fibers were predominantly seen in its deep cortical layers (layer V–VI) where they followed a rostro-caudal course towards the occipital cortex (fig. 2f). DA-labeled terminals were also demonstrable within the hippocampal formation especially in its temporal part (fig. 2g).

The specificity of immunoreactions visualized by using our DA antiserum was controlled by several absorption tests and ELISA. In the ELISA method, we used many related compounds bound onto poly-L-lysine (MW 300,000) via GA, as fixed antigens. Under these conditions, the strongest cross-reaction was revealed only toward NA (< 0.1%). The antiserum preabsorbed with NA-GA-BSA conjugate was then applied alternatively for staining, in order to exclude possible labeling in noradrenergic systems, but no alteration in staining was confirmed even after this treatment. From these procedures, we believe that our antiserum revealed a highly specific immunoreaction to DA in GA-fixed tissue.

After the destruction of the noradrenergic system, dense fluorescent (presumably dopaminergic) terminals could be visualized in the prefrontal (anteromedial, suprarhinal and anterior cingulate) and limbic (pyriform and entorhinal) cortices. The detailed organization of these dopaminergic systems has been well documented in many studies[2, 4, 8, 9, 12]. Few systematic surveys, however, have been performed on the distribution of dopaminergic input into the remaining cortical structures. Recent study, using both fluorescence histochemical and tyrosine hydroxylase-immunohistochemical techniques combined with pharmacological manipulations, reported the presence of dopaminergic terminals within restricted neocortical fields (sensorimotor, visual and retrosplenial cortices)[3]. Our immunohistochemical study confirms the above observations, and we have also detected additional dopaminergic terminals within the dorso-lateral frontal cortex (Krieg's area 10), the parieto-temporal cortex (area 1, 2, 40, 41 and 20) and some areas of the occipital cortex (area 17 and 18a)[7].

Though these neocortical dopaminergic innervations show a markedly lower density than the prefrontal or limbic dopaminergic input, it is suggested that dopamine might be involved in synaptic transmission at neocortical sites.

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2 Berger, B., Thierry, A. M., Tassin, J. P., and Moyne, M. A., Brain Res. *106* (1976) 133.
3 Berger, B., Verney, C., Alvarez, C., Vigny, A., and Helle, K. B., Neuroscience *14* (1985) 983.
4 Björklund, A., and Lindvall, O., in: Handbook of Chemical Neuroanatomy, vol. 2, p. 55. Eds A. Björklund, T. Hökfelt and M. J. Kuhar. Elsevier, Amsterdam 1984.
5 Geffard, M., Buijs, R., Seguela, P., Pool, C. W., and Le Moal, M., Brain Res. *294* (1984) 161.
6 Geffard, M., Henrich-Rock, A.-M., Dulluc, J., and Seguela, P., Neurochem. int. *7* (1985) 403.
7 Krieg, W. J. S., J. comp. Neurol. *84* (1946) 221.
8 Lindvall, O., and Björklund, A., in: Chemical Neuroanatomy, p. 229. Ed. P. C. Emson. Raven Press, New York 1983.
9 Lindvall, O., Björklund, A., and Divac, I., Brain Res. *142* (1978) 1.
10 Sakai, M., Yoshida, M., Karasawa, N., Teramura, M., Ueda, H., and Nagatsu, I., Experientia *43* (1987) 298.
11 Sternberger, L., in: Immunocytochemistry, p. 90. Wiley, New York 1986.
12 Van Eden, C. G., Hoorneman, E. M. D., Buijs, R. M., Matthijssen, M. A. H., Geffard, M., and Uylings, H. B. M., Neuroscience *22* (1987) 849.
13 Yoshida, M., Karasawa, N., Ito, M., Sakai, M., and Nagatsu, I., Neurosci. Res. *3* (1986) 356.
14 Yoshida, M., Teramura, M., Karasawa, N., Sakai, M., and Nagatsu, I., Biogenic Amines *4* (1987) 135.
15 Yoshida, M., Teramura, M., Sakai, M., Karasawa, N., Nagatsu, T., and Nagatsu, I., Brain Res. *410* (1987) 169.

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The use of iodinated density gradient media for the isolation of rod outer segments

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Summary. Sucrose, nycodenz, metrizamide and a mixture of equal volumes of sucrose and metrizamide were used as density gradient media for the isolation of retinal rod outer segments. The high osmolarity of sucrose had a strongly negative effect on the nature of the rod outer segments, whereas they were much better preserved using iodinated density gradient media such as nycodenz and metrizamide for their isolation.
Key words. Retina; rod outer segments; density gradient; iodinated density gradient media; sucrose; nycodenz; metrizamide; electron microscopy.

Rod photoreceptor cells of the mammalian retina process light to initiate neurotransmission; light induces shedding of the rod outer segments (ROS). One of the basic functions of the retinal pigment epithelium cells is the phagocytosis of the continuously shed ROS during the visual cycle[1, 2]. A technical problem currently being investigated is the isolation of rod outer segments, in order to study phagocytosis in vitro. Generally ROS are isolated by using sucrose density gradient media[3–6]. In our experience, ROS were strongly osmotically influenced in sucrose containing media. In order to avoid this, we tried iodinated density gradient media such as metrizamide and nycodenz, which are described as being less osmotic than sucrose[7].

Materials and methods. Light adapted cattle eyes collected at the local slaughterhouse were kept for 4–5 h at 4 °C before use. The retinas were carefully removed from the eye cup using blunt forceps. 10 retinas were incubated in 100 ml EDTA-buffer (NaCl 137 mmol, EDTA 1.08 mmol, $Na_2HPO_4 - 12\,H_2O$, 7.90 mmol, $NaH_2PO_4 - H_2O$, 1.28 mmol, KCl 2.7 mmol, phenol red 0.01 mmol, pH 7.4) at 4 °C for 15 min. This suspension was shaken by hand for 1 min and centrifuged at 45 × g for 5 min. The supernatant

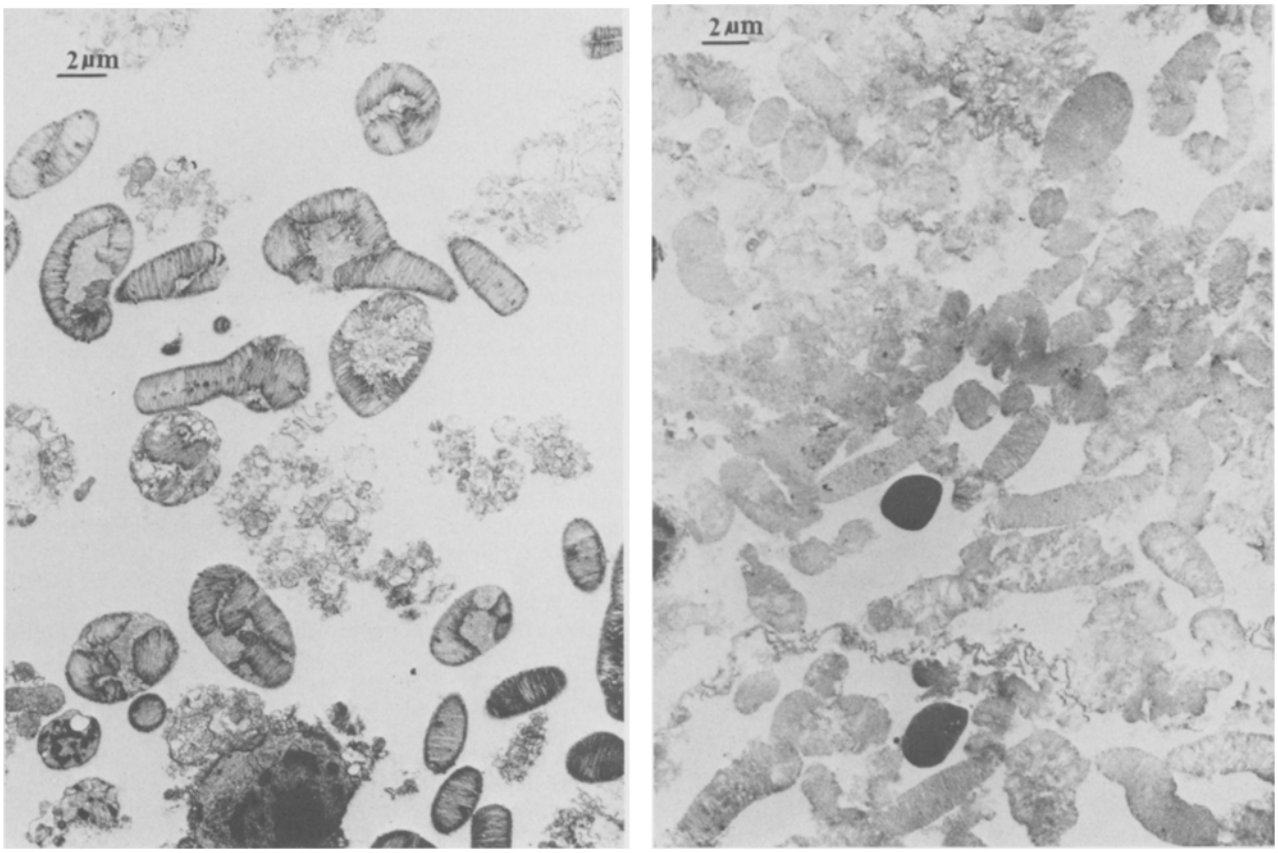

Figure 1. Electron microscopic sections of bovine rod outer segments (ROS) isolated through metrizamide density gradient centrifugation. *A* Upper band fraction showing ROS and well-preserved discs in the plasmalemma. *B* Middle band fraction showing both well-preserved and partially disrupted ROS.

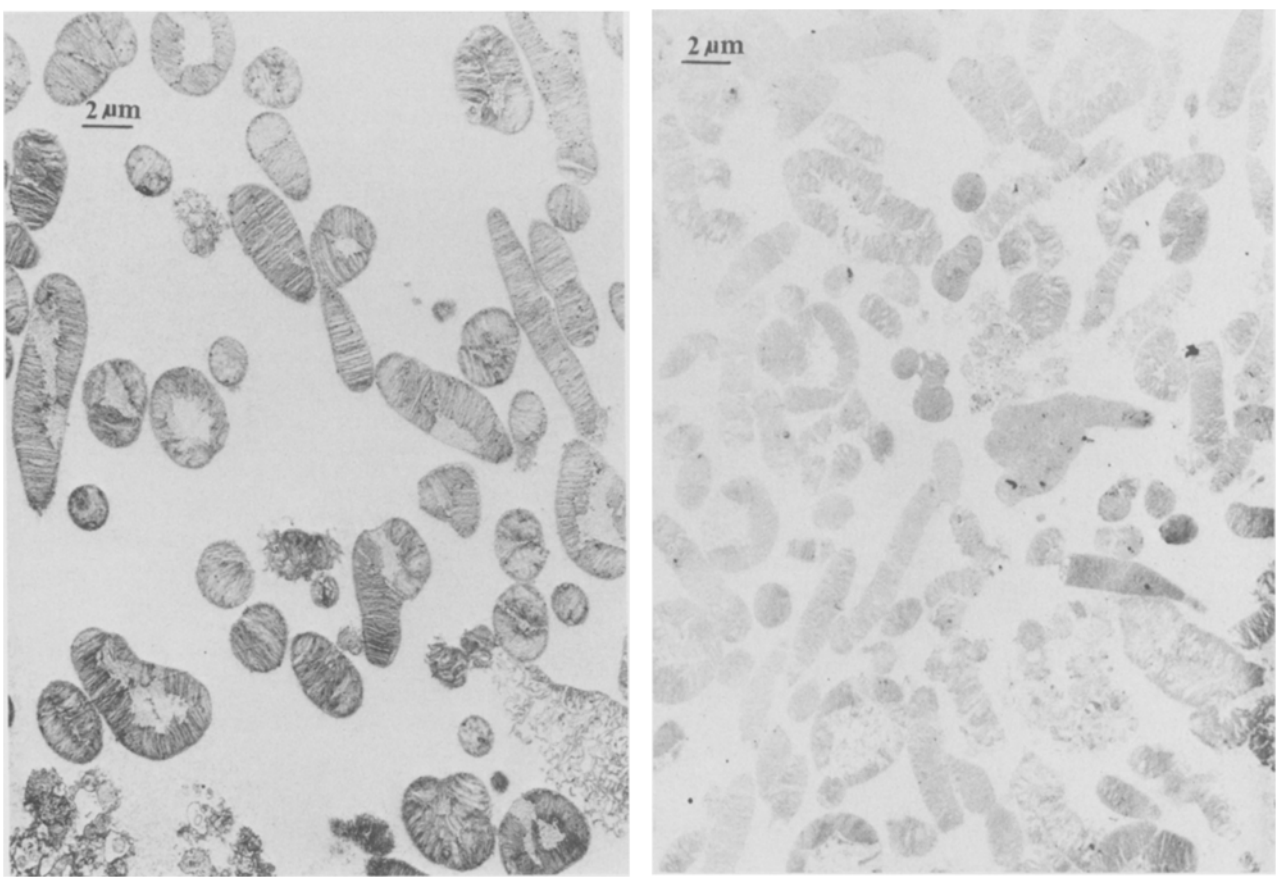

Figure 2. Electron microscopic sections of bovine rod outer segments (ROS) isolated through sucrose-metrizamide density gradient centrifugation. *A* Upper band fraction showing almost exclusively well-preserved discs and plasmalemma. *B* Lower band fractions showing elongated ROS together with partially disrupted ROS.

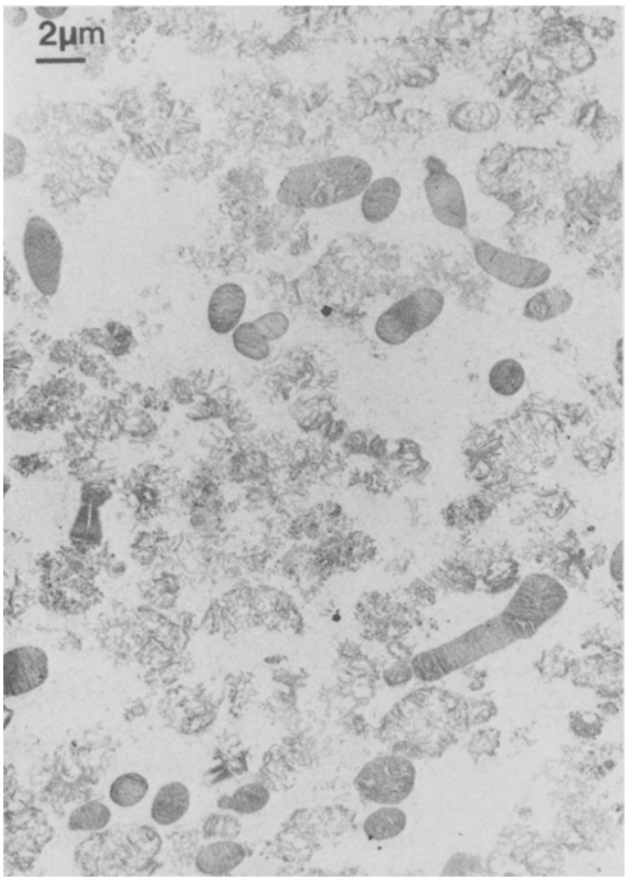

Figure 3. Electron microscopic section of bovine rod outer segments (ROS) isolated through sucrose density gradient centrifugation. Severely disrupted ROS predominantly showing drastically disorganized discs.

was carefully collected and centrifuged at 1600 × g for 5 min. The pellet was resuspended in 5 ml EDTA-buffer (crude ROS).

5–50% linear density gradients were made in EDTA-solution containing either metrizamide, nycodenz, sucrose or the mixture sucrose-metrizamide (1:1). 1 ml crude ROS was floated on the surface of the gradient and centrifuged at 272 807 × g for 45 min (Beckman model L3-50 ultracentrifuge, SW41 rotor). After fractionation of the gradients, each fraction was studied by light microscopy. Fractions containing rods were examined by electron microscopy as previously described [8, 9].

Results. In the metrizamide as well as in the nycodenz gradients the ROS banded near the top of the gradient and in the midregion. The upper band consisted of a mixture of regular refractive rod-like ROS and especially non-refractive folded ROS, while in the midregion of the gradient regular refractive rod-like ROS were predominant. Electron microscopic sections of the rods of the upper band showed several pleomorphous ROS which had membranes without any apparent structural degradation. The plasmalemma still contained discs. Also, cytoplasmic fragments could be observed (fig. 1 A). These probably corresponded to the rods. The rods in the midregion were less pleomorphous and longer than those in the upper band, though several disrupted ROS were observed (fig. 1 B).

Sucrose-metrizamide gradient centrifugation resulted in two different bands, both located at about the middle of the gradient. The upper band consisted of non-refractive irregularly folded ROS while the lower band showed regular refractive rod-like ROS.

Electron microscope sections of the upper band showed several pleomorphous ROS with well-preserved membrane structures. Predominantly rounded ROS occurred (fig. 2 A). The lower band on the contrary showed relatively well-preserved elongated ROS and partially disrupted ROS (fig. 2 B). In sucrose gradients both the regular rod-like ROS and the irregularly folded ROS sedimented in one band near the bottom of the gradient.

Electron microscopy of this band showed that the majority of the ROS were drastically disrupted. The few remaining ROS conserved their typical lamellar structure (fig. 3).

Discussion. The purification of ROS is generally carried out by sucrose gradient centrifugation. The purified ROS are very often used to study phagocytosis by retinal pigment epithelial cells in vitro [5, 6, 10].

The mechanism of action of phagocytosis of ROS by retinal pigment epithelial cells is not yet well known.

Under our experimental conditions the ROS purified by sucrose gradient centrifugation were drastically disrupted. We assumed that if a better approach to physiological conditions could be obtained the purified ROS should maintain as much as possible their native structure. By testing iodinated density gradient media we found that in metrizamide or in nycodenz the normal ROS were better preserved than in sucrose gradients. We also observed that the most suitable conditions to purify ROS are obtained by mixing metrizamide with sucrose as described in materials and methods. Oxidation would play a negative role during purification of ROS under normal atmospheric conditions since structural unsaturated fatty acids in membranes can relatively easily be oxidized [11]. This could be reflected in structural alterations of the lipid bilayer which consequently can result in disruption of ROS. Supporting these findings the generally used antioxidant tocopherol was shown to protect ROS membranous structures.

Though we cannot give any explanation yet, we can conclude that in our experimental condition the morphology of the ROS is certainly better preserved in the presence of either metrizamide or nycodenz or the mixture of sucrose and metrizamide if compared to the sucrose gradients.

1 Bok, D., Invest. Ophthalm. vis. Sci. *26* (1985) 1659.
2 Bok, D., and Young, R. W., in: The Retinal Pigment Epithelium, p. 148. Eds M. F. Marmor and K. M. Zinn. Harvard University Press, Cambridge 1979.
3 Papermaster, D. S., and Dreyer, W. J., Biochemistry *13* (1974) 2438.
4 O'Brian, P. J., and Muellenberg, C. G., Exp. Eye Res. *18* (1974) 241.
5 Chaitin, M. H., and Hall, O. M., Invest. Ophthalm. vis. Sci. *24* (1983) 812.
6 Colley, N. J., and Hall, O. M., Exp. Eye Res. *42* (1986) 323.
7 Rickwood, D., in: Iodinated Density Gradient Media, p. 1. Ed. D. Rickwood. IRL Press, Oxford 1983.
8 Raynold, E. S., Cell Biol. *17* (1963) 208.
9 Spurr, A. R., J. Ultrastruct. Res. *26* (1969) 35.
10 Masterson, E., Goldman, A. I., and Chader, G. I., Vision Res. *21* (1981) 143.
11 Farnsworth, C. C., and Dratz, E. A., Biochim. biophys. Acta *443* (1976) 556.

0014-4754/88/080702-03$1.50 + 0.20/0