solution was observed in the DK-2 spectrophotometer against a blank containing only the Carr-Price reagent. (A positive test gave a band with λ_{max} at 662 m μ .)

SONICATION: Particles were sonicated as required, using the Branson probe sonifier at maximum amplitude for 15 seconds.

ELECTRON MICROSCOPY: Specimen grids were covered with thin parlodion film followed by a light coat of carbon. Suspensions of particles were dropped on the grid with a pipette, and excess fluid was removed with filter paper. Immediately, 1 or 2 per cent phosphotungstic acid, pH 7.3, freshly filtered through a 100 A Millipore filter, was dropped onto the surface of the grid, where it remained for 15 seconds to 2 minutes before it was blotted dry with filter paper. The micrograph in Fig. 5 was taken on a Kodak medium contrast plate at 50 kv in the RCA EMU-3E microscope in the Department of Anatomy at the University of Wisconsin, by courtesy of Dr. David B. Slautterback. The other micrographs were taken on Kodak contrast plates at 75 kv in the Hitachi HU-11B microscope at the Enzyme Institute.

Thin sections of osmium tetroxide-fixed pellets sedimented from suspensions were examined during early stages of the isolation effort. Intact mitochondria, and ROS fragments which still retained stacked discs, were unequivocally identifiable in sections. Isolated discs and mitochondrial fragments were, however, extremely difficult to differentiate. After vigorous homogenizing, this task was essentially hopeless. Negative staining was adopted as the method of choice, not merely because it was much quicker but because it permitted more accurate determination of the composition of fractions.

RESULTS

Fig. 1 is a photograph of the gradients at the end of an experiment. The top band (1.12) in the layered gradient (right) was brilliant red, with pink material trailing down through the lower portion of the 1.12 layer. The bands at 1.14 and 1.16 were white, and the pellet (1.18) was tan. In the continuous density gradient, the thick band at the top (1.11) was again red, the pellet tan, and the intermediate material pink. No gross differences were observed between the 45 and 30 per cent suspensions with respect to their appearance on either gradient.

Table I summarizes the DPNH, succinate, and ascorbate oxidizing activities of particles drawn from each band when the 45 per cent suspension was fractionated. The absence of both DPNH oxidase and succinoxidase activities in the red band

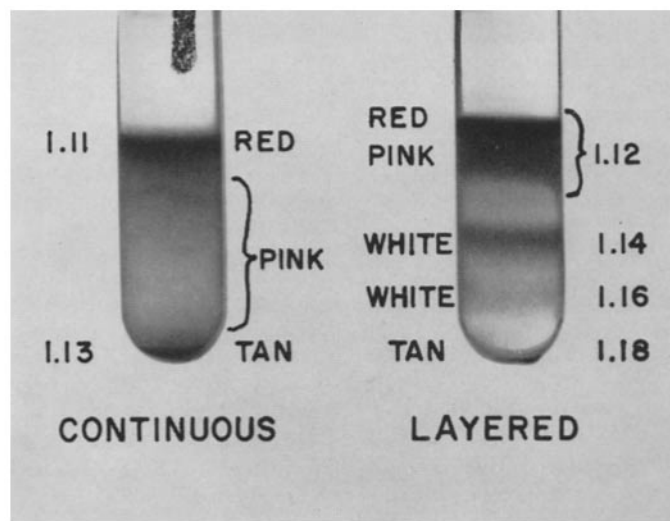


FIGURE 1 Photograph of 5-ml cellulose centrifuge tubes after completion of 1.5-hour run at 131,000*g* in the Spinco SW-39-L rotor. The sucrose-Tris gradient on the left was continuous from densities 1.11 to 1.13. The gradient on the right combined layers of densities 1.12, 1.14, 1.16, and 1.18.

demonstrated the lack of appreciable mitochondrial content. However, this band contained a vigorous ascorbate oxidizing activity. In the 1.14 band substantial mitochondrial content was indicated, in addition to the presence of a particle which oxidized ascorbate more rapidly than did the red particle. Neither HBHM nor ETP_H oxidized ascorbate in the assay. The possibility that retinal mitochondria differ in this capacity from heart mitochondria seems remote, since in the 1.18 pellet, where intact heavy retinal mitochondria had sedimented, the ascorbate oxidation relative to DPNH oxidation was fractional. The 1.16 band had only very low activities in any of the three assays.

TABLE I

Specific Respiration (Expressed as $m\mu$ Atoms of Oxygen Taken up per min. per mg of Protein) Observed in Each of the Four Bands of Fig. 1 (right)

Layer	Substrate		
	DPNH	Succinate	Ascorbate
1.12	0	0	213
1.14	262	138	246
1.16	9	0	26
1.18	137	75	38

Small amounts of residual mitochondrial contamination could not be satisfactorily estimated by the polarographic technique. For this purpose, either the succinate-cytochrome *c* reductase or cytochrome oxidase assay was used because of its higher sensitivity. Table II shows the estimated per cent of mitochondrial contamination in the red band as a function of time of centrifugation in the continuous density gradient of Fig. 1. Total protein sedimented below the red band after $\frac{1}{2}$ hour of centrifugation was about 26 per cent of the protein in the original uncentrifuged suspension. The first order rate constant for the cytochrome oxidase activity of the uncentrifuged 45 per cent suspension, expressed as \ln change in absorbance $\text{min.}^{-1} \text{mg}^{-1}$ protein, was 22.53. The corresponding rate constants for the other suspensions were divided by this number, and the quotients were multiplied by 26 per cent to obtain the estimated contamination. Since this method assumes that all the lower particles are of mitochondrial origin, the estimate represents a maximum. It is clear from Table II that the suspension in 30 per cent sucrose

TABLE II

Estimated Maximum per cent (by weight) of Mitochondrial Contamination Found in Two Sucrose Suspensions of ROS, as a Function of the Length of Time of Centrifugation in the SW-39L Rotor at Maximum Speed

Sucrose concentration	Duration of centrifugation	Maximum contamination
<i>per cent</i>	<i>min</i>	<i>per cent</i>
45	0	26.0
	15	1.29
	30	0.74
30	90	0.61
	0	22.9
	15	0.35
	30	0.16
	90	0.12

Particles originated in either 45 per cent or 30 per cent sucrose suspensions.

retained a major mitochondrial content not substantially lower than that of the less rigorously prepared suspension in 45 per cent sucrose. However, with either preparation, contamination dropped to 1 per cent or less within 15 minutes at 131,000 *g*. Removal of detectable mitochondrial contaminant remaining after 90 minutes was completed after rehomogenization and resedimentation of the red material on the continuous density gradient.

Comparison of the cytochrome oxidase activity of the 1.14 band to that of the 1.18 pellet revealed, as in the case of the polarographically assayed activities of Table I, that the particles in the 1.14 band were more active than those in the pellet. Estimate of per cent mitochondrial content by the same method as above would therefore lead to a figure greater than 100 per cent. The increased electron transport activity of the 1.14 particles (a common attribute of mitochondrial fragments) constitutes a second reason for regarding the estimates of Table II as maxima, since the mitochondrial fragments in the isolated fractions were probably also more active.

Microsomal content of the crude suspensions, as determined by the glucose-6-phosphatase assay, was negligible: free inorganic phosphate attributable to a protein aliquot of 1 mg in the absence of substrate was no greater than 24 $m\mu$ moles; free phosphate found in the substrate was 48 $m\mu$ moles for an aliquot of 25.6 μ moles of substrate. Free

phosphate measured after incubation of protein and substrate together in no case exceeded the total of these two figures, 72 $m\mu$ moles. Liver microsomes in the same assay system liberated 6.6 μ moles of phosphate per mg protein in a 15 minutes incubation period. The TPNH-cytochrome *c* reductase activity of the crude suspensions was also zero. Liver microsomes reduced cytochrome *c* at a rate of 56 $m\mu$ moles min^{-1} mg^{-1} protein.¹

Both the biquinoline test and the ESR signal failed to demonstrate the presence of copper in the red band. This band contained 2 $m\mu$ moles of iron per mg protein.

pigment absorbing in the visible range (probably largely lipid, see reference 37) but different from rhodopsin or the products of its bleaching.

A sharp "opsin" peak at 278 $m\mu$ was in evidence in the red band. The ratio of extinction at 278 $m\mu$ to that at 500 $m\mu$ ranged between 4 and 8 in independent determinations with the preparations of 4 different days. The figure 2.2 is provided for pure rhodopsin by Wald (8).

Chloroform extraction and saponification of the red particles yielded a clear yellow solution containing substantial amounts of retinal ($\lambda_{\text{max}} = 390$ $m\mu$ in chloroform) and retinol (325 $m\mu$). A posi-

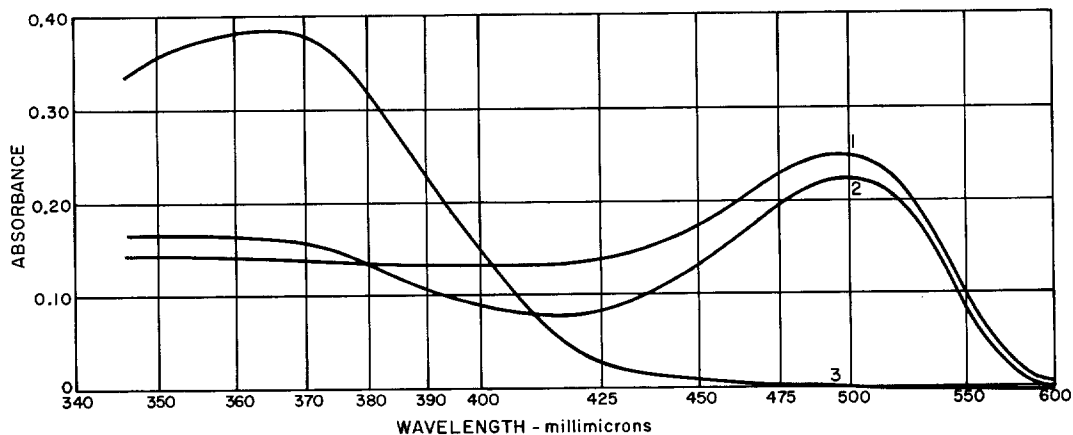


FIGURE 2 Absorption spectra of the red particle from the 1.12 band (Fig. 1) after protein dispersion with deoxycholate: 1, particle in sucrose-Tris + DOC; 2, the same as 1 + NH_2OH ; 3, the same as 2 + light bleaching.

The visual pigment of the red particle from the 1.12 band is depicted in Fig. 2. The spectral changes between (1) and (2) indicate formation of the oxime between hydroxylamine and free retinal ($\lambda_{\text{max}} = 365$ $m\mu$). The latter is derived from indicator yellow (35, 36), a bleached form of rhodopsin. Bleaching (3) released the remainder of the retinal as the oxime. The ratio of the absorbancies at 400 and 500 $m\mu$ (calculated from 2), often used as an inverse index of the purity of rhodopsin extracts, was almost double that found in digitonin extracts of dried, lipid-extracted outer segments (around 0.2) (8). This indicates the presence in the red particle of a substantial quantity of

¹ Observations (to be published) made subsequent to acceptance of the present report indicate that glycolytic activities, possibly microsomal in origin, are present in the crude ROS, but are eliminated by subsequent isolation procedures.

Carbonyl test was obtained. Only trace amounts of carotenoids were detected in corresponding extracts of the three lower bands, and the Carr-Price test was negative. The concentrations of retinal and retinol per mg starting protein were determined from the molar extinctions of the 1.12 band extracts. These ratios added to 3.4×10^{-9} M carotenoid per mg protein, or about $\frac{1}{4}$ the ratio of retinal to protein provided for pure rhodopsin by Hubbard (15). Probable loss of carotenoid during repeated extractions and washings prevents use of this figure in preference to that derived from direct spectra above (between $\frac{1}{2}$ and $\frac{1}{4}$), for estimating the visual pigment content of the ROS discs relative to rhodopsin.

Difference spectra in the presence of dithionite or ferricyanide provided no evidence of the presence of hemes in the red band.

No indication of visual pigment was found in

the direct spectra of deoxycholate-dispersed particles taken from the lower bands, either in the visible or in the ultraviolet. In the UV, λ_{\max} for each of these three bands was well under 278 $m\mu$. Hydroxylamine and bleaching by light did not alter the spectra of these bands, but in the 1.14 band and the tan pellet, small humps were detected corresponding to the Soret bands of mitochondrial hemes.

The ATPase activities of the four bands are given in Table III. The activities of the middle bands were substantially higher than those of the top and bottom bands. Table IV shows that both the total ATPase and its oligomycin sensitivity were sharply reduced in the red ROS suspension as mitochondria were sedimented from it. By comparison with the crude suspension, total ATPase of

TABLE III
ATPase Activity of the Particles Found in the Four Bands of the Density Gradient of Fig. 1 (right)

Layer	Liberation of phosphate in $m\mu$ moles $\text{min}^{-1} \text{mg}^{-1}$ protein
1.12	130
1.14	248
1.16	203
1.18	169

The particles originated in a 45 per cent sucrose suspension of outer segments.

the 1.12 band on the layered gradient was reduced 59 per cent, oligomycin sensitivity by half, and the protein:rhodopsin extinction ratio by half, while mitochondria were reduced from 16 to 2 per cent. Use of the continuous gradient reduced total ATPase an additional 7 per cent, oligomycin-sensitive ATPase to a statistically non-significant amount, and mitochondria to less than 1 per cent. The slight increase in the extinction ratio in the 1.11 band is probably due to increased lipoprotein (see below) in this lighter fraction. While the extinction ratio underwent a 50 per cent reduction as the crude suspension was fractionated on the layered gradient, the corresponding ratio in which protein was estimated in milligrams (Folin's test, reference 32) was reduced only about 30 per cent: a figure more consistent with the determination of total protein sedimented below the red band (26 per cent, Table II).

The lipid phosphorus content of the four bands of the layered gradient is estimated in Table V.

TABLE IV

Comparison of Total ATPase Activity, Its Component Inhibited by Oligomycin, Mitochondrial Content, and Protein:Rhodopsin Extinction Ratio in Each of Three Progressively Refined ROS Suspensions

ROS suspension	(1) Total ATPase	(2) Inhibition by oligomycin	(3) Mitochondria	(4) $\epsilon_{278}/\epsilon_{500}$
	<i>per cent of crude</i>	<i>per cent</i>	<i>per cent</i>	
Crude	100	68 ± 3 ($p < 10^{-3}$)	16.1	12.6
1.12 Layered	41	36 ± 2 ($p < 10^{-3}$)	2.2	6.3
1.11 Continuous	34	22 ± 13 ($p = 0.14$)	0.6	7.7

Gradients were prepared as described in the text, the centrifugation being carried out for $\frac{1}{2}$ hour. The top red band was analyzed from both a layered and a continuous gradient derived from the same crude 45 per cent suspension. All material on a third gradient of the same origin was sedimented after dilution, and resuspended in 0.25 M sucrose-0.01 M Tris-acetate, pH 7.5, to reconstitute the original crude suspension. Total ATPase (without oligomycin) is represented as a per cent of the specific activity of the crude suspension. Per cent inhibition was calculated by comparing specific activities of the ATPase with and without oligomycin. The figure in parenthesis refers to the probability (confidence level) associated with a t for independent measures; degrees of freedom in each case were 6. Maximum per cent mitochondria was estimated by comparing the cytochrome oxidase rate constant of each suspension with that of the tan pellet, assuming the latter contained 100 per cent mitochondria. The ratio (4) compares the extinction coefficient (ϵ) of protein absorbing at 278 $m\mu$ with that of unbleached rhodopsin, as measured by the difference spectrum (2 minus 3, Fig. 2) in the presence of NH_2OH .

The red 1.12 band contained the highest phospholipid content and the 1.18 pellet the lowest. The fact that the standard errors for these two bands were also substantially below those calculated for the other two bands indicates greater uniformity in these particles than in those of the two intermediate bands. The mean lipid phosphorus of the red band corresponds to a figure of about 42 per cent total lipid (dry weight determinations of neutral and total lipids for the highly purified ROS preparation (37) indicate about 33 μg phosphorus per mg

TABLE V

Ratios of Lipid Phosphorus to Protein (expressed as $\mu\text{g P}$ per mg protein) in the Particles Found in the Four Bands of the Density Gradient of Fig. 1 (right)

Layer	$\mu\text{g P}/\text{mg protein}$ (average)	SE
1.12	23.7	1.9
1.14	18.0	4.7
1.16	21.5	11.6
1.18	10.6	2.2

Particles were derived from a 45 per cent suspension of retinal outer segments. Three independent determinations (different preparations and separate gradients) were made, the means appearing in the second column, and the standard errors in the third.

lipid). For the 1.11 band of the continuous gradient, the figures were $33.2 \pm 3.6 \mu\text{g P}/\text{mg protein}$, corresponding to 50 per cent total lipid.

ELECTRON MICROSCOPY: Fig. 3 is a micrograph at low magnification of a typical field consisting of particles from the 1.12 layered band. In the center can be seen a labyrinth of twisted and elongated fragments of the ROS discs. At other points in the field (arrows) can be observed fragments in which morphology is less deranged. Such a group of fragments is seen at high magnification in Fig. 4. Despite the breaking up by homogenizing of the stacked arrangement of the intact discs, the surfaces of the fragments still display the characteristic mosaic (1, 2) of tightly interdigitated subunits, the centers of which are usually separated by about 50 A, though wider spacing can be observed. At the edges of some fragments (arrows) 2 or 3 distinct rows of 50 A subunits can be seen. These may represent the margins of adjacent lobules of a single disc. In the intact receptor the average diameter of the discs (as revealed by unpublished thin sections) was $1.5 \mu : 37.5$ times the 400 A marker in this micrograph.

Occasional fields were observed in which morphologically more deranged fragments were encountered than are to be seen in Fig. 3. These forms resembled myelin figures which were observed in negatively stained preparations of mitochondrial phospholipid (38). Typical ROS myelin figures can be seen in Fig. 5, together with a fragment of an intact ROS. Examples of the latter were rarely seen after thorough homogenizing, and the frequency of the myelin forms increased with

homogenizing. These forms demonstrated well defined edges, unlike the mitochondrial phospholipid, and much more variability in shape than the latter. A second form of the mitochondrial phospholipid exhibited a superficial similarity to the ROS fragments. However, the concentrated phospholipid forms were more diffuse, their edges were incomplete, and the subunit sizes were, in general, no larger than 35 A and in some cases considerably smaller (38).

Sonication of the most carefully isolated suspensions of ROS fragments (Fig. 6) resulted in deterioration of the neat mosaic, together with the appearance of numbers of subunits of about 100 A diameter (arrow).

The white 1.14 band contained 3 categories of particles as viewed under the electron microscope. Perhaps 40 per cent of the particles (as estimated from several grids prepared from each of at least 4 different days' samples) were quite similar to those of Fig. 6, and in some cases were even less disorganized. These particles were tentatively identified as disrupted ROS fragments. To a second category, comprising perhaps 20 per cent of the total, were assigned fragments which appeared clearly recognizable as mitochondrial in origin, with tubular cristae and elementary particles (EP) (39). The remainder of the particles could not clearly be placed in either of the foregoing categories. Numerous subunits were observed on membranes in various stages of disorganization, but judging only by electron microscopic appearance these could have originated from either the discs or the mitochondria, and even elsewhere in the retina, had the minimal homogenizing given the retinas in the first step caused major fragmentation of any but the visual receptor cells.

In the white 1.16 band, the same 3 categories of particles were observed, except that particles tentatively designated as ROS fragments were judged with considerably less conviction than in the 1.14 band. One such ambiguous particle (*D*) appears in Fig. 7. The lower fragment appears to be that of a mitochondrial crista with recognizable EPs (arrow).

When the tan pellet at density 1.18 was taken up in 0.25 M sucrose-Tris and homogenized, a few fragments were identified which could have been called ROS fragments or their derivatives. The overwhelmingly predominant particle was an intact, compact mitochondrion which sequestered or "puddled" the phosphotungstic acid so as to effectively prevent examination of internal detail.

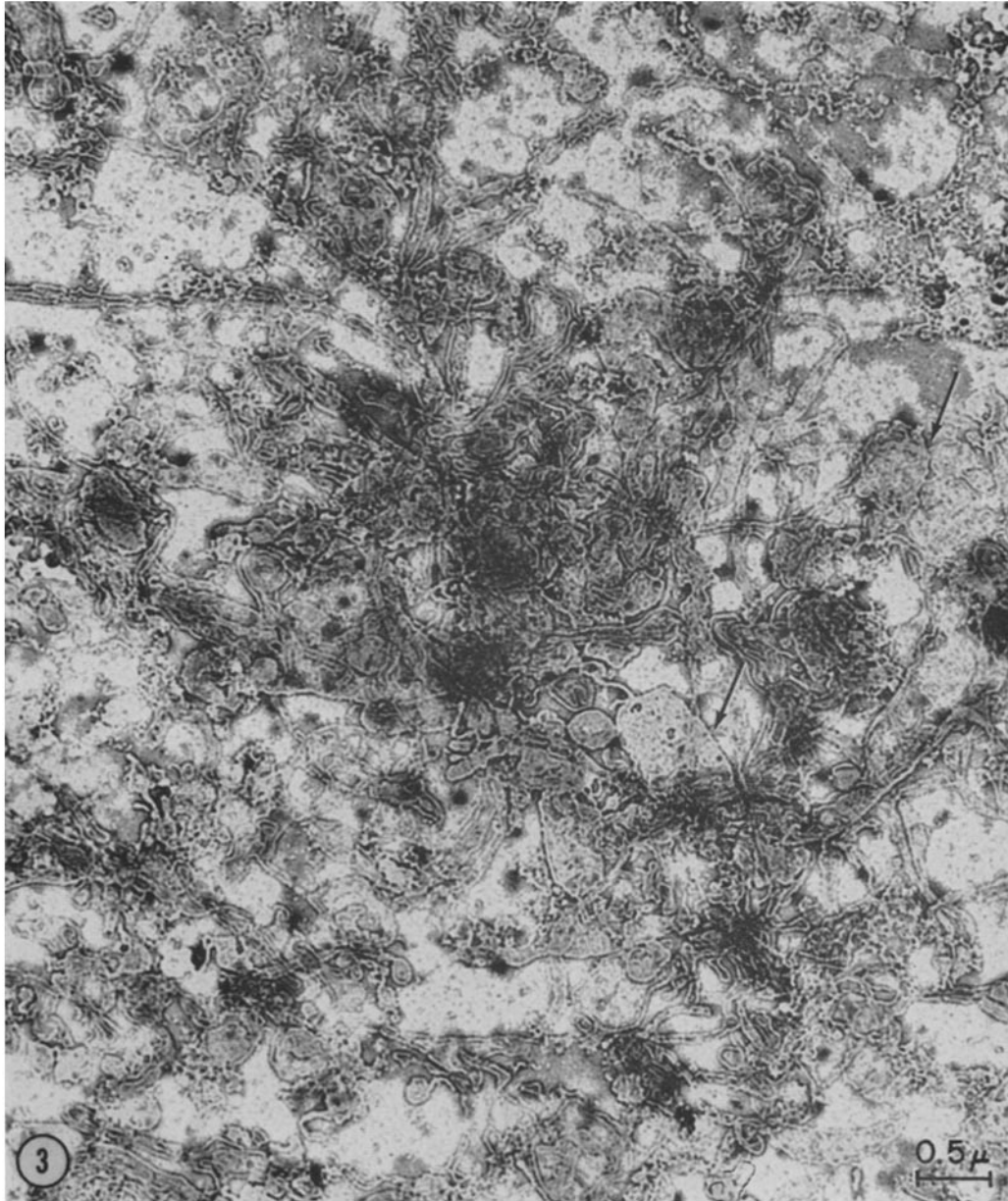


FIGURE 3 Representative field of particles from 1.12 band of layered gradient. Arrows denote ROS disc fragments in which characteristic morphology has survived homogenization. $\times 21,000$.

This detail was easily revealed when sonication of the mitochondrial suspension preceded staining.

The composition of each of these bands, as judged by electron microscopy, was not distinguishably different whether the original suspension was in 45 or 30 per cent sucrose during the 1100 *g* sedimentations.

DISCUSSION

The fact that only minor differences (Table II) were found in the extent of mitochondrial contamination, whether debris was sedimented once from a 45 per cent sucrose suspension or repeatedly from a 30 per cent suspension, testifies to the diffi-



FIGURE 4 ROS disc fragment from the 1.12 red band at the top of the layered gradient. Arrows point to parallel rows of subunits at edges of the lobules. $\times 210,000$.

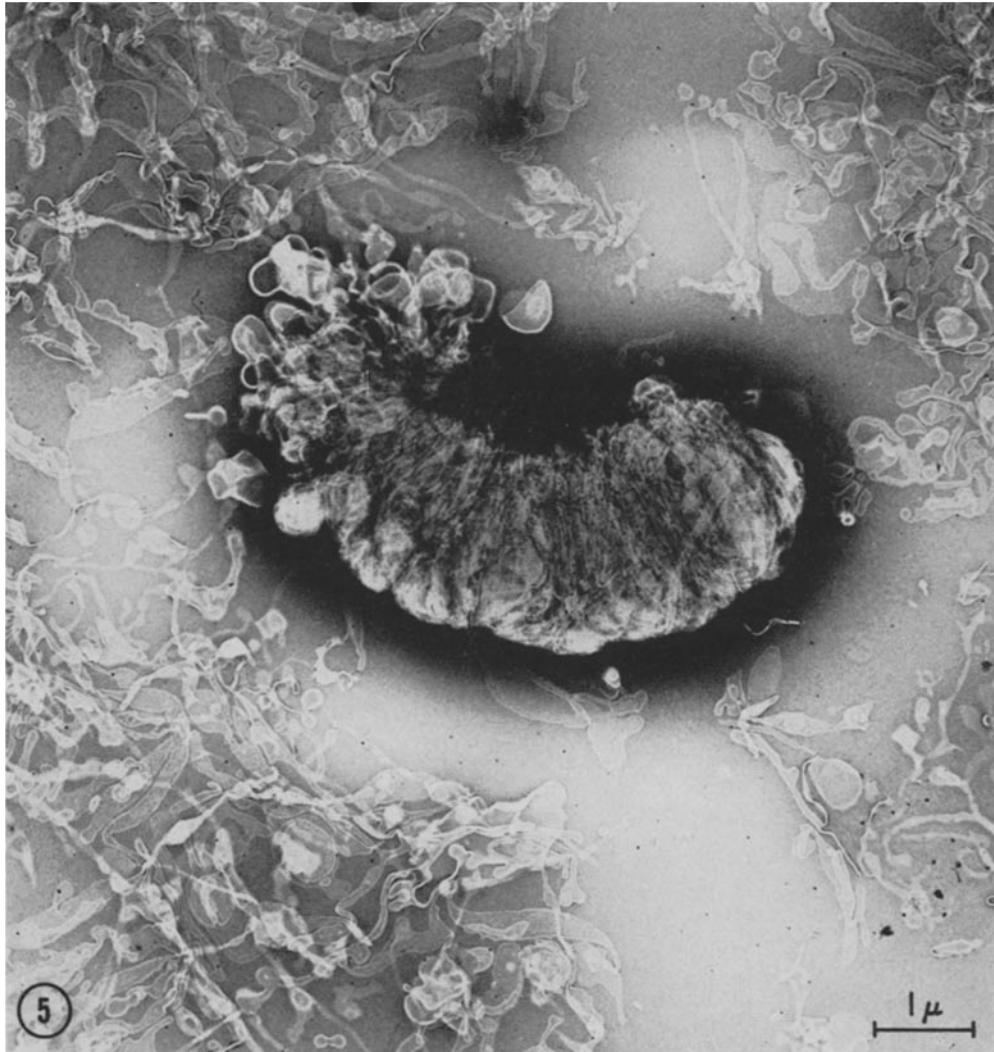


FIGURE 5 Myelin figures and a fragment of an intact ROS from the 1.12 band of the layered gradient. Individual discs can be seen breaking free from the stacked arrangement prevailing within the intact ROS. $\times 13,650$.

culty of separating mitochondria from outer segment suspensions in the absence of a high centrifugal field.

The results of the critical assays permit us to exclude mitochondria and microsomes as important contaminants of the red band prepared by careful fractionation. If other contaminants survive, they are not discernible in the micrographs.

A disproportionately large contribution by mitochondria to the ATPase activity of crude ROS suspensions is apparently reflected both by the reduction of total ATPase as fractionation proceeds

on the gradient, and by the concomitant loss in sensitivity to oligomycin. The residual ATPase of the purified ROS fraction is nonetheless substantial. The fact that the activity is lower in this fraction than elsewhere on the gradient may be due not only to the greater activity of mitochondria but to the possibility that intact discs or disc lobules hydrolyze ATP less vigorously than their disrupted fragments. Such a relationship was established for mitochondria by Lardy and Wellman (40).

The possibility cannot be entirely excluded that the disproportionality between mitochondrial con-

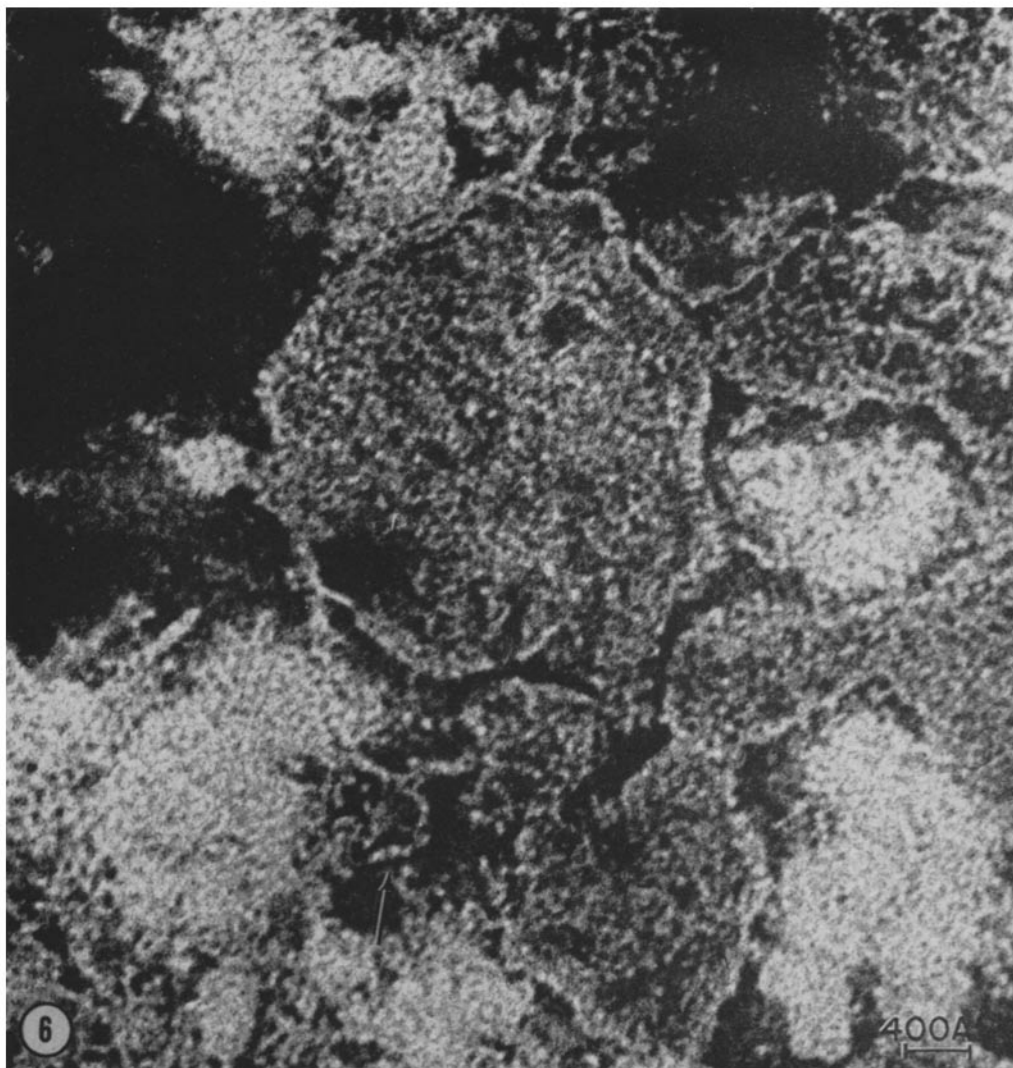


FIGURE 6 ROS disc fragments which have been isolated on the continuous gradient of Fig. 1, frozen for storage, and, after several days, thawed and sonicated. Morphology of the discs is only slightly de-ranged by freezing and thawing alone. Arrow points to three adjacent 100 A subunits, more of which can be seen in the interior of the central fragment. $\times 225,000$.

tent (as determined by the cytochrome oxidase assay) and oligomycin sensitivity results from a contribution to the latter by either of two species of particles which might sediment below the red band: (a) a mitochondrial fragment which no longer transports electrons but retains a high oligomycin-sensitivity. Results of several investigations (41-44) have been interpreted to mean that the two properties are inextricable facets of the same phosphorylation mechanism; that one can-

not hold in the absence of the other. If this is true, the presence of such a particle on the gradient seems unlikely; (b) an oligomycin-sensitive fragment of ROS origin. The identification of such a particle would, by analogous reasoning, confer phosphorylating capacity on the particle.

Increasing the ratio of magnesium to ATP molarities from 0.3 to 1.0 (the latter is used by Frank and Goldsmith, see reference 45) does not alter the relationships of Tables III and IV. How-

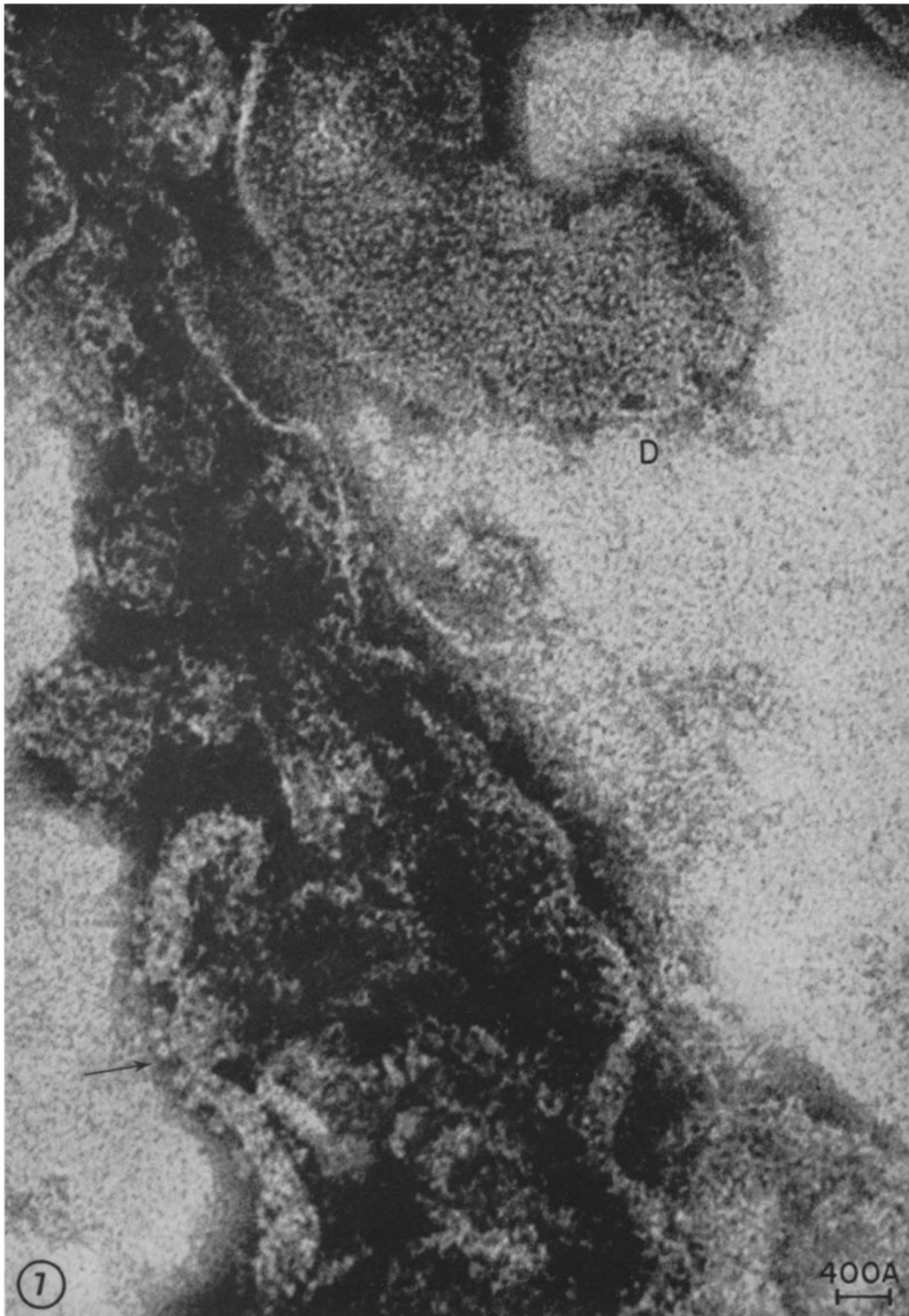


FIGURE 7 Particles sampled from 1.16 band of layered density gradient in Fig. 1. Arrow points to headpiece of elementary particle on what is probably a mitochondrial fragment. The particle labeled *D* was tentatively identified as a degraded form of ROS disc fragment. $\times 210,000$.

ever, aging of the preparations *can* destroy oligomycin sensitivity of the ATPase. It is therefore obvious that the mere absence of oligomycin sensitivity in ATPase does not necessarily imply that a preparation is free from mitochondrial fragments. Multiple criteria should be invoked before such a claim is made. This point is germane to reports by Frank and Goldsmith (45) and previously by Incefy (46) that partial separation of ATPase activity and rhodopsin can be achieved in digitonin extracts of outer segments. These investigators argue that the separation casts doubt on the suggestion by McConnell and Scarpelli (19) that rhodopsin is itself an ATPase, but they provide no data concerning the purity of their ATPase. The mitochondrial contribution to their activities may therefore have been quite large, as it appears to have been in the case of the crude fraction in the present report.

It seems probable that the ROS disc is the locus of the ascorbate-oxidizing activity. A detailed report to follow shows that an identically prepared particulate suspension of cattle forebrain, from which the retina is embryologically derived, does not exhibit this activity, and moreover that the activity of the ROS preparation is markedly increased by bleaching, boiling, or sonication (47). The first finding argues against the activity's localization in some extraneous but otherwise undetected contaminant from elsewhere in the retina. The second finding, particularly in the case of bleaching, suggests that the site of the activity is in the interior of the disc fragments and is exposed by their disruption. Boiling of the disc fragments also results in loss of lipid with a corresponding increase in their density, though similar effects by bleaching or sonication are small (47). Thus it seems plausible that what appear in electron micrographs of the 1.14 band to be disrupted disc fragments are fragments in which carotenoid and part of the lipid have been removed or perhaps not yet incorporated. If the existence of such a species of disc can be corroborated, it may be related to the migration of Vitamin A between the outer segment and pigment epithelium (48), a phenomenon which gains additional credibility from the report by Andrews and Futterman (14) that esterification of retinol by long-chain fatty acids occurs in microsomes, and not in the outer segments themselves.

At the present time, the absence of copper in the disc excludes attributing the oxidation of ascorbate to a typical ascorbate oxidase of the kind first described in plants by Szent-Györgyi (49). The activity may result from non-enzymatic oxidation of ascorbate in the presence of some metal associated with the disc.

Although we are by no means the first to report the high lipid content of the ROS disc, an extensive analysis to be reported elsewhere (37) confirms that lipid in the rigorously isolated fragments is at least 50 per cent (twice that of mitochondria) whereas Collins *et al.* (50) reported about 30 per cent. This implies that the ratio of lipid to protein in the ROS disc is 3 times that in mitochondria. There is a corresponding increase in the tendency of disc fragments to form myelin figures. While occasional myelin figures can be observed in aged preparations of intact mitochondria, they do not occur as readily as in the ROS disc. That any of the ROS species observed represent pure phospholipid, however, can be excluded on two accounts. First, such figures would all have to dissociate from the protein after centrifugation on the gradient, since phospholipid would not sediment on the gradient. Second, the morphology of the figures which do appear differs in important respects from that of the pure phospholipid.

The ultimate exclusion from the red band of particles which oxidized cytochrome *c* was of more than passing significance, since strong histochemical reactions, ostensibly for cytochrome oxidase, have been reported by Niemi and Merenmies (51) and by the present author (36). Use of a more specific test for cytochrome oxidase results in a negative histochemical finding in the outer segment (52).

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