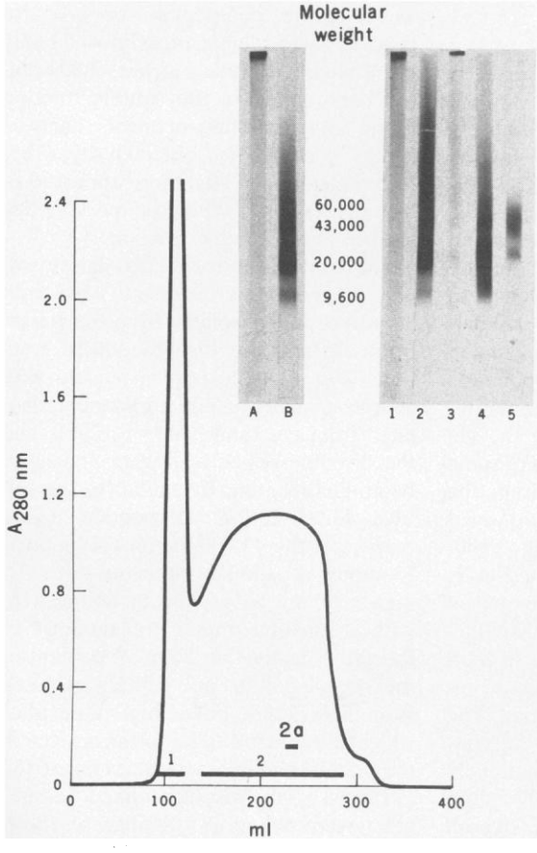
An Extrinsic Membrane Polypeptide Associated with High-Molecular-Weight Protein Aggregates in Human Cataract

Abstract. *A 43,000-dalton polypeptide has been isolated from the high-molecular-weight disulfide-rich fraction of the water-insoluble protein of human cataractous lenses. On the basis of immunochemical reactivity and fluorescent antibody binding, this polypeptide is localized in the membrane region of the lens cell. This observation suggests an interaction between the soluble lens proteins and membrane-associated polypeptides in the formation of large protein aggregates which may cause cataract.*

A major hypothesis to explain the development of cataract associated with aging and opacification of the inner region of the lens is based on changes in the structure of the soluble lens proteins (*1-4*). According to this concept the soluble protein, the crystallins, form giant macromolecules greater than 50×10^6 daltons and large water-insoluble components (*1, 3-5*). Such huge aggregates are believed to act as scatterpoints of light and cause the loss of lens transparency. Support for this viewpoint has come from theoretical and experimental observations. On the basis of light-scatter theory, a random array of macromolecules greater than 50×10^6 daltons, if present in sufficient concentration, with a significantly different refractive index from their environment, can cause opacification (*2*). Such large protein macromolecules have been detected in both animal and human lenses (*3-6*). The early work with bovine lenses suggested that alpha-crystallin (*1, 3, 7*), one of the major soluble structural proteins, was the major component involved in the transformation to high-molecular-weight and water-insoluble protein (*8*). However, studies with human material have shown that these macromolecules are more complex than had initially been suspected (*9*). Polypeptides arising from many of the soluble lens proteins may be present in such aggregates. The similarity in the polypeptide composition of the high-molecular-weight and water-insoluble protein in both bovine and human lenses led to the suggestion that the high-molecular-weight aggregates may be a precursor to the water-insoluble fraction (*3, 5, 9*).

The problem of the origin of these macromolecules is further complicated by the presence of components that are not usually found in the three major soluble structural lens proteins. Such components include (i) a large-sized protein component linked by disulfide (*10*), (ii) a 9600-dalton polypeptide, consisting primarily of a cleaved alpha-crystallin A chain (*11*), and (iii) a 43,000-dalton polypeptide distinctly different from any of the characterized crystallin polypeptides (*12*). We have partially characterized these fractions (*12*).

The large disulfide-linked component has only been found in cataractous lenses and represents a significant fraction of the isolated high-molecular-



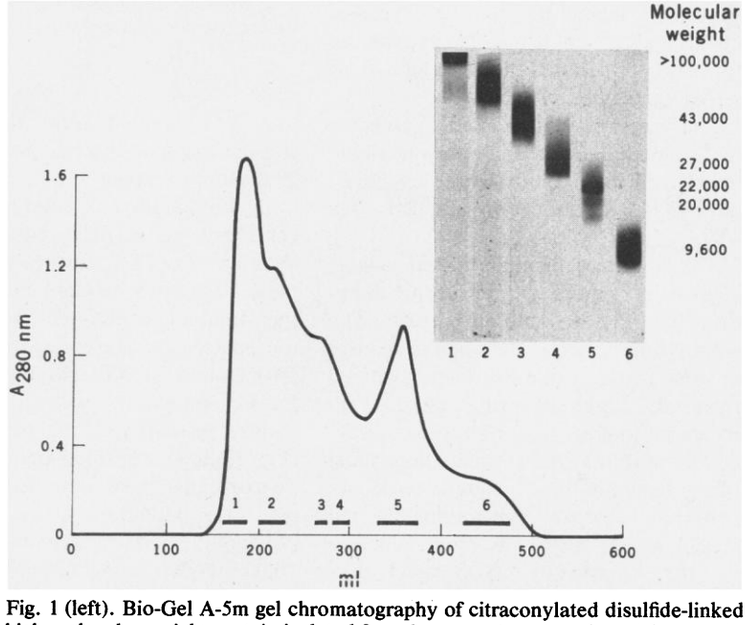


Fig. 1 (left). Bio-Gel A-5m gel chromatography of citraconylated disulfide-linked high-molecular-weight protein isolated from human cataractous lens. The protein was isolated from the water-insoluble fraction of human cataractous lenses (*10*), solubilized in 7.2*M* urea. This fraction was treated with citraconic anhydride (*26*) and subjected to gel filtration (Bio-Gel A-5m) at 4°C in a solution of 0.01*M* tris, *p*H 8.0, 0.1*M* KCl, 0.001*M* EDTA (column, 1.5 by 150 cm). Portions (2 ml) were collected. Agarose polyacrylamide electrophoresis was carried out in the presence of 0.1 percent SDS (*10*). Reduction was performed with DTT or 2-mercaptoethanol (*27*). Agarose acrylamide gel electrophoresis of the disulfide-linked high-molecular-weight starting material before and after reduction, respectively (gels A and B), the two pooled nonreduced peaks 1 and 2, respectively (gels 1 and 3), peaks 1 and 2 after reduction (gels 2 and 4), and the reduced fraction 2a (gel 5) are shown in the insets. In all cases equal amounts of reduced and nonreduced material were used. The gels were calibrated with reference polypeptides of known molecular weight. Fig. 2 (right). Sephadex G-200 chromatography of reduced and alkylated disulfide-linked high-molecular-weight polypeptide isolated from human cataractous lenses (*10*). Reduction was performed in 8*M* guanidine HCl, 0.1*M* tris, *p*H 8.6, 0.002*M* EDTA under nitrogen with 50-fold excess of DTT for 15 minutes at 100°C and 4 hours at 37°C. Alkylation was carried out with iodoacetamide at 37°C for 2 hours. Gel filtration was conducted in a column (2.0 by 200 cm) equilibrated with 7.2*M* urea and 10 percent acetic acid at room temperature. SDS polyacrylamide gel electrophoresis of the fractions 1 to 6 was performed after dialysis and lyophilization (*27*). The gels were calibrated by comparison with reference polypeptides of known molecular weight.

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weight and water-insoluble protein (*10*). The behavior of this material in dissociating media such as urea, guanidine, and sodium dodecyl sulfate (SDS) and light-scattering studies suggest that some of the disulfide-linked macromolecules themselves may be considerably larger than 1×10^6 daltons. From 65 to 75 percent of the thiol is in the disulfide state. This large component after reduction has been shown to contain polypeptides in the 20,000-dalton range as well as 43,000- and 60,000-dalton species and a heterogeneous noncharacterized fraction (*10*). In general, polypeptides of similar size are associated with the disulfide-linked large aggregate and with the remainder of the water-insoluble fraction. The 43,000-dalton polypeptide has also been found in the soluble fraction (*12*).

We now report that the 43,000-dalton polypeptide in the water-soluble and disulfide-linked fraction are immunochemically identical. This polypeptide is localized in the membrane region of the lens cell. We present data to explain its release into the soluble fraction. We show that this extrinsic membrane polypeptide is linked to putative crystallin chains and present a model to explain the formation of some of the large light-scattering aggregates.

The large disulfide-linked aggregates were isolated from the water-insoluble fraction of cataractous lenses by Sephadex G-200 fractionation in 7.2*M* urea (*10*).

The isolated disulfide-linked aggregates were treated with citraconic anhydride in order to solubilize them. The solubilized material continued to behave in some respects like the nonderivatized material. Light-scattering studies indicate an average size of approximately 5×10^6 daltons. Before reduction, most of the disulfide-linked aggregates do not penetrate agarose polyacrylamide gel (Fig. 1, gel A). However, after reduction of the aggregates, SDS gel electrophoresis revealed the expected components in the 20,000-dalton range, 43,000- and 60,000-dalton polypeptides, a diffusely streaky low-mobility component, and a weak 9600-dalton band not previously observed (Fig. 1, gel B). The 43,000-dalton component represents approximately 25 percent of the total material. The solubilized nonreduced material was fractionated further on a Bio-Gel A-5m gel filtration column. Two major peaks were obtained (Fig. 1). Peak 1 contains approximately 35 percent of the starting material, with the remainder in the broad second peak. Gel electrophoresis of reduced peak 1 indicated that it contained only the diffusely streaky low-mobility component and the 20,000-dalton-range species (Fig. 1, gel 2). Nonreduced peak 1 did not enter the gel (Fig. 1, gel 1). After reduction and alkylation of peak 1, further fractionation by gel filtration resulted in isolation of the streaking component, a 26,000-dalton species, and previously undetected 60,000- and 43,000-dalton components. Before reduction, peak 2 material remained primarily at the top of the gel (Fig. 1, gel 3), but small amounts of some polypeptides were detected within the gel. After reduction, peak 2 contained polypeptides of approximately 9600, 20,000, 43,000 and 60,000 daltons (Fig. 1, gel 4). This peak appears to be composed of a heterogeneous group of disulfide-linked polypeptides, on the basis of SDS gel electrophoresis after reduction of respective fractions of the material. The 43,000-dalton component was distributed through much of peak 2. Fraction 2a contained only 43,000- and 20,000-dalton polypeptides (Fig. 1, gel 5). The overall result suggests a population of large aggregates composed of strongly interacting units stabilized by disulfide linkages.

Because of the small amount of material available in fraction 2a, the unfractionated disulfide-linked species was used for the isolation of the 43,000-dalton polypeptide. The disulfide linkages were reduced with dithiothreitol (DTT) and the sulfhydryl groups were protected by treatment with iodoacetamide. The material was then fractionated on Sephadex G-200 in the presence of 7*M* urea; a complex profile was obtained (Fig. 2). Fractions were pooled, and subjected to polyacrylamide gel electrophoresis. Pooled fraction 3 contained only polypeptides in the 43,000-dalton range. This fraction was used in the following immunochemical experiments.

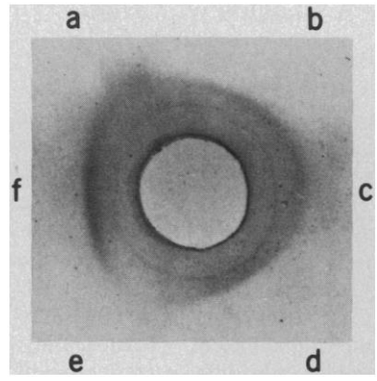

Fig. 3. Double immunodiffusion (*16*) of the total water-soluble protein and the 43,000-dalton polypeptide isolated from various lens fractions, tested with antiserum to the 43,000-dalton polypeptide. The center well contained 5 μl of the antibody (titer, 1 mg/ml). Portions (5 μl) of the following solutions were placed in the outer wells: (a) total water-soluble protein, 1 mg/ml; (b) 43,000-dalton polypeptide from the water-soluble fraction, 0.75 mg/ml; (c) 43,000-dalton polypeptide isolated from the urea-soluble fraction, 0.5 mg/ml; (d) 43,000-dalton polypeptide isolated from the disulfide-linked high-molecular-weight fraction, 0.5 mg/ml; (e) 43,000-dalton polypeptide isolated from the water-soluble fraction, 2.0 mg/ml; and (f) 43,000-dalton polypeptide isolated from the disulfide-linked high-molecular-weight fraction, 0.5 mg/ml.

The 43,000-dalton polypeptide isolated from the soluble fraction of the lens (*12*) was used to prepare a rabbit antiserum by multiple subcutaneous injections of 0.5 mg of the antigen after an initial injection of 1 mg of the antigen dissolved in Freund's complete adjuvant. The antiserum was purified by precipitation with ammonium sulfate (*13*). Radial immunodiffusion (*14*) indicated that the antibody preparation was monospecific against serial dilutions of the total water-soluble protein fractions. The antibody titer, based on experiments with either pure 43,000-dalton polypeptide or preparations of total soluble protein was 1 mg/ml. The concentration of the 43,000-dalton polypeptide in the soluble fraction found by immunochemical analysis agrees with results obtained by other techniques (*15*). Thus, there appeared to be only one 43,000-dalton polypeptide antigen in the soluble fraction.

The antibody to the 43,000-dalton soluble polypeptide cross-reacts with similar polypeptides isolated from the water-insoluble and the disulfide-linked fractions (Fig. 3). All samples reacted with complete identity. The components isolated from the total water-insoluble and the disulfide-linked aggregate appear to be immunochemically similar to the soluble 43,000-dalton polypeptide. Comparison of the 43,000-dalton polypeptide fractions at similar concentrations by means of the halo plate technique (*16*) with a constant amount of antibody in the gel indicates that 80 to 90 percent of the 43,000-dalton polypeptide isolated from the disulfide-linked high-molecular-weight protein is similar to the antigen in the water-soluble fraction. Most of the citraconylated disulfide-linked aggregates were bound to a Sepharose-4B affinity column prepared with antibody to the 43,000-dalton polypeptide. Thus it appears that most disulfide-linked units contain 43,000-dalton components. There does not appear to be any similarity between the lens 43,000-dalton polypeptide fraction and actin (*17*). In partic-

ular, the 43,000-dalton polypeptide antibody neither reacts with actin nor is there a similarity in amino acid composition between actin and this lens polypeptide.

The indirect-staining immunofluorescent technique was used to localize the 43,000-dalton polypeptide in the lens fiber cells. This method has been used to localize chick lens polypeptides (*18*). For our experiments, 13 normal human lenses (*19*) were obtained at autopsy within 24 hours of death; the donors ranged in age from 19 to 41 years except for one who was 73 years of age. Both fresh and fixed tissue (*20*) were cut into 4- to 8-μm sections for fluorescent staining (*21*). The antibody to the 43,000-dalton polypeptide was used for this work.

Immunofluorescence was only found in the fiber cell borders in both the longitudinal and cross-sectional preparations (Fig. 4, A and B). Similar observations were made with the lenses of various ages. The enclosed cytoplasm is generally nonreactive but, because of the thickness of the sections, some fluorescence of membranes beneath the surface layer of the section contributes to apparent lighter cytoplasmic areas. Focusing on these deeper layers clearly shows that only the membrane region is involved. To eliminate the possibility of nonspecific fluorescence or autofluorescence, a counterstaining procedure (Evan's blue) and a dilution series of the antibody were used. Both procedures showed that the fluorescence was dependent on the concentration of the antibody to the 43,000-dalton polypeptide. To demonstrate specificity of the staining, an antibody preparation to total soluble lens proteins was applied to the lens section. This resulted in a positive fluorescence throughout the lens fibers.

The usual manner of isolating soluble lens protein is to homogenize the lens with an alkaline KCl buffer. Such treatment causes the release of some of the 43,000-dalton polypeptide into the soluble phase. Mostafapour and Reddy (*22*) reported that isolation of soluble protein by hypotonic treatment of the lens results in the release of little 43,000-dalton polypeptide. We have confirmed this result (Fig. 5, gel 1). These observations suggest that physical insult to the tissue causes release of some 43,000-dalton polypeptide. If 2 m*M* EGTA is present in the hypotonic solution there is a large increase in the release of 43,000-dalton polypeptide [Fig. 5, gel 2 (see arrow)], suggesting that the polypeptide is probably dependent on calcium for its binding to the membrane. Two additional observations are of interest with respect to these experiments. A 43,000-dalton polypeptide does not appear to be released from the membrane fraction by homogenization in very young lenses (*15*). Furthermore, the ratio of D- to L-aspartic acid, an indication of the aging of the material, suggests that the insoluble material has a much higher relative abundance of D-aspartate (*23*). Hence, a complex set of reactions appears to be involved in the age-dependent modification of the membrane structure.

The 43,000-dalton polypeptide may be depicted in two states (Fig. 6). In the first state it is primarily bound to the membrane and is not strongly interacting with constituents within the cell, reflecting a situation typical of young lenses where little water-insoluble protein and no disulfide-linked aggregates have been detected. All of the polypeptide thiol is in the reduced form. The polypeptide can be released from the membrane by removal of calcium (Fig. 6, dotted arrow). It is not clear whether the calcium is involved in the direct binding of the polypeptides to the membrane by electrostatic interaction or whether the loss of calcium changes the overall archi-

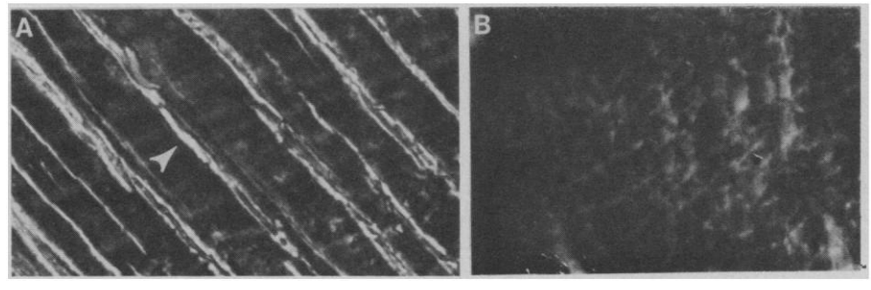

Fig. 4. Immunofluorescence of midcortical sections of normal human lens. (A) Longitudinal section from a 36-year-old lens (×450), (B) cross section from a 41-year-old lens (×300). These preparations were treated with the antibody to the 43,000-dalton polypeptide antibody and fluorescein-conjugated goat antiserum to rabbit immunoglobulin (Miles Yeda Ltd). The fluorescence appears in the fiber cell membranes (see arrows), the only region where the antibody to the 43,000-dalton polypeptide is bound.

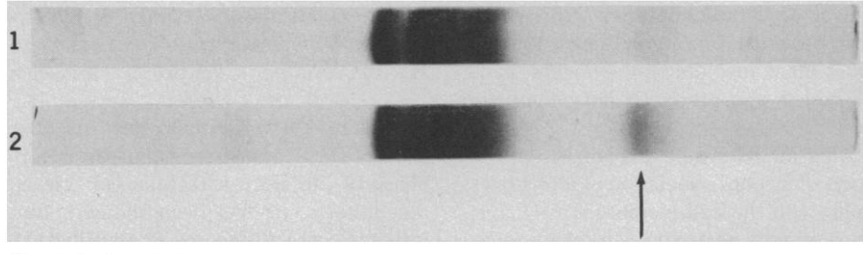

Fig. 5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of soluble lens proteins: (gel 1) released by hypotonic treatment (*28*) of a 54-year-old decapsulated normal human lens; (gel 2) subsequent treatment of the same lens with 2 m*M* EGTA. Portions (25 μl) of a solution (2 mg/ml) of the mercaptoethanol reduced proteins were subjected to electrophoresis (*27*) with 10 percent acrylamide and 0.27 percent cross-linkage. Arrow denotes position of 43,000-dalton polypeptide. Identification is based on the mobility of standard markers. Top of gels is on the right side of the figure.

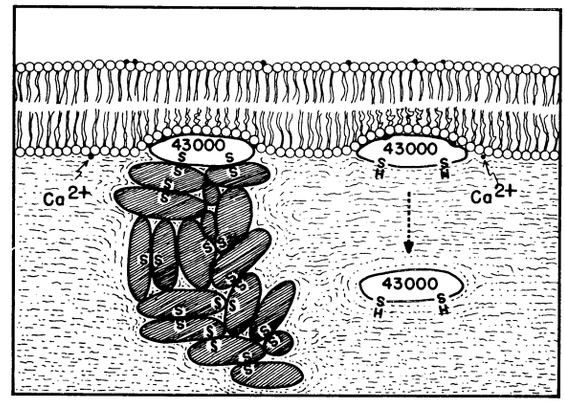

Fig. 6. Diagram of the plasma membrane structure of a small region of the lens fiber. Two proposed states of the extrinsic membrane 43,000-dalton polypeptide are depicted. The arrow illustrates the release of the 43,000-dalton polypeptide by disruption of the membrane by either mechanical (homogenization) or chemical means (EGTA). The divalent calcium cations are shown as the filled circles. The interior of the fiber cell is represented by the stippled area.

tecture of the membrane so that the polypeptide is released. In the second state, found only in cataractous lenses, it is presumed that the 43,000-dalton polypeptide has formed disulfide links with putative crystallin polypeptides. Other polypeptides such as the 9600-dalton species and the components larger than 43,000 daltons are also observed in the complex. In this state, the 43,000-dalton polypeptide may act as a nucleation site on the membrane for the formation of these large aggregates. The individual aggregates are probably composed of units that are stabilized by disulfide linkage and are associated with each other by strong noncovalent forces. Some noncovalently linked individual polypeptides may also be trapped in the matrix of the aggregate. An intermediate stage in which the aggregate is held primarily by noncovalent linkage is not shown. This intermediate condition reflects the situation observed in older normal lenses where aggregates with similar polypeptide composition are observed but are not disulfide linked.

The model does not attempt to be all-inclusive. No definition is presented of the heterogeneous streaky component of peak 1 (Fig. 1) and its association with 20,000-dalton components. It is not known whether such aggregates are associated with the membrane. Other membrane components may also be involved as interaction sites with soluble lens proteins. The model does not consider other insoluble components, some of which are probably not membrane bound.

This model would explain earlier reports of strong association of alpha-crystallin with the cell membrane (*24*). Further support also comes from measurements of Bragg diffraction of laser light caused by large fluctuation in the index of refraction (*25*). The evidence indicates that the amplitude of the change in the index of refraction increases with cataract formation. The scatter elements have the same periodicity as the lens fiber cell membranes.

ABRAHAM SPECTOR
MARGARET H. GARNER
WILLIAM H. GARNER
DEBDUTTA ROY
Biochemistry and Molecular Biology Laboratory, Department of Ophthalmology, College of Physicians and Surgeons, Columbia University, New York 10032
PATRICIA FARNSWORTH
SUSAN SHYNE
Department of Physiology and Ophthalmology, New Jersey College of Medicine and Dentistry, Newark 07103

References and Notes

1. A. Spector, T. Freund, L.-K. Li, R. C. Augusteyn, *Invest. Ophthalmol.* **10**, 677 (1971).
2. G. B. Benedek, *Appl. Opt.* **10**, 459 (1971).
3. A. Spector, *Is. J. Med. Sci.* **8**, 1577 (1972).
4. J. Jedziniak, J. H. Kinoshita, E. M. Yates, L. O. Hocken, G. B. Benedek, *Exp. Eye Res.* **15**, 185 (1973).
5. K. N. Liem-The and J. H. Hoenders, *ibid.* **18**, 143 (1974).
6. A. Spector, J. Stauffer, J. Sigelman, *Ciba Found. Symp.* **19**, 185 (1973); A. Spector, S. Li, J. Sigelman, *Invest. Ophthalmol.* **13**, 795 (1974); J. Jedziniak, J. H. Kinoshita, E. M. Yates, G. B. Benedek, *Exp. Eye Res.* **20**, 367 (1975); F. J. Giblin, B. Chakrapani, V. N. Reddy, *ibid.* **26**, 507 (1978).
7. R. Clark, S. Zigman, S. Lerman, *Exp. Eye Res.* **8**, 172 (1969).
8. D. Roy and A. Spector, *ibid.* **26**, 429 (1978) (discussion of soluble and insoluble lens proteins).
9. ———, *ibid.* **7**, 164 (1976).
10. A. Spector and D. Roy, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3244 (1978).
11. D. Roy and A. Spector, *Exp. Eye Res.* **26**, 445 (1978).
12. W. H. Garner, M. H. Garner, A. Spector, *ibid.*, in press.
13. H. Harboe and A. Ingild, *Quantitative Immunoelectrophoresis*, N. H. Axelson, J. Kroll, B. Week, Eds. (Universitetsforlaget, Oslo, 1975), pp. 161–164.
14. W. Becker, *Immunochemistry* **6**, 539 (1969).
15. W. H. Garner and A. Spector, *Doc. Ophthalmol.* **18**, 91 (1979).
16. Ö. Ouchterlony and L.-A. Nilsson, *Handbook of Experimental Immunology*, D. M. Weis, Ed. (Blackwell, Oxford, 1978), pp. 1917–1944.
17. M. Elzinga and R. C. Lu, *Contractile Systems in Normal Muscle Tissues*, S. V. Perry, A. Margreth, R. S. Adelstein, Eds. (Elsevier/North-Holland, Amsterdam, 1976), pp. 29–37.
18. P. R. Waggoner and H. Maisel, *Exp. Eye Res.* **27**, 151 (1978); P. R. Waggoner, N. Lieska, J. Alcala, H. Maisel, *Ophthalmol. Res.* **8**, 292 (1976).
19. L. T. Chylack, *Arch. Ophthalmol.* **96**, 888 (1978).
20. P. N. Farnsworth, S. C. J. Fu, P. A. Burke, I. Bahia, *Invest. Ophthalmol.* **13**, 274 (1974).
21. A. Ikeda and J. Zwaan, *ibid.* **5**, 404 (1966); *Dev. Biol.* **15**, 348 (1967); J. Zwaan, and A. Ikeda, *Exp. Eye Res.* **7**, 301 (1968).
22. M. K. Mostafapour and V. N. Reddy, *Doc. Ophthalmol.* **46**, 193 (1978).
23. W. H. Garner and A. Spector, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3618 (1978).
24. P. G. Bracchi, F. Carta, P. Fasella, G. Maraini, *Exp. Eye Res.* **12**, 151 (1971).
25. G. Benedek, J. Clark, E. W. Serrallach, C. Y. Young, L. Mengell, T. Sauke, A. Bragg, K. Benedek, *Philos. Trans. R. Soc. London*, in press.
26. H. B. F. Dixon and R. N. Perham, *Biochem. J.* **109**, 312 (1968).
27. K. Weber and M. Osborn, *J. Biol. Chem.* **244**, 4406 (1969).
28. O. U. Blumenfeld and B. Zvilichovsky, *Methods Enzymol.* **28**, 245 (1972).
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Rhythms in Human Performance: $1^1/_2$- Hour Oscillations in Cognitive Style

Abstract. *Performance on verbal and spatial matching tasks was assessed every 15 minutes for 8 hours. Significant 90- to 100-minute oscillations were observed for each task. These oscillations were 180° out of phase, supporting the hypothesis that in humans the basic rest activity cycle involves alternating activation of processing systems residing in the two cerebral hemispheres.*

In the 1950's Kleitman and his colleagues (*1*) demonstrated that the sleep phase of the sleep-wakefulness cycle is not uniform, as had been thought, but rather is characterized by an ultradian (*2*) rhythm with striking variations in many behavioral and physiological functions. The two phases of this rhythm—which differ in electroencephalogram (EEG) pattern, heart and respiration rate, and muscle tone—are referred to as rapid-eye-movement (REM) sleep and non-rapid-eye-movement (NREM) sleep because transient eye movements occur during one phase but not during the other (*3*). In the human adult, a full cycle (period) of REM/NREM alternation lasts approximately 80 to 120 minutes. Kleitman (*4*) subsequently proposed that the alternation between REM and NREM sleep is one expression of a phylogenetically old basic rest activity cycle (BRAC), which continues throughout the day and on which the sleep-wakefulness cycle is superimposed. Many studies have since supported this proposal, although the choice of the terms "rest" and "activity" to describe the alternate phases has been questioned (*5*).

It has recently been hypothesized that the REM/NREM rhythm and its continuation during wakefulness (the BRAC) involves an alternation in the relative efficiency or activation of the two cerebral hemispheres (*6, 7*). Broughton (*7*) cites the following evidence in support of this hypothesis: (i) the subjective qualities of dreams reported on wakening from REM and NREM sleep differ radically with vividly visual dreams of an illogical nature reported following REM sleep awakenings and less vivid, more thoughtlike and rational dreams reported after NREM sleep awakenings (*8*); (ii) the right cerebral hemisphere shows greater EEG desynchronization than the left during REM sleep (*9*); (iii) 90- to 110-minute rhythms during wakefulness have been reported for a variety of measures, including oral behavior (*10*), the magnitude of the spiral aftereffect (*11*), heart rate (*12*), vigilance performance (*13*), and the fantasy content of day dreams (*14*); (iv) some of these rhythms may be in

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