Biochemistry

 Volume 17, Number 19 September 19, 1978

Independent Structural Domains in the Membrane Protein Bovine Rhodopsin†

Arlene D. Albert and Burton J. Litman*

ABSTRACT: Rhodopsin, in retinal rod outer segment disk membranes, was proteolyzed by treatment with papain. This treatment leaves three fragments of apparent molecular weights 26 000, 19 000, and 10 000 in the membrane. These fragments copurify by chromatography on concanavalin A (Con A)–Sepharose 4B. The circular dichroism (CD) of solubilized, proteolyzed rhodopsin, in both the far-ultraviolet and visible spectral regions, was essentially identical with that of native rhodopsin. Maintenance of the visible CD spectrum after papain treatment demonstrates that the retinal binding site is unchanged by proteolysis. The far-ultraviolet CD spectrum of papain-treated rhodopsin shows that this species, which is thought to be largely imbedded in the hydrocarbon region of the membrane, retains more than 90% of the helical content of native rhodopsin. Although the proteolysis fragments form a tight complex, such that the structural characteristics of native rhodopsin are retained, the two lower molecular weight fragments can be eluted from Con A–Sepharose 4B when proteolyzed rhodopsin is bleached in the presence of the detergent cetyltrimethylammonium bromide. The 26 000 molecular weight fragment, which contains the carbohydrate groups of rhodopsin, can be subsequently eluted from the column with α-methyl mannoside. Far-ultraviolet CD measurements, on the light and α-methyl mannoside releasable fractions, indicate that the helical features of native rhodopsin are largely maintained, even when the proteolysis fragments are dissociated. This finding indicates that independently stabilized folding domains exist within the tertiary structure of rhodopsin.

The visual pigment rhodopsin is an intrinsic membrane protein which constitutes 80–90% (Papermaster and Dreyer, 1974) of the rod outer segment disk membrane protein. The absorption of light by the rhodopsin chromophore, 11-*cis*-retinal, initiates the visual transduction process (Hubbard and Kropf, 1958; Wald, 1968). Since structure and function are intimately related, it is of importance to establish the position of rhodopsin within the membrane and to determine the structural elements of the protein.

Membrane proteins such as cytochrome b_5 (Strittmatter et al., 1972; Spatz and Strittmatter, 1971) and glycophorin (Tomita and Marchesi, 1975; Jackson et al., 1973) have been shown to have hydrophilic domains, which are exposed to the aqueous phase, and a hydrophobic segment, which penetrates the lipid bilayer, while bacteriorhodopsin has been shown to consist of seven transmembrane, helical segments connected by random-coil regions (Henderson and Unwin, 1975). Structural domains such as those observed in cytochrome b_5 and glycophorin have been separated by proteolytic digestion. Recently it was shown that the cytoplasmic enzyme tryptophan synthetase could be cleaved by trypsin without loss of activity. The fragments were separated and individually unfolded and then refolded. When these fragments were recombined, enzymatic activity was restored (Hogberg-Raibaud and Goldberg, 1977). While such a dramatic demonstration of independent structural folding domains may not be presently possible with membrane proteins, the demonstration of such a substructure in an integral membrane protein, whose major mass is thought to be within the membrane, is of great interest.

Several proteolytic enzymes have been used to study rhodopsin in the disk membrane (Saari, 1974; Trayhurn et al., 1974a,b; Pober and Stryer, 1975; Sale et al., 1977). Proteolyzed rhodopsin has been shown to retain its native absorption spectrum when solubilized in detergent (Trayhurn et al., 1974a). Upon bleaching in the membrane, proteolyzed rhodopsin shows the same ability to recombine with 11-*cis*-retinal and regenerate a native absorption spectrum as native opsin (Trayhurn et al., 1974b; Van Breugel et al., 1975). We have used the enzyme papain to determine the effect of proteolytic cleavage on the secondary and tertiary structure of rhodopsin, to investigate the existence of independent structural domains in rhodopsin, and to determine if structural analogies between bovine rhodopsin and bacteriorhodopsin exist.

† From the Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia 22903. *Received March 6, 1978.* This research was supported by National Science Foundation Grant GB-41313 and National Institutes of Health Grant EY00548. A.D.A. received support from National Institutes of Health predoctoral training grant GM07294. A preliminary report of this research was given at the 1977 Meeting of the Biophysical Society [*Biophys. J. 17*, 100a (1977)].

0006-2960/78/0417-3893$01.00/0

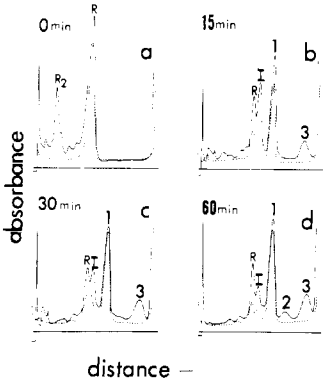

FIGURE 1: Time course of papain proteolysis of rhodopsin in the disk membrane. $NaDodSO_4$ gel electrophoresis of (a) control disks and disks proteolyzed for (b) 15, (c) 30, and (d) 60 min. The gels were stained first with the carbohydrate stain, PAS (- - -), and then with Coomassie blue (—). R, R_2, I, 1, 2, and 3 stand for rhodopsin, rhodopsin dimer, intermediate of apparent molecular weight 34 000, and proteolytic fragments of apparent molecular weight 26 000, 19 000, and 10 000, respectively. The gels contracted slightly after the Coomassie staining procedure; therefore, it was appropriate to align the peaks rather than the ends of the scans.

Materials and Methods

Preparation of Disks. Disks were prepared from bovine retinas (Hormel) as described by Smith et al. (1975). The rods were hypotonically lysed by suspension in 5% Ficoll and spun in an SW-27 swinging-bucket rotor at 25 000 rpm for 2 h within an hour of suspension.

Protein Concentration Determination. The change in absorbance at 500 nm upon bleaching was used to determine the rhodopsin concentration. A 0.1-mL aliquot of the rhodopsin or disk solution was solubilized in 0.4 mL (1.4% CTAB,[1] 0.1 M hydroxylamine) of 0.1 M phosphate buffer, pH 7. After the absorbance was measured, the sample was bleached for 30 s under a microscope lamp and another absorbance measurement was made. Absorbance measurements were made on an Hitachi Model 100-60 double-beam spectrophotometer. The molar extinction coefficient of rhodopsin was taken to be 40 000. Total protein content was measured by the Lowry method (Lowry et al., 1951).

Proteolysis. Papain, two times crystallized, was purchased from Sigma. Papain proteolysis was performed as described by Trayhurn et al. (1974). The rhodopsin concentration used was between 1 and 2 mg/mL. The protein to enzyme ratio was 20:1. Incubation was carried out at 37 °C.

$NaDodSO_4$ Polyacrylamide Gel Electrophoresis. Discontinuous disk gel electrophoresis was performed using the methods of Laemmli (1970) with modifications described by Smith et al. (1975). All gels were 12% acrylamide. The gels were stained for carbohydrate using the periodic acid–Schiff (PAS) stain (Segrest and Jackson, 1972). The gels were stained first with PAS and then with Coomassie blue. The retinal–opsin Schiff base linkage was reduced with $NaCNBH_3$ to provide a fluorescent marker on the gels as described by Fager et al. (1972). A solution of 2 M $NaCNBH_3$ in 1 M acetate buffer (pH 5) and 100 mM OG[1] was prepared immediately before using. The $NaCNBH_3$ solution was added to the disk suspensions in a ratio of 1:1 under dim red light. The reaction was allowed to proceed at least 30 min in the dark. The solution was then dialyzed against 0.1 M phosphate buffer, pH 7.

Gels stained with Coomassie blue or PAS were scanned at 550 nm using the linear-transport attachment on a Gilford Model 240 spectrophotometer. Fluorescent bands were scanned using the Gilford fluorescence scanner attachment.

Rhodopsin can spontaneously polymerize when solubilized in $NaDodSO_4$ and was present in both monomer and dimer forms in varying ratios on all gels. In many cases, trimer and tetramer could also be seen. This polymerization was increased by heating (Smith and Litman, unpublished observation). This characteristic of rhodopsin was used for a molecular weight estimation of the proteolytic fragments. Rhodopsin and its polymers were used to determine the slope of the log molecular weight vs. R_f line. This slope was used to calculate a molecular weight for the proteolytic fragments within a series of gels. This procedure assumes all the gels in a given set have the same calibration constants and was designed to give estimates of the molecular weights of the fragments observed in a highly reproducible gel-banding pattern. The molecular weight of rhodopsin was taken to be 38 000.

Rhodopsin Purification. Rhodopsin was purified from bovine rod outer segment disks by affinity chromatography on a concanavalin A (Con A)–Sepharose 4B (Pharmacia Fine Chemicals) column (Steinemann and Stryer, 1973). The detergent, OG, at a concentration of 30 mM was substituted for 1.4% CTAB and Tris–acetate buffer (pH 7.0) was substituted for the sodium acetate buffer (pH 5.0). α-Methyl D-mannoside, 0.1 M, was used to elute the bound rhodopsin. The A_{280}/A_{500} spectral ratio obtained for purified rhodopsin was 1.6.

Separation of Proteolysis Fragments. Light release of peptides from proteolyzed rhodopsin bound to the Con A–Sepharose 4B column was carried out by stopping the column flow (after all the material which did not bind was washed off) and bleaching for 1 min with a microscope lamp. Room lights were on for 15 min before the column flow was restarted. In this experiment 1.4% CTAB was used in the eluant. The column was monitored by the absorbance of the collected fractions at 280 nm. The column was 8-cm long and 0.9 cm in diameter. The flow rate of the column was approximately 1 mL/min. No more than 1 mg of rhodopsin was loaded per milliliter of column bed volumn. Column fractions were of uniform size within a given run and varied from 1.5 to 3.5 mL from one column run to another.

Circular Dichroism Measurements. The CD measurements were made using a Cary 60 spectropolarimeter with a 6001 CD atachment. All samples were measured at 27 °C. For far-ultraviolet spectra, the protein concentration was between 0.1 and 0.2 mg/mL. The cell path length was either 0.5 or 1 mm. For visible CD spectra, the protein concentrations were in all cases about 1 mg/mL and the cell path length was 1 cm. The dynode voltage was maintained below 500 V in all measurements. A mean residue weight of 115 was employed in ellipticity calculations. The helical content of rhodopsin was estimated by the formula, percent helix = $[100([\theta]_{222} + 2340)/-30\,300]$ (Chen et al., 1972).

Results

Proteolysis of Rhodopsin in the Disk Membrane. Papain proteolysis has been employed here to study the accessibility of rhodopsin to a soluble protein and to generate a set of fragments which would help elucidate the substructure of rhodopsin. The time course of papain proteolysis of rhodopsin was monitored by $NaDodSO_4$–polyacrylamide gel electrophoresis. The reaction was stopped at various times between 2 min and 2 h. The gel scans shown in Figure 1 were typical of several time course experiments. An initial rapid cleavage of rhodopsin occurred, which produced an intermediate, I, of molecular weight 34 000. The decay of this intermediate as the

[1] Abbreviations used: OG, octyl glucoside; CTAB, cetyltrimethylammonium bromide.

reaction proceeded resulted in the appearance of three new bands, consisting of a polypeptide of molecular weight 26 000 (fragment 1) and two smaller polypeptides of molecular weight 19 000 (fragment 2) and 10 000 (fragment 3), respectively. Although a good initial species–intermediate–product time course relationship could be determined for rhodopsin, the intermediate I, and fragment 1, the weak Coomassie blue staining of fragments 2 and 3 did not allow good quantitation of these bands with respect to time course or relative concentration. The time course of proteolysis (Figure 1) seems to indicate that the development of the fragment 2 is delayed relative to fragments 1 and 2, which appear close in time to one another. In agreement with observations by Trayhurn et al. (1974a,b), some of the disk rhodopsin was not affected by proteolytic attack. To test whether the residual rhodopsin was acutally not subject to attack or if the enzyme had become inactive during the incubation periods, disks which had already been subjected to proteolysis for 1 h were resuspended in buffer, and additional enzyme was added. No further proteolysis was observed. We have, however, found that the percent of uncleaved rhodopsin varied with different papain preparations. The presence of inverted disks in our preparations is a possible source of the uncleaved rhodopsin in our experiments.

Since rhodopsin has at least two carbohydrate chains (Hargrave, 1978), it was of interest to determine which fragments contain carbohydrate. The gels were treated with the carbohydrate stain PAS. As noted by Trayhurn et al. (1974a,b), we find that fragment 1 stained positively for carbohydrate. Consistent with the 34 000 molecular weight species being a proteolytic intermediate, it also stained positively for carbohydrate. In Figure 1, the progress of proteolysis, as monitored by both Coomassie blue and PAS stain, is shown.

In rhodopsin, the Schiff base linkage between retinal and a lysine residue can be reduced by sodium borohydride (Bownds, 1967; Akhtar et al., 1965) or cyanoborohydride (Fager et al., 1972) to yield a fluorescent product, covalently linked to opsin. Cyanoborohydride, a more hydrophobic reagent than sodium borohydride, was chosen because the reduction could be carried out on unbleached rhodopsin. Sodium borohydride is known to reduce disulfide bonds; this is less likely to occur with cyanoborohydride, since it is a weaker reducing agent than sodium borohydride. Attempts to establish the location of the retinal on one of the three fragments by cyanoborohydride reduction were not conclusive. When reduction followed proteolysis, fluorescence was observed associated with both fragments 1 and 3 of rhodopsin. If reduction preceded proteolysis, the fluorescence was associated with fragments 2 and 3. In both cases, the same gels were stained with Coomassie blue after the fluoresent bands were located on the gels. When the reduction followed proteolysis, the Coomassie blue staining pattern was the same as that observed for the unreduced control. However, when reduction preceded proteolysis, the Coomassie staining pattern was altered. The intensity of the stain corresponding to the residual rhodopsin, the first proteolytic intermediate, and fragment 1 was considerably reduced. The staining intensity of fragment 2 was dramatically increased. This indicates that some altered cleavage pattern results when reduction precedes proteolysis.

Absorption Spectra. For all optical measurements reported here, papain proteolysis was carried out for at least 1 h. The gel scan in Figure 1d was typical of the proteolyzed disk samples employed in these studies. As observed by Trayhurn et al. (1974a,b), the proteolyzed disk sample and the control disk sample exhibited the same absorbance at 500 nm. A 3–4% decrease in the 280-nm absorbance was observed after papain treatment.

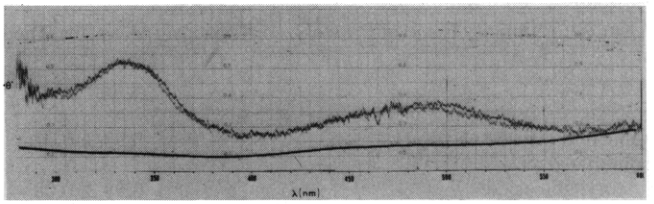

FIGURE 2: Visible CD spectrum of native disks and proteolyzed disks, solubilized in 0.2 M OG. The concentration of rhodopsin in the native disk solution is 0.019 mM and that in the proteolyzed disk solution is 0.020 mM. Full scale chart expansion is 0.04° and the cell pathlength is 1 cm. The curve which appears slightly lower is the native disk spectrum.

Visible Circular Dichroism of Solubilized Disks. The CD spectrum of rhodopsin in the visible region is associated with the 11-*cis*-retinal chromophore and is characterized by two intense bands, one at 345 and the other at 485 nm. Since the visible CD spectrum is determined by the chromophore interaction with opsin, this region of the spectrum should be sensitive to conformation changes near the chromophore binding site. We, therefore, examined this region of the spectrum to determine if interactions necessary for the maintenance of the integrity of the chromophore binding site were changed as a result of proteolysis. The visible CD spectrum of proteolyzed and control disk samples is shown in Figure 2. The disks were solubilized in 0.2 M OG. The concentration of the control disk solution was 95% of that of the proteolyzed disk solution. It can be seen that the spectra are essentially superimposable. The ratio of ellipticities at 345 and 485 nm is 2.5 for both preparations. This compares favorably with the value of 2.7 found for purified rhodopsin (Stubbs et al., 1976).

Far-Ultraviolet CD of Rhodopsin. The UV CD spectral region between 190 and 250 nm is associated with secondary structural features of proteins (Chen et al., 1974). An α-helical structure is characterized by troughs in the region of 210 and 222 nm. The ellipticities of both proteolyzed and unproteolyzed control disk samples were compared at these wavelengths. If a loss in helical structure occurred during proteolysis, a loss of ellipticity at these wavelengths should be observed. UV CD spectra were obtained for proteolyzed and control samples in the form of sonicated disks, solubilized disks, and column-purified rhodopsin. The UV CD spectrum of sonicated disks was characterized by a red-shifted long-wavelength trough (minimum at 224 nm) and a marked decrease in ellipticity in the 210-nm region; this spectral profile is characteristic of the scattering artifact observed in many membrane systems (Litman, 1972). As has been observed by others (Shichi, 1973; Rafferty et al., 1977), no change in the UV CD spectrum occurred in the native disks upon bleaching. We have observed that papain-proteolyzed disks also maintain their UV CD spectrum upon bleaching. There was less than 10% loss of ellipticity for the proteolyzed disk system compared to the control disks.

The 210-nm trough has been found to be more sensitive to scattering than the 222-nm trough. The ratio of ellipticities of these troughs has been shown to become constant as particle size, and hence scattering, decreased below some critical value (Litman, 1972). In this study, disks were solubilized in OG to eliminate scattering artifacts. When an increase in concentration of detergent had no effect on the ellipticity ratio, the sample was taken to be fully solubilized and the UV CD spectra no longer subject to scattering artifacts. OG/phospholipid ratios above 30 gave a consistent $[\theta]_{210}/[\theta]_{222}$ ratio

TABLE I: Ellipticity Ratio of Proteolyzed Rhodopsin to Native Rhodopsin.

	unbleached		bleached		
	222 nm	210 nm	222 nm	210 nm	n^a
sonicated disks[b]	0.93 ± 0.02		0.93 ± 0.02		2
solubilized disks					
octyl glucoside[c]	0.98 ± 0.08	0.98 ± 0.15	0.95 ± 0.07	0.94 ± 0.10	14
1.4% CTAB	0.98 ± 0.10		0.98 ± 0.03		3
12% emulphogene	0.96 ± 0.10	0.94 ± 0.04	1.01 ± 0.19	0.94 ± 0.07	3
purified rhodopsin (in 30 mM OG)	1.03 ± 0.01	1.03 ± 0.04	1.03 ± 0.03	0.92 ± 0.01	2

[a] n = number of determinations. [b] The trough position in these samples is shifted to 224 nm. [c] OG concentration varied from 30 to 200 mM.

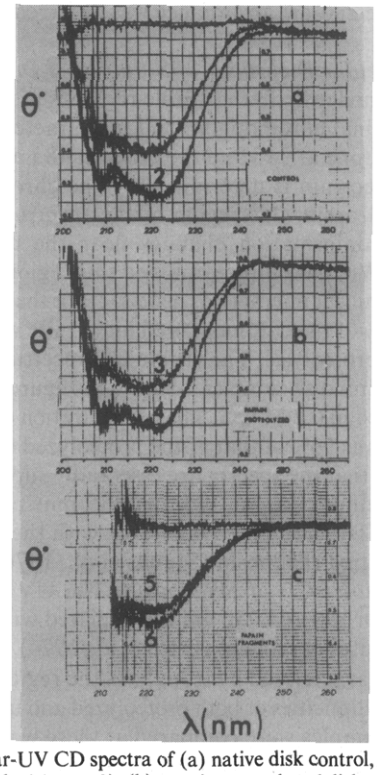

FIGURE 3: Far-UV CD spectra of (a) native disk control, bleached (curve 1) and unbleached (curve 2); (b) papain-proteolyzed disks, bleached (curve 3) and unbleached (curve 4); and (c) proteolytic fragments 2 and 3 (curve 5) and fragment 1 (curve 6). The concentrations of rhodopsin in the proteolyzed and control disk samples were 0.21 and 0.020 mM, respectively. Both samples were solubilized in 75 mM OG. The concentrations of the fragment 2 and 3 mixture and fragment 1 sample were 0.21 and 0.30 mg/mL, respectively. Full scale chart expansion employed was 0.04°, 0.04°, and 0.1° for the spectra in frames a–c, respectively. The path lengths of cells used in obtaining the spectra in frames a–c were 0.5, 0.5, and 1.0 mm, respectively.

of 0.9. Table I shows the ratio of ellipticities of solubilized, papain-proteolyzed disks to solubilized, control disks at 210 and 222 nm for both bleached and unbleached rhodopsin. The ellipticities of the samples being compared differed by no more than 6% for both wavelengths. Figure 3 shows the UV CD spectra typically observed for native and proteolyzed solubilized disks.

The far-UV CD spectra of disks solubilized in the detergents CTAB and emulphogene were also examined. The UV CD spectrum of rhodopsin in emulphogene was, within experimental error (±5%), the same as that obtained in OG. The UV CD spectrum of rhodopsin in CTAB could only be measured down to about 215 cm. Below this wavelength the detergent began to absorb strongly. The CTAB data agreed well with that obtained in both emulphogene and OG. In CTAB, OG, and emulphogene, the solubilized, proteolyzed disks showed little loss of ellipticity in the spectral region examined, relative to solubilized, native disks. Due to scattering, spectra of sonicated disks are not directly comparable to detergent-solubilized disk spectra.

Purification of Rhodopsin and Proteolyzed Rhodopsin. Although rhodopsin is the major protein of the disk membrane, there are other proteins which could contribute to the far-UV CD spectrum of solubilized disks and which would certainly contribute to protein concentration determination. It was desirable, therefore, to study purified papain-proteolyzed and native rhodopsin. After proteolysis, the disks were solubilized in OG and purified on a Con A–Sepharose 4B column. Proteins or peptides which possess a carbohydrate group, and peptides which are tightly associated with carbohydrate-containing species, will bind to the Con A–Sepharose 4B column. After lipids and other protein components were washed off, rhodopsin or proteolyzed rhodopsin was eluted from the column with 0.1 M α-methyl mannoside. The gel patterns obtained for purified, proteolyzed rhodopsin and native rhodopsin were essentially the same as those obtained for the equivalent disk samples in the region of rhodopsin and its proteolytic fragments (Figure 4). The relative peak heights of the various fragments showed some variation in different preparations, but the pattern of 1 > R > I was always the same. PAS staining (Figure 1) showed that fragment 1 contained carbohydrate. Since the small peptides undergo copurification with the carbohydrate-containing fragment, it would appear that the proteolytic fragments solubilize as a noncovalently bonded complex. If proteolysis altered the region near the carbohydrate group, the binding of proteolyzed rhodopsin to the column, relative to native rhodopsin, may have also been altered, and a separation of the rhodopsin and its proteolytic fragments might be achieved by a gradient elution with α-methyl mannoside. A column containing both native and proteolyzed rhodopsin was eluted with a gradient of 0–0.075 M α-methyl mannoside. Only a single peak was resolved, and the banding patterns from $NaDodSO_4$–polyacrylamide gel electrophoresis of fractions on the ascending and descending sides of the peak were identical. Hence, carbohydrate exposure in the solubilized protein does not seem to be affected by proteolysis.

Far-UV CD Spectrum of Purified Proteolyzed and Native Rhodopsin. Spectra of proteolyzed and control samples which had been purified were compared. These results are noted in Table I. The spectra indicated, as was the case for the solubilized disks, that no significant loss of secondary structure occured due to papain cleavage.

Light-Released Fragments of Proteolyzed Rhodopsin. It has been shown that when the proteolytic product of thermolysin cleavage of rhodopsin is bleached, while bound to a Con A–Sepharose 4B column, a small peptide is released (Pober and Stryer, 1975). This procedure was investigated for the papain-proteolyzed system as a means of purifying the papain

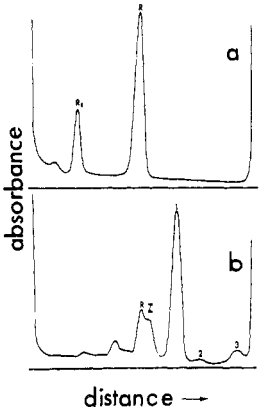

FIGURE 4: $NaDodSO_4$ gel electrophoresis of rhodopsin purified on a Con A-Sepharose 4B column: (a) control disks and (b) papain-proteolyzed disks. α-Methyl mannoside eluted fractions were concentrated prior to $NaDodSO_4$-polyacrylamide gel electrophoresis.

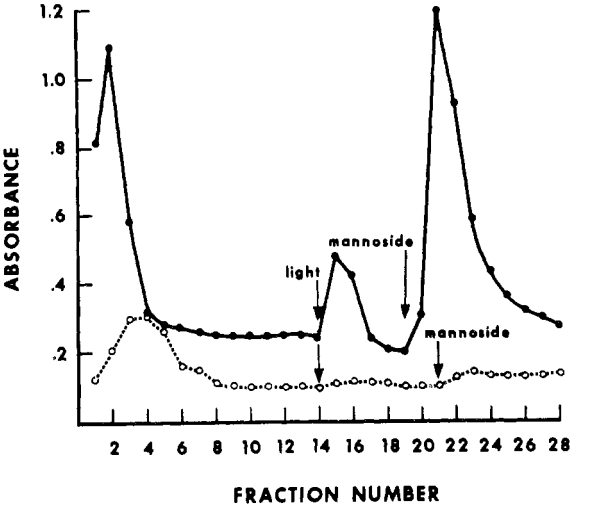

FIGURE 5: Elution of proteolysis fragments of rhodopsin from a Con A-Sepharose 4B column by exposure to light and by 0.1 M α-methyl mannoside. Detergents used were 1.4% CTAB (—) and 30 mM OG (- - -). See Materials and Methods for details.

fragments of proteolyzed rhodopsin. Papain-proteolyzed rhodopsin, solubilized in CTAB, was bound to a Con A-Sepharose 4B column, which was then washed to remove lipids and other proteins. The column flow was stopped and the bound rhodopsin was exposed to light for 15 min. Following light exposure, peptide-containing fractions eluted from the column. Further peptide release was achieved by elution with 0.1 M α-methyl mannoside. The elution profile of this column is shown in Figure 5. The column fractions associated with the light and α-methyl mannoside released peak were pooled. The total protein yield in both fractions relative to the rhodopsin loaded on the column was approximately 50%. Twenty percent of this was eluted upon exposure to light and the remaining 30% by α-methyl mannoside. CTAB was removed by chloroform extraction. The samples were then examined by $NaDodSO_4$-polyacrylamide gel electrophoresis. These gels indicated that fragments 2 and 3 were released upon exposure to light, while fragment 1 was released by α-methyl mannoside.

The detergent used in this experiment played a major role. When this same procedure was carried out using OG, no peptide release could be detected following light exposure. Even washing the column with 0.1 M α-methyl mannoside produced no protein release. It appears that after bleaching in OG, rhodopsin and its proteolytic fragments irreversibly bind to the Con A-Sepharose 4B column. This is consistent with our finding that when a disk solution known to contain both bleached and unbleached rhodopsin is purified on a Con A-Sepharose 4B column, an A_{280}/A_{500} spectral ratio of 1.6 is obtained, demonstrating the removal of bleached material from the α-methyl mannoside eluted fraction.

Far-UV CD spectra were taken of both the light- and mannoside-released fragments from the Con A-Sepharose 4B column (Figure 3). These measurements were made prior to the chloroform-CTAB extraction. The samples were in Con A-Sepharose 4B column buffer which contained 1.4% CTAB for the spectral measurements.

Both fractions exhibited intense CD bands at 222 nm. Because of the presence of CTAB, meaningful data at 210 nm could not be obtained. $[\theta]_{222}$ was used to estimate the helical content of proteolyzed opsin, the light-released fragments, and the mannoside-released fragment; these values were 37, 36, and 29% for proteolyzed opsin, fragments 2 and 3, and fragment 1, respectively. From this data, it can be seen that relatively little secondary structure is lost, even when rhodopsin proteolytic fragments are physically separated.

Discussion

Studies of water-soluble, globular proteins have suggested that the tertiary structure of these proteins is comprised of several independently folded domains (Goldberg, 1969; Reed et al., 1975; Hogbert-Raibaud and Goldberg, 1977). Proteolysis experiments, on a class of nonpenetrating membrane proteins such as is represented by cytochrome b_5, have indicated a substructure characterized by a hydrophobic tail, whereby the protein interacts with the bilayer and a globular region containing the active site (Spatz and Strittmatter, 1971; Strittmatter et al., 1972). A membrane-penetrating protein, glycophorin, has been shown to have a hydrophobic region which passes through the bilayer (Segrest et al., 1973). In both of these examples, the major mass of the protein is external to the bilayer. Bacteriorhodopsin, whose major mass resides in the membrane, consists of seven transmembrane helical segments connected by random-coil regions (Henderson and Unwin, 1975). It is of interest therefore to investigate the substructure of a protein, such as rhodopsin, whose major mass is also thought to reside in the membrane (Saibil et al., 1976) and whose function is suggested to be a translocation of calcium across the rod outer segment disk membrane (Hagins, 1972; Hagins and Yoshikami, 1974; Yoshikami and Hagins, 1973; Smith et al., 1977).

We have utilized papain proteolysis of intact rod outer segment disk membranes as a means of generating rhodopsin fragments which can be examined so as to obtain information concerning the substructure of this protein. The fragmentation pattern was monitored as a function of time by $NaDodSO_4$-polyacrylamide gel electrophoresis. Within 10 min, about 80% of the rhodopsin was cleaved to a 34 000 molecular weight intermediate, I. The rhodopsin remaining at this point seemed to be resistant to papain attack. This intermediate contains both the retinal binding site and the carbohydrate group. A similar intermediate has been reported by Hargrave and Fong (1977) in their studies of the action of thermolysin on rhodopsin. Coincident with the disappearance of the intermediate is the appearance of the carbohydrate-containing fragment 1. This finding is also in agreement with thermolysin treatment (Pober and Stryer, 1975). Two additional fragments of molecular weight 19 000 (fragment 2) and 10 000 (fragment 3) appear in about the same time frame as fragment 1, the poorly staining fragment 2 apparently appearing after fragments 1

and 3. While fragment 1 produced a sharp, intensely staining Coomassie band on the gels, fragment 2 and 3 bands were more diffuse, making it difficult to quantitatively relate these two fragments either by mass percent or in time course of the development with the fragment 1. The fragment 3 correlates better with the appearance of the fragment 1 than does fragment 2.

Thermolysin proteolysis seems to produce only the equivalent of fragments 1 and 3 but not a species corresponding to fragment 2. Papain fragment 2 is weakly staining and diffuse, possibly due to heterogeneity, and thus the equivalent thermolysin fragment may have been missed. If fragment 2 represents a further breakdown product of fragment 1, then it would appear to have lost the carbohydrate groups, since it no longer stains positively with PAS. The lack of a carbohydrate associated with the fragment 2 is further supported by the fact that this fragment is released from a Con A-Sepharose 4B column by light rather than by α-methyl mannoside. Alternatively, fragment 2 may arise from some minor alternate cleavage pattern or may in fact be a dimer of fragment 3. The copurification of fragment 2 with unbleached, proteolyzed rhodopsin seems to imply that it is a direct proteolysis fragment of rhodopsin rather than a further breakdown product of an earlier fragment. Additional evidence for the three-fragment pattern comes from the work of Sale et al. (1977); these authors observe the equivalent of our fragments 1-3 in their gel patterns of papain-cleaved rhodopsin.

The visible CD of rhodopsin arises from an optical activity of the 11-*cis*-retinal chromophore and is thought to arise either from a geometric distortion of the chromophore planarity upon binding to opsin (Mommaerts, 1969; Sperling and Rafferty, 1969; Burke et al., 1973; Ebrey and Yoshizawa, 1973), by interaction of the chromophore with an asymmetric electrostatic field induced by the opsin side chains in the retinal binding site region (Johnston and Zand, 1972; Kropf et al., 1973; Waggoner and Stryer, 1971), or the stereospecific binding of a single enantiomer of 11-*cis*-retinal by opsin (Honig et al., 1973). Given the sensitivity of optical activity to local geometry, the retention of the visible CD spectrum upon solubilization of proteolyzed rhodopsin is strong evidence that the molecule is solubilized as a noncovalently, associated form of the fragments, with an unaltered retinal binding site. This observation indicates that any residues removed from rhodopsin during proteolysis must be distant from the chromophore site and have had no influence on the tertiary structure of the site. It would appear that the orientation of the interacting fragments is essentially that of the native protein. The ability to bleach and regenerate proteolyzed rhodopsin in the disk membrane supports the conclusion that this form exists in the membrane also. Additional evidence for the association of rhodopsin proteolytic fragments, upon solubilization, comes from the binding of proteolyzed rhodopsin to a Con A-Sepharose 4B column, with subsequent elution of purified proteolyzed rhodopsin (plus some intact rhodopsin); this material yields the same fragment banding pattern on $NaDodSO_4$-polyacrylamide gel electrophoresis as solubilized disks and a native 500-nm absorption band and visible and UV CD spectrum. Since the carbohydrate moieties are associated with fragment 1, copurification of the fragments on Con a-Sepharose 4B and maintenance of native optical properties provide a strong demonstration of their state of association in solubilized, proteolyzed rhodopsin.

The far-UV CD spectrum was used to monitor changes in the rhodopsin secondary structure after proteolysis. The α-helical structure produces the greatest contribution to the CD spectrum in the region of 207 to 240 nm (Chen et al., 1974). The random coil has essentially no contribution above 207 nm and other workers have found no evidence for β structure in rhodopsin (Rafferty et al., 1977; Rothschild et al., 1976). According to computed spectra based on protein X-ray data, the ratio of $[\theta]_{210}/[\theta]_{222}$ for an α-helical structure is 0.88. The $[\theta]_{210}/[\theta]_{222}$ ratio of 0.9, which was obtained for rhodopsin in the absence of scattering, is consistent with a minimal amount of β structure. Consistent with earlier reports (Shichi et al., 1969; Shichi and Shelton, 1974; Stubbs et al., 1976), we have found rhodopsin to contain about 60% α-helical secondary structure. A helical content of 29% was recently reported by Rafferty et al. (1977). These measurements were made on solubilized rod outer segments. Figures presented by these authors for the percent of rhodopsin, relative to the total protein content of their preparations, indicate that their measurements were made on samples which contained from 30 to 50% bleached rhodopsin. Since opsin undergoes a 25% loss in helicity upon solubilization (Shichi, 1973; Stubbs et al., 1976), this would reduce the observed ellipticities in detergent solutions and lead to a lower estimate of the helical content of the remaining rhodopsin. In intact rhodopsin preparations, chromatographically purified on a Con A Sepharose 4B column using the detergent OG, only rhodopsin was eluted in the α-methyl mannoside fraction. Spectra taken of these solutions are not complicated by the presence of opsin and support a 60% α-helical content for rhodopsin. After proteolysis, rhodopsin lost little or no helical secondary structure as measured by the UV CD. This implies that the helical portions were buried in the membrane, protected from proteolytic attack, and that they were stable and independent of interactions with the portion of the protein which was removed. Proteolyzed rhodopsin retained its native UV CD spectrum in OG, emulphogene, and CTAB and is therefore not destabilized relative to native rhodopsin in these different detergents. Upon bleaching, proteolyzed rhodopsin showed the same 25% loss of helical structure exhibited by native solubilized rhodopsin.

In order to determine the structural independence of the rhodopsin subdomains represented by the proteolytic fragments, we undertook their separation and purification. Pober and Stryer (1975) had shown that thermolysin-proteolyzed rhodopsin could be dissociated and separated by bleaching rhodopsin bound to a Con A-Sepharose 4B column. Similar treatment of papain-proteolyzed rhodopsin resulted in a release by light of fragments 2 and 3 followed by α-methyl mannoside release of fragment 1, thus allowing a partial separation of the three fragments. UV CD measurements made on the light-released and α-methyl mannoside released fragments indicate that the helical content of these fragments is very similar to the helical content of purified opsin. These data demonstrate that these fragments represent essentially independently folded subdomains in the tertiary structure of rhodopsin. Whether these fragments represent independently nucleated folding domains, which form as the molecule grows on the polyribosome, cannot be determined at this point. They may initially be thought of as random fragments generated by papain cleavage. However, the similar fragmentation pattern observed with several proteolytic enzymes implies that there exist specific exposed regions of the protein which are susceptible to cleavage and serve to connect the three fragments. These connection regions likely coincide with random-coil sections connecting helical segments which penetrate through the membrane. Since proteolysis is carried out in a fashion which exposes only one side of the membrane to the protease, only turns on the extradiscal side are available for cleavage.

It is of interest to compare the available structural information for glycophorin and bacteriorhodopsin with that for

bovine rhodopsin. The membrane-spanning segment of glycophorin consists of 23 residues in the form of an α helix (Segrest and Kohn, 1973); this corresponds to a length of 34.5 Å, in good agreement with the estimated thickness of the hydrocarbon region of biological membranes. Bacteriorhodopsin has a molecular weight of 26 000, 70% of which is estimated to be in the form of an α helix (Reynolds and Stoeckenius, 1977); this helical structure has been shown to be in the form of seven transmembrane segments (Henderson and Unwin, 1975). On the basis of the helical content, molecular weight, and number of transmembrane segments, one can estimate for bacteriorhodopsin that each helical segment is the order of 23 residues long, in good agreement with the membrane spanning the segment of glycophorin. Bovine rhodopsin has a molecular weight of about 38 000 and is estimated to contain about 60% α-helical secondary structure; this corresponds to about 195 residues in α-helical segments. Assuming 23 residues per transmembrane helical segment, one can estimate that there are between eight and nine such segments in bovine rhodopsin. Since the amino and carboxy termini of rhodopsin are located on opposite surfaces of the disc membrane, an odd number of transmembrane helical spans are required in the tertiary folding of rhodopsin. Bovine rhodopsin is, therefore, likely to have either seven or nine transmembrane helical spans. By analogy with bacteriorhodopsin, one would expect these helices to interact in a manner which would give rhodopsin a cylindrical shape; these helices, along with the extramembranous mass of rhodopsin, would cause rhodopsin to appear as a prolate ellipsoid in hydrodynamic studies. This is in agreement with the observations of Wright (1974).

While this manuscript was in preparation, Sale et al. (1977) reported their studies of the effect of papain proteolysis on rhodopsin. They concluded that papain-proteolyzed rhodopsin consists of three fragments which remain associated in an active form. Their work associates the retinal exclusively with the intermediate molecular weight fragment. We observed fluorescence associated with the first and third fragments. These authors report that proteolyzed rhodopsin undergoes a meta I to meta II transition in the temperature range normally associated with this photolytic transition and concluded that the proteolyzed visual pigment was functionally active. These results are in agreement with our preliminary results that papain-proteolyzed disk membranes show a light-stimulated release of calcium similar to that which this laboratory has reported for native disks (Smith et al., 1977).

A variety of experiments suggest that rhodopsin undergoes a conformation change upon bleaching in the disk membrane (Saibil et al., 1976; Liebman et al., 1974; Downer and Englander, 1977; McDowell and Williams, 1976; Ostroy, 1977). However, it has been a long-standing observation that no change in helical content, as estimated by the UV CD, occurs on bleaching under similar conditions (Shichi, 1973). The results obtained here provide a possible explanation for this observation. It is clear that the papain-generated fragments remain associated in an essentially native tertiary conformation, both in the membrane and after solubilization in detergent. The retinal binding site is apt to be located in a hydrophobic interfacial region where these subdomains interact. In the intact visual pigment, these subdomains are connected by random-coil sections. If bleaching, with the concomitant 11-cis to all-trans isomerization of retinal, were to modify the interactions at this interaction surface, then a change in conformation in the tertiary structure of rhodopsin could occur through motion of the subdomains at their interaction surface, without any change in secondary conformation. It is suggested that such a motion occurs on bleaching and is responsible for both the exposure of new reactive groups on bleaching and the calcium translocation thought to be the coupling mechanism in visual transduction.

The results reported here imply a model for rhodopsin of a series of transmembrane helical segments connected by random-coil regions, thus suggesting considerable structural analogy with bacteriorhodopsin. Both molecules function as ion translocaters. Whether this type of structure will be of a general nature for translocating membrane proteins remains to be seen. The independent nature of the secondary folding of the subdomains of rhodopsin generated by papain proteolysis has been demonstrated here. Maintenance of the native visible absorption and CD spectra and the UV CD spectra of purified proteolyzed rhodopsin, relative to native rhodopsin, provides strong evidence that rhodopsin can be solubilized as a hydrophobically associated unit of the papain fragments in an essentially native tertiary conformation.

Acknowledgments

The authors thank Drs. P. A. Hargrave and J. H. McDowell for their critical comments.

References

Akhtar, M., Blosse, P. T., and Dewhurst, P. B. (1965), *Life Sci. 4,* 1221.
Bownds, D. (1967), *Nature (London) 216,* 1178.
Burke, M. J., Pratt, D. C., Faulkner, R. R., and Moscowitz, A. (1973), *Exp. Eye Res. 17,* 557.
Chen, Y., Young, J. T., and Martinez, H. M. (1972), *Biochemistry 11,* 4120.
Chen, Y., Yang, J. T., and Chau, K. H. (1974), *Biochemistry 13,* 3350.
Downer, H. W., and Englander, S. W. (1977), *J. Biol. Chem. 252,* 8101.
Ebrey, T. G., and Yoshizawa, T. (1973), *Exp. Eye Res. 17,* 545.
Fager, R., Sejnowski, P., and Abrahamson, E. W. (1972), *Biochem. Biophys. Res. Commun. 47,* 1244.
Goldberg, M. E. (1969), *J. Mol. Biol. 46,* 441.
Hagins, W. A. (1972), *Annu. Rev. Biophys. Bioeng. 1,* 131.
Hagins, W. A., and Yoshikami, S. (1974), *Exp. Eye Res. 18,* 299.
Hargrave, P. A. (1978), *Biochim. Biophys. Acta* (in press).
Hargrave, P. A., and Fong, S. L. (1977), *J. Supramol. Struct. 6,* 559.
Henderson, R., and Unwin, P. N. T. (1975), *Nature (London) 257,* 28.
Hogberg-Raibaud, A., and Goldberg, M. E. (1977), *Biochemistry 16,* 4014.
Honig, B., Kahn, P., and Ebrey (1973), *Biochemistry 12,* 1637.
Hubbard, R., and Kropf, A. (1958), *Proc. Natl. Acad. Sci. U.S.A. 44,* 130.
Jackson, R. L., Segrest, J. P., Kahan, I., and Marchesi, V. T. (1973), *Biochemistry 12,* 3131.
Johnston, E. M., and Zand, R. (1972), *Biochem. Biophys. Res. Commun. 47,* 712.
Kropf, A., Whittenberger, P., Goff, S. P., and Waggoner, A. S. (1973), *Exp. Eye Res. 17,* 591.
Laemmli, U. K. (1970), *Nature (London) 227,* 680.
Liebman, P. A., Jagger, W. S., Kaplan, M. W., and Bargott, F. G. (1974), *Nature (London) 251,* 31.
Litman, B. J. (1972), *Biochemistry 11,* 3243.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
McDowell, J. H., and Williams, T. P. (1975), *Vision Res. 16,*

643.
Mommaerts, W. F. H. M. (1969), The Retina, Straatsma, B. R., et al., Ed., Los Angeles, Calif., University of California Press, p 225.
Ostroy, S. E. (1977), *Biochim. Biophys. Acta 463,* 91.
Papermaster, D. S., and Dreyer, W. J. (1974), *Biochemistry 13,* 2438.
Pober, J. S., and Stryer, L. (1975), *J. Mol. Biol. 95,* 477.
Rafferty, C. N., Cassim, J. Y., and McConnell, D. G. (1977), *Biophys. Struct. Mech. 2,* 277.
Reed, R. G., Feldhoff, R. C., Clute, O. L., and Peters, T. (1975), *Biochemistry 14,* 4578.
Reynolds, J. A., and Stoeckenius, W. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 2803.
Rothschild, K. J., Andrew, J. R., DeGrip, W. J., and Stanley, H. E. (1976), *Science 191,* 1176.
Saari, J. C. (1974), *J. Cell Biol. 63,* 480.
Sailbil, H., Chabre, M., and Worcester, D. (1976), *Nature (London) 262,* 266.
Sale, G. J., Towner, P., and Akhtar, M. (1977), *Biochemistry 16,* 5641.
Segrest, J. P., and Jackson, R. L. (1972), *Methods Enzymol. 28,* 54.
Segrest, J. P., and Kohn, L. D. (1973), *Protide Biol. Fluids; Proc. Colloq. 21,* 183–189.
Segrest, J. P., Kahan, I., Jackson, R. L., and Marchesi, V. T. (1973), *Arch. Biochem. Biophys. 155,* 167.
Shichi, H. (1973), *Exp. Eye Res. 17,* 533.
Shichi, H., and Shelton, E. (1974), *J. Supramol. Struct. 2,* 7.
Shichi, H., Lewis, M. S., Irreverre, F., and Stone, A. L. (1969), *J. Biol. Chem. 244,* 529.
Smith, H. G., Stubbs, G. W., and Litman, B. J. (1975), *Exp. Eye Res. 20,* 211.
Smith, Jr., H. G., Fager, R., and Litman, B. J. (1977) *Biochemistry 16,* 1399.
Spatz, L., and Strittmatter, P. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 1042.
Sperling, W., and Rafferty, C. N. (1969), *Nature (London) 224,* 591.
Steinemann, A., and Stryer, L. (1973), *Biochemistry 12,* 1499.
Strittmatter, P., Rogers, M. J., and Spatz, L. (1972), *J. Biol. Chem. 247,* 7188.
Stubbs, G. W., Smith, H. G., Jr., and Litman, B. J. (1976), *Biochim. Biophys. Acta 426,* 46.
Tomita, M., and Marchesi, V. T. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2964.
Trayburn, P., Mandel, P., and Virmaux, N. (1974a), *FEBS Lett. 38,* 351.
Trayburn, P., Mandel, P., and Virmaux, N. (1974b), *Exp. Eye Res. 19,* 259.
Van Breugel, P. J. G. M., Daemen, F. J. M., and Bonting, S. L. (1975), *Exp. Eye Res. 21,* 315.
Waggoner, A. S., and Stryer, L. (1971), *Biochemistry 10,* 3250.
Wald, G. (1968), *Nature (London) 219,* 800.
Wright, A. K. (1974), *Biophys. J. 14,* 243.
Yoshikami, S., and Hagins, W. A. (1973), *Biochem. Physiol. Visual Pigm.* 245.

Reversible Dissociation of the $\alpha\beta$ Dimer of Tubulin from Bovine Brain†

H. William Detrich III‡ and Robley C. Williams, Jr.*

ABSTRACT: The reversible, concentration-dependent dissociation of the $\alpha\beta$ dimer of bovine brain tubulin (purified by phosphocellulose chromatography) has been demonstrated by equilibrium ultracentrifugation. The dissociation constant is approximately 8×10^{-7} M at 4.6 °C in PM buffer (0.1 M piperazine-*N,N'*-bis(2-ethanesulfonic acid), 2 mM ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid, 1 mM $MgSO_4$, 0.1 mM guanosine triphosphate, 2 mM dithioerythritol, at pH 6.9). This result was confirmed by observation of an appropriate dependence of the sedimentation coefficient of very dilute (<0.5 mg/mL) tubulin on its concentration. Small zone gel filtration experiments on Bio-Gel P-150 also demonstrated an increase in peak elution volume with decreasing column load concentration. Reversibility of the dissociation was demonstrated directly by sedimentation velocity and gel filtration experiments on tubulin reconcentrated from dilute solution by pressure ultrafiltration. Control experiments accompanying the sedimentation equilibrium experiments showed that this tubulin retained, under the conditions of the experiments, both its ability to form microtubules and more than 70% of its initial colchicine-binding activity.

The ordered assembly, maintenance, and disassembly of cytoplasmic microtubules appear to play critical roles in the functioning of eukaryotic cells (Porter, 1966; Olmsted & Borisy, 1973; Roberts, 1974; Snyder & McIntosh, 1976; Pipeleers et al., 1976; Yahara & Edelman, 1975; Edelman, 1976). An understanding of these roles will depend in part upon detailed knowledge of the properties of the major protein constituent and functional subunit of the microtubule, the tubulin dimer. This structure is probably an $\alpha\beta$ heterodimer of two related polypeptide chains (Bryan & Wilson, 1971; Ludueña et al., 1977) held together by noncovalent forces (Lee et al., 1973).

† From the Department of Molecular Biology, Vanderbilt University, Nashville, Tennessee 37235. *Received March 29, 1978.* This work was supported by Research Grants HL 12901 and HL 20873 and by Training Grant HD 00032 of the National Institutes of Health, as well as Biomedical Research Support Grant RR 07089-11 of the Public Health Service.

‡ Formal affiliation: Yale University, New Haven, Conn.

0006-2960/78/0417-3900$01.00/0