Lynch, W. P., Riseman, V. M., & Bretscher, A. (1987) *J. Biol. Chem. 262*, 7429-7437.
Marston, S. B., & Lehman, W. (1985) *Biochem. J. 231*, 517-522.
Marston, S. B., & Smith, C. W. J. (1985) *J. Muscle Res. Cell Motil. 6*, 669-708.
Ngai, P. K., & Walsh, M. P. (1984) *J. Biol. Chem. 259*, 13656-13659.
Ngai, P. K., & Walsh, M. P. (1985a) *Biochem. Biophys. Res. Commun. 127*, 533-539.
Ngai, P. K., & Walsh, M. P. (1985b) *Biochem. J. 230*, 695-707.
Owada, M. K., Hakura, A., Iida, K., Yahara, I., Sobue, K., & Kakiuchi, S. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3133-3137.
Pepinsky, R. B. (1983) *J. Biol. Chem. 258*, 11229-11235.
Sobue, K., Muramoto, Y., Fujita, M., & Kakiuchi, S. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5652-5655.
Sobue, K., Morimoto, K., Inui, M., Kanda, K., & Kakiuchi, S. (1982) *Biomed. Res. 3*, 188-196.
Sobue, K., Tanaka, T., Kanda, K., Ashino, N., & Kakiuchi, S. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5025-5029.
Szpacenko, A., & Dabrowska, R. (1986) *FEBS Lett. 202*, 182-186.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350-4354.
Umekawa, H., & Hidaka, H. (1985) *Biochem. Biophys. Res. Commun. 132*, 56-62.
Zweig, S. E., & Singer, S. J. (1979) *Biochem. Biophys. Res. Commun. 88*, 1147-1152.

# Molecular Characterization of the $AP_{180}$ Coated Vesicle Assembly Protein†

Kondury Prasad* and Roland E. Lippoldt
*Clinical Endocrinology Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892*

*Received December 1, 1987; Revised Manuscript Received March 17, 1988*

ABSTRACT: Recently, a new clathrin assembly protein ($AP_{180}$) has been purified from coated vesicles of bovine brain (Ahle & Ungewickell, 1986). This protein has been shown to promote polymerization of clathrin into a homogeneous population of baskets under conditions where pure clathrin does not polymerize by itself. We have purified this protein from coated vesicles by a simpler method than has been reported. The method involves a gel filtration step on a Sephacryl S-300 column, in 0.5 M Tris-HCl, pH 8.0, and a hydroxylapatite column eluted with 10 mM sodium phosphate/0.5 M Tris-HCl, pH 7.0. By running SDS gels over an extended period of time (5-15% gradient gel, 10 mA for the first 12 h followed by 20 mA for the next 3-4 h) after the marker dye entered the electrode buffer, we have been able to separate $AP_{180}$ from clathrin heavy chain on the gels. This enabled us to determine its stoichiometry to clathrin heavy chains in isolated coated vesicles and assembled baskets, and was helpful in the purification procedure. The apparent molecular weight of the pure protein on SDS gels was about 180000, yet gel filtration yielded values of about 120000. Thus, we undertook the molecular weight determination by another independent method, sedimentation equilibrium analysis, and found a molecular weight of 115000 and a sedimentation coefficient of 3.50 ± 0.05 S. Circular dichroism data revealed that it has 30% helical structure, 14% β-structure, 27% β-sheet, and the rest random peptides. A scan of the Coomassie Blue stained electrophoretic gel pattern of the polymerized baskets showed that every $AP_{180}$ protein molecule can polymerize approximately one clathrin triskelion into baskets, at 0.1 M Mes-NaOH, pH 6.5. $AP_{180}$ is also involved in binding of clathrin to stripped vesicles, and in this respect it resembles the other assembly proteins of 110-100-50-47 kDa of coated vesicles.

Clathrin-coated vesicles are involved in a variety of cellular processes, namely, membrane recycling, receptor-mediated endocytosis, and transfer of proteins across and between membranes (Brown et al., 1983; Goldstein et al., 1985; Keen, 1985; Heuser & Reese, 1973). The principal protein of the coat of coated vesicles is clathrin with three identical subunits of 180 kilodaltons (kDa)[1] and three light chains of two sizes, i.e., 33 and 36 kDa (Kirchhausen & Harrison, 1981; Pretorius et al., 1981). Clathrin can be dissociated from coated vesicles by a variety of processes, namely, treatment with 0.5 M Tris-HCl, pH 8.0, dialysis against 0.01 M Tris-HCl, pH 8.5, and treatment with 2 M urea (Keen et al., 1979; Pearse & Robinson, 1984; Pretorius et al., 1981; Schook et al., 1979; Woodward & Roth, 1979). Depending on the procedure used to obtain the clathrin-containing fraction, a certain proportion of a group of proteins of 110-100-50-47 kDa remain partly associated with the vesicle and partly with clathrin. Clathrin can then be separated from the above associated proteins by gel filtration in 0.5 M Tris-HCl, pH 8.0, on a Sephacryl S-300 or Sepharose 4B-Cl column (Keen et al., 1979; Prasad et al., 1986). Clathrin thus purified can be polymerized by reducing the pH to 6.0-6.2 in the presence of 0.1 M Mes-NaOH or in the pH range of 6.2-6.5 by the addition of millimolar $Ca^{2+}$ concentrations (Keen et al., 1979; Prasad et al., 1985). The polymerized clathrin resembles the lattice coat of the isolated coated vesicles but with a variety of sizes of basket-like

† Dedicated to the memory of the late Dr. Harold Edelhoch, in whose laboratory the authors have begun their work on clathrin.

* Address correspondence to the author at the National Institutes of Health, Building 10, Room 8N313, 9000 Rockville Pike, Bethesda, MD 20892.

[1] Abbreviations: CV(s), coated vesicle(s); UV(s), uncoated vesicle(s); ANM, AN-maleimide or *N*-(1-anilinonaphthalenyl)maleimide; AN, anilinonaphthalene; Tris, tris(hydroxymethyl)aminomethane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; SDS, sodium dodecyl sulfate; kDa, kilodalton(s); CHC, clathrin heavy chain; LCs, light chains.

structures, the smallest being of the order of 60 nm and the largest being about 120 nm.

The 110–100–50–47-kDa group of proteins described above can be separated and purified from either clathrin or the vesicle by gel filtration in 0.5 M Tris-HCl, pH 8.0. These proteins are capable of polymerizing clathrin at more physiological conditions (e.g., at pH 7.0–7.4) into a homogeneous population of baskets of the size range of 60–80 nm (Zaremba & Keen, 1983) and are also involved in the binding of clathrin triskelions to the stripped vesicles (Prasad et al., 1985). It has been shown that of the several 110–100-kDa proteins some are capable of polymerizing clathrin into predominantly large structures while some induce polymerization into smaller structures, though the combined group enhances the polymerization into smaller structures (Pearse & Robinson, 1984). We have recently reported the purification of one of the above 100-kDa proteins (Prasad et al., 1986).

Recently, Ahle and Ungewickell (1986) reported the purification of a new clathrin assembly protein that has a mobility nearly identical with clathrin heavy chains on SDS gels but has a much lower molecular weight when determined by hydrodynamic methods. The protein was identified and purified by using a monoclonal antibody (MAB) to the new protein. In view of its anomalous mobility on SDS gels (giving an apparent molecular weight of 180000), we determined to study its molecular weight by another independent method, sedimentation equilibrium analysis. We have also purified it by a simpler procedure and are able to identify it on SDS gels along with clathrin. We report here some of its molecular properties and its interaction with clathrin to form baskets.

## Materials and Methods

*Chemicals.* Mes was from Sigma. Tris-HCl was from Schwarz/Mann. Sodium dodecyl sulfate (SDS) was a very pure grade from BDH Poole, Ltd. All other reagents were Fisher analytical grade. Glass-distilled water was used everywhere. Hydroxylapatite, fast flow, was obtained from Calbiochem. *N*-(1-Anilinonaphthalenyl)maleimide (ANM) was obtained from Polysciences.

*Purification of Clathrin.* Coated vesicles (CVs) were prepared by the method of Nandi et al. (1982). Clathrin was obtained by dissociating CVs in 0.01 M Tris-HCl, pH 8.5, and centrifuging the contents in a 70.1 Ti Beckman rotor at 55000 rpm for 1 h. It was further separated from the 110–100–50–47-kDa and $AP_{180}$ proteins by gel filtration on a Sephacryl S-300 column (see below). The purity of the clathrin preparation was routinely checked for the contamination with $AP_{180}$ or other associated proteins by SDS gels described in this section. Purified clathrin does not polymerize at 0.1 M Mes–NaOH, pH 6.5.

*Column Chromatography.* The gel filtration of proteins was carried out in a Sephacryl S-300, 1.5 × 196 cm, column equilibrated with 0.5 M Tris-HCl, pH 8.0. A flow rate of 10 mL/h was used. Two-milliliter fractions were collected, and the protein concentration was determined by using tryptophan fluorescence.

*Hydroxylapatite Chromatography.* The proteins were adsorbed onto a hydroxylapatite column (dry weight, 2 g; height, 0.6 cm; diameter, 2.5 cm) equilibrated in 0.5 M Tris-HCl, pH 7.0. After the column was washed with the equilibration buffer, it was eluted in the same buffer, containing appropriate concentrations of sodium phosphate. Typically, about 100-mL samples were loaded at the rate of 1 mL/min, and elution was carried out at a rate of 1 mL/5 min. A slow elution was found to be necessary to avoid the contamination of $AP_{180}$ with clathrin. When the elution was carried out with 10 mM phosphate/0.5 M Tris-HCl, pH 7.0, $AP_{180}$ eluted as a sharp peak between 14 and 19 mL in the elution, with a significant trail until 30 mL of the eluate.

*Gel Electrophoresis.* Polyacrylamide gels (5–12% or 5–15%) were employed. Electrophoresis in SDS was performed according to the procedure of Laemmli (1970). Gels were typically run at 10 mA for 12–15 h, followed by 20–30 mA for the next 3–4 h. The experimental conditions for each of the gels described in this paper are given under the respective figure legends and described in the text. Gels were stained with a solution consisting of 0.25% Coomassie Brilliant Blue stain R-250 in 50% trichloroacetic acid for 45 min and destained with a solution consisting of 2% ethanol and 7.5% acetic acid. They were scanned for Coomassie Blue stain at 590 nm with a Beckman DU8 spectrophotometer equipped with a gel scanner.

*Fluorescence Measurements.* Fluorescence intensities were measured in a Perkin-Elmer MPF-3 spectrofluorometer. Relative protein concentration was determined by using the intrinsic tryptophan fluorescence by exciting at 290 nm and measuring the emission at 340 nm. *N*-(1-Anilinonaphthalenyl)maleimide (ANM)-labeled proteins were measured by excitation at 350 nm and emission at 426 nm.

*Sucrose Gradient Centrifugation.* Solutions of polymerized clathrin or re-formed CVs were sedimented on a 10–30% sucrose gradient in an SW 40 rotor at 27000 rpm for 110 min in an L8M-Beckman ultracentrifuge. Nine-drop fractions from the gradient were collected from the bottom of the tube by using a peristaltic pump. They were assayed by monitoring the intrinsic tryptophan fluorescence or AN fluorescence of the labeled proteins.

*Circular Dichroism.* The circular dichroism spectra were obtained on a Jasco J500C spectropolarimeter equipped with a temperature-controlled cell block set at 25 °C. The light path was 0.20 cm, and the scan was at 1 nm/min with a sensitivity of 1 mdeg/cm. The protein concentration was 0.0617 mg/mL.

*Protein Concentration.* The concentrations of $AP_{180}$ and clathrin were determined by measuring the ultraviolet absorbance at 215 and 280 nm and using the equation

$$C\ (\mathrm{mg/mL}) = (A_{215} - 3.667A_{280})/11.55$$

that was reported by us earlier (Prasad et al., 1986). An extinction coefficient of $E_{1\%}^{280\mathrm{nm}} = 3.8$ is obtained for the purified $AP_{180}$. For pure clathrin, an extinction coefficient of $E_{1\%}^{280\mathrm{nm}} = 12.1$ was determined.

*Sedimentation Velocity and Sedimentation Equilibrium.* The sedimentation velocity and equilibrium studies were carried in a Beckman Model E analytical ultracentrifuge equipped with an ultraviolet scanner. For the velocity runs, temperature was controlled at 25 °C by an RTIC-type device from Arden Instruments. For the equilibrium study, the sedimentation pattern analyzed here was obtained after 48 h at 13000 rpm and at a temperature of 7.9 °C. The sample volume was 120 μL. The solution height in the cell was 3.9 mm, and the light path was 1.2 cm. The optical density of the solution was 0.21 at 280 nm.

*Preparation of Stripped Vesicles or Uncoated Vesicles (UVs).* A suspension of CVs in 0.1 Mes–NaOH, pH 6.5 (2 mg/mL), was dialyzed against 0.01 M Tris-HCl, pH 8.5, for 15 h. The solution was then layered on 10–30% sucrose gradients and centrifuged at 35000 rpm for 3.5 h at 20 °C in an SW 40 rotor. Under these conditions, the stripped vesicles or uncoated vesicles (UVs) were well separated from the soluble fraction of clathrin and other associated proteins (data not shown). Peak fractions corresponding to UVs were

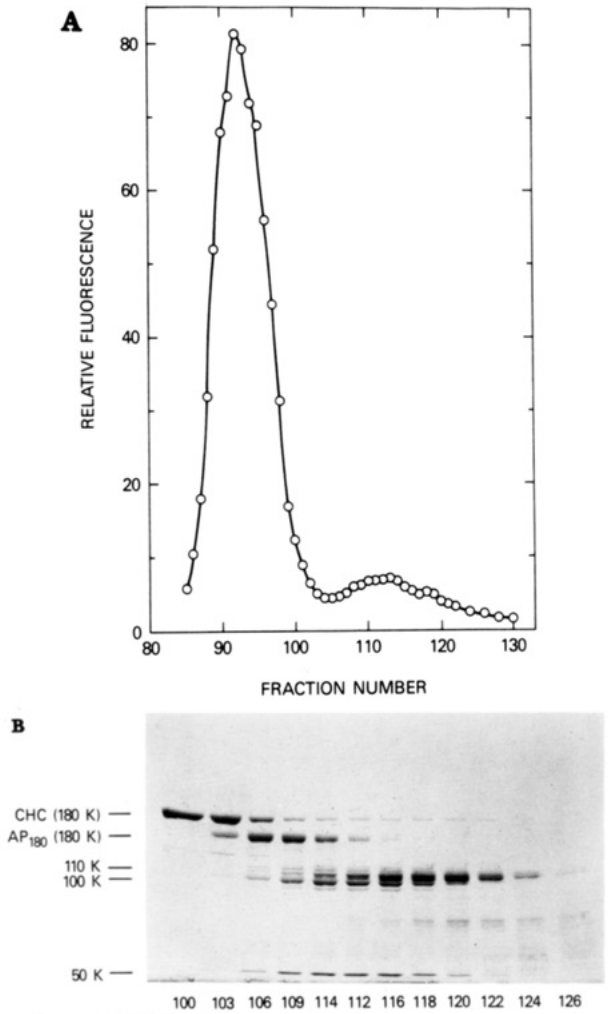


FIGURE 1: (A) Sephacryl S-300 chromatography elution profile of a 0.01 M Tris-HCl, pH 8.5, extract of CVs in 0.5 M Tris-HCl, pH 8.0. Fractions of 2 mL were collected. (B) SDS gel electrophoretic pattern of various fractions of the above column: numbers below the gel represent the fraction numbers in the column. The gel is a 5–15% gradient gel, run with a current of 10 mA for 12 h followed by 20 mA for the next 3 h.

pooled and dialyzed against 0.5 M Tris-HCl, pH 8.0, for 12 h. The resulting solution was then layered on 10–30% sucrose gradients, and contents were centrifuged at 35000 rpm for 4.5 h, at 20 °C, in the same rotor. Substantial amounts of associated proteins were well separated from UVs (data not shown). The UVs were then dialyzed against 0.01 M Tris-HCl, pH 8.0, to remove sucrose and high concentrations of Tris. To distinguish UVs from clathrin and $AP_{180}$, UVs were labeled with (anilinonaphthalenyl)maleimide (ANM). The labeling procedure was described elsewhere (Prasad et al., 1984). A densitometric scan of the Coomassie Blue stained SDS gels of CVs and 0.5 M Tris-HCl stripped vesicles revealed that for the same concentration of the vesicles (or for the same concentration of the tubulin band in the vesicles that is not extractable by 0.5 M Tris-HCl, pH 8.0, treatment; Pfeffer et al., 1983) clathrin, 110K–100K proteins, and 50K proteins are depleted to an extent of 96%, 77%, and 76%, respectively, in 0.5 M Tris-HCl, pH 8.0, stripped vesicles.

## RESULTS

*Purification of $AP_{180}$.* CVs were prepared in 0.1 M Mes–NaOH, pH 6.5, from bovine brain (Nandi et al., 1982). They were dialyzed against 0.01 M Tris-HCl, pH 8.5, for 15 h, and the contents were centrifuged in a 70.1 Ti rotor for 1 h at

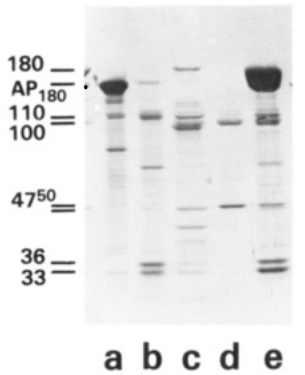


FIGURE 2: SDS gel electrophoretic pattern of fractionation of proteins by hydroxylapatite column chromatography. Fractions corresponding to fractions 101–124 of Figure 1A were pooled and, after being adjusted to pH 7.0, were loaded onto a hydroxylapatite column. The column was eluted with a gradient of 0.5 M Tris-HCl/0–500 mM phosphate, pH 7.0. Fraction corresponding to lane a, 10 mM phosphate; lane b, 25 mM phosphate; lane c, 150 mM phosphate; lane d, 500 mM phosphate; lane e, 0.01 M Tris-HCl, pH 8.5, extract of CVs. The gel is a 5–15% gradient gel, run with a current of 10 mA for 8 h followed by 25 mA for the next 4 h.

55000 rpm to separate the soluble proteins containing clathrin and the associated proteins from the pellet of the vesicles. The supernatant is then dialyzed against 0.5 M Tris-HCl, pH 8.0, and gel filtered on a Sephacryl S-300 column equilibrated in the same buffer. The column profile and SDS gel electrophoretic pattern of the fractions are shown in panels A and B, respectively, of Figure 1. The first peak at fraction 91 represents the clathrin fraction. The second broad peak represents the total associated proteins, with $AP_{180}$ preceding the other associated 110–100–50–47-kDa proteins, but nevertheless overlapping with them. Also to be noted is the separation of the 110-kDa group of proteins at the tail end of the column (fractions 122–124). In this gel, the proteins are electrophoresed for a longer time (5–15% gradient gel, 10 mA for the first 12 h followed by 20 mA for the next 3 h) after the dye entered the electrode buffer. A shorter run gel (5–15% gradient gel, 10 mA for 12 h) does not separate the $AP_{180}$ from clathrin. The 110–100–50–47-kDa proteins are partially separated from $AP_{180}$, as seen in Figure 1B. When the total of the associated proteins (including $AP_{180}$), representing fractions 101–124 in the above column, were pooled and adsorbed on a hydroxylapatite column in 0.5 M Tris-HCl, pH 7.0, and the column was eluted with a gradient of 0–500 mM phosphate, the $AP_{180}$ preferentially eluted at about 10 mM phosphate, and 110-kDa proteins eluted at about 25 mM phosphate, but in both cases there was a contamination of one from the other (Figure 2, lanes a and b). At higher phosphate concentrations, the 110–100–50–47-kDa proteins are resolved into two groups. One group contains a 47-kDa protein and about equivalent amounts of 100- and 110-kDa proteins at around 150 mM phosphate, and another group contains a 50-kDa protein with mostly 100-kDa proteins at around 500 mM phosphate (lane d). A similar fractionation of the 110–100–50–47-kDa proteins is recently reported by Manfredi and Bazari (1987) and Keen (1987). We believe the HA-I (peak I of hydroxylapatite column elution) group of proteins reported by Pearse and Robinson (1984) is the one eluted between 10 and 25 mM phosphate. Therefore, to avoid contamination of $AP_{180}$ with the 110-kDa group of proteins in the purification of $AP_{180}$, and since they share the same characteristic of eluting in low phosphate, we pooled the fractions in the S-300 column pattern that are richer in $AP_{180}$ (fractions 101–115) and discarded fractions 116–124 that are richer in 110-kDa and other associated proteins. When these fractions are adsorbed

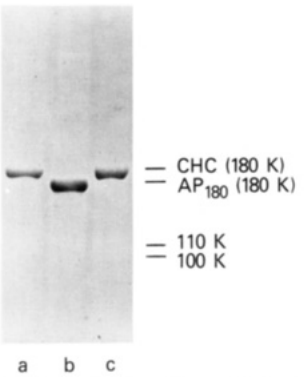


FIGURE 3: SDS gel electrophoretic analysis of the pure $AP_{180}$ characterized. Lane a, clathrin; lane b, $AP_{180}$; lane c, clathrin. The light chains of clathrin are not seen as they migrated into the running buffer. The gel is a 5–15% gradient gel run with 10 mA for 12 h followed by 20 mA for the next 3 h.

onto a hydroxylapatite column and the column eluted with 10 mM phosphate, $AP_{180}$ was eluted with greater than 97–98% purity. The purified protein is represented in Figure 3 (lane b) along with clathrin (lanes a and c). Earlier work of Ahle and Ungewickell (1986) suggested that $AP_{180}$ is sensitive to degradation. That the protein isolated here is actually $AP_{180}$, but not a degraded product of it (170 kDa), is clear for two reasons. First, as seen in SDS gels (Figures 2 and 3), there is no other band above the $AP_{180}$ band from which the present component could have been derived. Second, when the purified $AP_{180}$ was stored in cold (at 4 °C) for 6 weeks, it did get degraded to an extent of 20% to a predominantly 170-kDa band as reported by Ahle and Ungewickell (1986).

$AP_{180}$ can also be purified from the 0.01 M Tris-HCl, pH 8.5, stripped vesicles. However, it is generally observed that $AP_{180}$ purified from the vesicles is somewhat degraded (showing a significant amount of protein at a molecular weight range of 170000 along with the $AP_{180}$ band) compared to that obtained from the 10 mM Tris-HCl, pH 8.5, extract of CVs (data not shown). In addition, when the CVs and 0.01 M Tris-HCl, pH 8.5, extract of CVs were scanned for the relative ratio of clathrin to $AP_{180}$, it was found that roughly 62% of $AP_{180}$ is extractable by 0.01 M Tris-HCl, pH 8.5, treatment of CVs. It therefore appears that 0.01 M Tris-HCl, pH 8.5, stripped vesicles would be a relatively poorer source for the $AP_{180}$ in comparison to the 0.01 M Tris-HCl extract of CVs.

*Identification and Estimation of the Concentration of $AP_{180}$ in Isolated Coated Vesicles.* Given the right loading conditions and the other electrophoretic conditions described in this paper, $AP_{180}$ is readily identifiable in CVs and in 0.01 M Tris-HCl, pH 8.5, extract of CVs (used in the above purification procedure) by SDS gel electrophoresis. At a low level of loading of CVs, $AP_{180}$ is seen as a faint band, and at high concentration of CVs, it is masked by the clathrin heavy-chain band (data not shown). A scan of the Coomassie Blue stained SDS gels of CVs showed that the stain ratio of clathrin heavy chains to $AP_{180}$ is approximately 15.4:1.0. Assuming a molecular weight that is obtained in the present paper, this gave a mole ratio of 4.4 clathrin triskelions to $AP_{180}$. However, it was observed that under our present staining conditions, $AP_{180}$ stains poorly by Coomassie Blue. When equivalent amounts of clathrin heavy chains and $AP_{180}$ (1, 2, and 3 $\mu$g) are loaded on the same SDS gel side by side, and the Coomassie stained gels wee quantitated by scanning densitometry, it was found that for the same mass of the protein clathrin heavy chains stained 3.8 times as intensely as $AP_{180}$. We therefore used this factor in determining the relative concentrations of $AP_{180}$ with respect to clathrin throughout this report whenever it was required. When this was done, the molar ratio of clathrin triskelions to $AP_{180}$ in isolated coated vesicles appears to be 1.0:0.87.

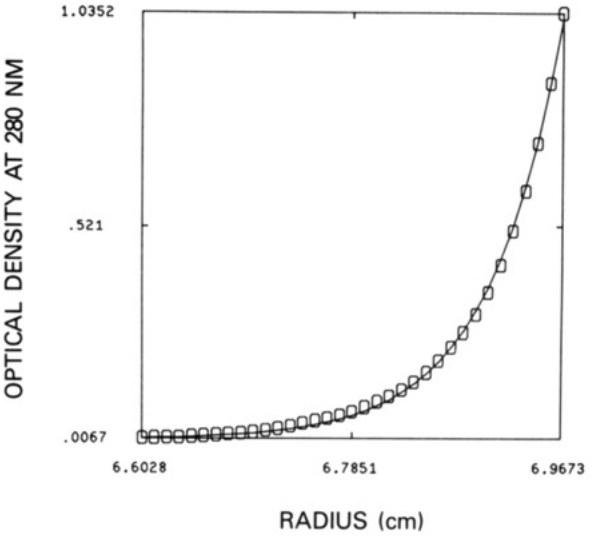


FIGURE 4: Equilibrium centrifugation pattern of purified $AP_{180}$ in 0.01 M Tris-HCl, pH 8.0. The circles represent the measured optical density. The line represents the fit of the data for a one-component system.

*Characterization of $AP_{180}$.* (*A*) *Sedimentation Velocity.* The purified $AP_{180}$ sediments as a symmetrical boundary with a sedimentation coefficient, $s_{20,w}$, of 3.50 ± 0.05 S in a solution of a concentration of 0.4 mg/mL, in 0.01 M Tris-HCl, pH 8.5, or 0.5 M Tris-HCl, pH 8.0. This value is in excellent agreement with the reported value of 3.5 S (Ahle & Ungewickell, 1986).

(*B*) *Sedimentation Equilibrium.* Sedimentation equilibrium analysis is carried out in 0.01 M Tris-HCl, pH 8.5, or in 0.5 M Tris-HCl, pH 8.0. In either of the solutions, the pattern is similar, and the former is shown in Figure 4. The line is a best-fit curve for a single component of molecular weight 115000 ± 800. The root mean square error for the fit was 0.0066. A partial specific volume of 0.73, reported by Ahle and Ungewickell (1986) obtained from their amino acid composition data, was used here. The fit was further improved by using a multicomponent analysis, but such an analysis gave an anomalously low value for the predominant component. However, since such heterogeneity was not seen in the SDS gels, the multicomponent analysis was discarded in favor of a single component. This molecular weight value of 115000 agrees with the value of 119000 or 123000 reported by Ahle and Ungewickell (1986) and confirms the notion that $AP_{180}$ has an anomalous mobility on SDS gels, and the true value lies closer to 115000. When the molecular weight of 115000 was combined with a sedimentation coefficient $s_{20,w}$ of 3.50 S, a frictional ratio of 2.43 was obtained.

(*C*) *Circular Dichroism.* The molecular ellipticity pattern of purified $AP_{180}$ in the far-ultraviolet wavelength region is similar to that of the 100-kDa protein reported by us earlier (Prasad et al., 1986). When the curve was analyzed by the modeling program of Provencher (1982a,b), it gave a composition of 30% helix, 14% $\beta$-structure, 27% $\beta$-sheets, and the rest random coil. The average deviation of the points from the fitted curve was ±0.38% of the value of the total curve height.

(*D*) *Polymerization of Clathrin.* Pure clathrin does not polymerize by itself in 0.1 M Mes–NaOH, pH 6.5, in the absence of divalent metal ions. However, the polymerization can be induced by the addition of the 110–100–50–47-kDa

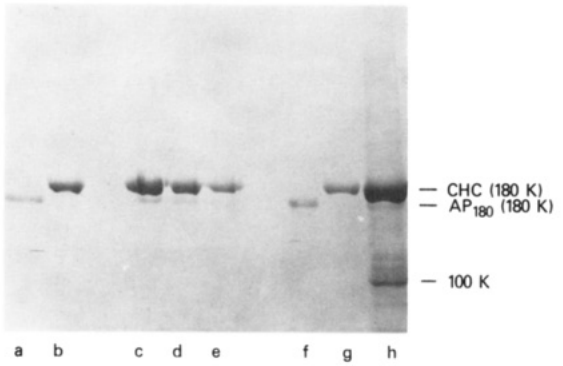


FIGURE 5: SDS gel electrophoretic analysis of the baskets formed from clathrin and $AP_{180}$. Lane a, $AP_{180}$; lane b, clathrin; lane c, baskets formed from 0.2 mg/mL clathrin plus 0.08 mg/mL $AP_{180}$; lane d, baskets formed from 0.2 mg/mL clathrin plus 0.055 mg/mL $AP_{180}$; lane e, baskets formed from 0.2 mg/mL clathrin plus 0.027 mg/mL $AP_{180}$; lane f, $AP_{180}$; lane g, clathrin; lane h, CVs. Clathrin in 0.01 M Tris-HCl, pH 8.0, is dialyzed overnight against 0.1 M Mes–NaOH, pH 6.5, to which a concentrated solution of $AP_{180}$ in 0.01 M Tris-HCl, pH 8.0, is added to give the final concentrations described above. Final buffer conditions are 0.002 M Tris-HCl/0.08 M Mes–NaOH, pH 6.5. The solutions (1 mL of each) were analyzed on 10–30% sucrose gradients. Peak fractions representing baskets were concentrated by vacuum dialysis and loaded on SDS gels. The purpose of this gel is to quantitate the relative ratio of clathrin to $AP_{180}$ under the varying concentration ratios described in the text. The gel is a 5–12% gradient gel, run with a current of 10 mA for the first 10 h followed by 30 mA for the next 2–3 h.

associated proteins. The new $AP_{180}$ is also capable of polymerizing clathrin in 0.1 M Mes–NaOH, pH 6.5, as reported by Ahle and Ungewickell (1986). We have studied this reaction in order to understand the nature of the reaction and to determine the stoichiometry of the products formed. Clathrin is first brought to 0.1 M Mes–NaOH, pH 6.5, by overnight dialysis from 0.01 M Tris-HCl, pH 8.0. Any residual polymerization (≈5%) is removed by centrifugation. Three aliquots of the solution are made each containing clathrin at 0.25 mg/mL in 0.1 M Mes–NaOH, pH 6.5. To each of them is added a concentrated solution of $AP_{180}$ in 0.01 M Tris-HCl, pH 8.0, to give a final concentration of 0.2 mg/mL clathrin and 0.08, 0.055, and 0.027 mg/mL $AP_{180}$, respectively. Final buffer conditions are 0.002 M Tris-HCl/0.08 M Mes–NaOH, pH 6.5. Sucrose gradient centrifugation of the three solutions showed that as the concentration of $AP_{180}$ is increased more clathrin is polymerized into baskets (data not shown). Velocity centrifugation in the analytical ultracentrifuge gave a sedimentation coefficient of 130 S (measured only at the highest concentration) for the baskets. The sedimentation boundary of the baskets is very sharp in contrast to the baskets formed from 100-kDa protein reported by us earlier that had an average sedimentation coefficient of 150–180 S.[2] The baskets formed are isolated from the sucrose gradients, concentrated by vacuum dialysis, and analyzed for their composition on SDS gels. In Figure 5, lanes c–e, are shown the data of the baskets corresponding to the three concentrations of $AP_{180}$ mentioned above. Also shown in the gel for comparison purposes are pure clathrin (lanes b and g), pure $AP_{180}$ (lanes a and f), and CVs (lane h). When the Coomassie-stained lanes c–e were scanned by densitometry, and a correction factor of 3.8 was used for the relative staining ratio of clathrin heavy chains to $AP_{180}$ by Coomassie Blue stain, a mole ratio of 1:1.1 was obtained for clathrin triskelion to $AP_{180}$ in the polymerized baskets.

(*E*) *Kinetics of Polymerization of Clathrin with* $AP_{180}$. The rate of the reaction between $AP_{180}$ and clathrin is analyzed by light scattering. When a concentrated solution of $AP_{180}$ in 0.01 M Tris-HCl, pH 8.0, is added to a clathrin solution at 0.1 M Mes–NaOH, pH 6.5, giving a final concentration of clathrin and $AP_{180}$ of 0.2 and 0.05 mg/mL, respectively, a sharp increase in the light scatter was observed. In the absence of $AP_{180}$, there was no increase in the light scattering. The curve has a biphasic nature, with 60% of the reaction taking place in about 50 s, followed by a slow increase that plateaued in about 10 min. In this respect, $AP_{180}$ resembles the 110–100–50–47-kDa proteins previously reported by us which also induced a large increase in the rate of polymerization of clathrin (Prasad et al., 1985).

(*F*) *Coat Formation on the Vesicles*. We have reported earlier that the 110–100–50–47-kDa assembly proteins present in the coated vesicles are very important in the binding of clathrin to the stripped vesicles (Prasad et al., 1985). Though $AP_{180}$ resembles these proteins in polymerizing clathrin, the baskets made from these two groups of proteins differ in their appearance in the electron microscope. In light of this, we examined the effect of $AP_{180}$ in the binding of clathrin to the stripped vesicles.

Three solutions were made: first, clathrin (0.19 mg/mL) plus AN-labeled UVs (0.08 mg/mL); second, clathrin (0.19 mg/mL) plus $AP_{180}$ (0.08 mg/mL); third, clathrin (0.19 mg/mL) plus $AP_{180}$ (0.08 mg/mL) plus AN-labeled UVs (0.08 mg/mL). All the solutions were made in 0.01 M Tris-HCl, pH 8.0, and after they were separately dialyzed against 0.1 Mes–NaOH, pH 6.5, for 12 h, they were analyzed on 10–30% sucrose gradients (see Materials and Methods). Under the above centrifugation conditions, clathrin sediments at fractions 3–4 (from the top), UVs (in 0.01 M Tris, pH 8.0) at fraction 9, baskets made of $AP_{180}$ at fractions 16–17, and CVs with total associated proteins at fractions 20–22. The positions of these when independently centrifuged under the present experimental conditions will serve as markers for the identification of the products formed in the present experiment and are therefore shown as arrows at the top of the figure (Figure 6A–C) where the present data are plotted.

In Figure 6A are plotted the data on the reaction between clathrin and stripped vesicles. When clathrin alone is present (circles), there is no appearance of a tryptophan fluorescence peak at fractions 16–17 corresponding to the basket fraction, and hence there was no polymerization of clathrin. When clathrin and UVs are present in the solution, very little clathrin combined with UVs as inferred by the lack of reduction in tryptophan fluorescence at fraction 4, and an increase in tryptophan fluorescence at fraction 20. However, UVs tend to aggregate under these conditions, shifting the peak of tryptophan fluorescence (triangles) or AN fluorescence (squares) from fraction 9, where the unaggregated UVs should be present, to fraction 16. The broad tryptophan fluorescence peak at fraction 16 therefore represents the contribution from aggregated UVs though a small amount of it could be due to clathrin reacting with UVs.

In Figure 6B, the data corresponding to the reaction between clathrin and $AP_{180}$ in the absence and presence of UVs are shown. In the absence of UVs (open circles), there is some polymerization of clathrin, giving rise to a tryptophan fluorescence peak at fractions 16–17, characteristic of baskets, and a reduction in the clathrin tryptophan fluorescence at fraction 4 (compare with the circles in Figure 6A). In the presence of UVs (closed circles), there is a further enhance-

[2] We have reported a value of 150 S in order to be consistent with our earlier terminology of 150 S or 300 S. We are now in the process of differentiating the various size baskets on the basis of their protein composition.

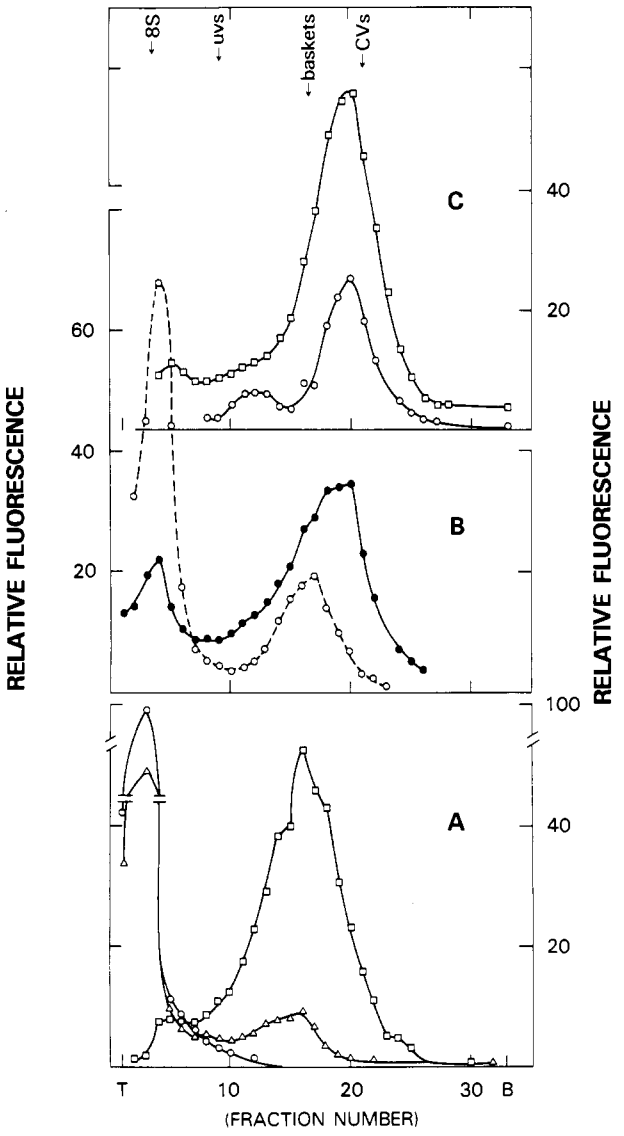


FIGURE 6: Analysis on 10–30% sucrose gradients of the products formed. (A) Clathrin (0.19 mg/mL), (○) tryptophan fluorescence; clathrin (0.19 mg/mL) plus AN-labeled UVs (0.08 mg/mL), (△)-tryptophan fluorescence, (□) AN fluorescence. (B) Clathrin (0.19 mg/mL) plus $AP_{180}$ (0.08 mg/mL), (○) tryptophan fluorescence; clathrin (0.19 mg/mL) plus $AP_{180}$ (0.08 mg/mL) plus AN-labeled UVs (0.08 mg/mL), (●) tryptophan fluorescence. (C) Clathrin (0.19 mg/mL) plus $AP_{180}$ (0.08 mg/mL) plus AN-labeled UVs (0.08 mg/mL), (□) AN fluorescence, (○) difference tryptophan fluorescence of the two curves in (B). The position of the various reactants when centrifuged separately under the same conditions is marked by the arrows at the top of the figure. 8 S represents unpolymerized clathrin and sediments at fraction 4 The position of the UVs is shown as fraction 9, under nonaggregating conditions, e.g., 0.01 M Tris-HCl, pH 8.0. The position of the CVs corresponds to the native CVs as isolated from brain tissue and contains the full complement of 110–100–50–47-kDa associated proteins in addition to $AP_{180}$.

ment of the tryptophan fluorescence peak at fractions 16–17 with a concomitant appearance of another large peak at fraction 20, characteristic of CVs. In addition, the peak at fraction 4 is further reduced in the presence of UVs than in the absence of UVs, showing a further interaction of clathrin presumably to form CVs. If the stripped vesicles had not reacted with clathrin and $AP_{180}$, the result would have been similar in both cases, but with a small additional tryptophan contribution of aggregated UVs in fractions 9–16.

That UVs actually reacted with clathrin and $AP_{180}$ is confirmed by the AN fluorescence of UVs (Figure 6C). It centers on fractions 19–20, that is a likely place for the CVs. The position of the native CVs is fractions 20–22; however, the native CVs probably contain a significant amount of associated proteins that increases the mass of the particle. In the present experiment, most of these proteins are absent, except for $AP_{180}$. In order to estimate the *minimum* amount of CVs that had formed here, a difference tryptophan fluorescence curve of the two curves in Figure 6B for fractions ranging from 5 to 30 is plotted in Figure 6C.[3] The tryptophan fluorescence peak centers at fraction 20, corresponding to the AN fluorescence peak. However, there would actually be more CVs than that shown here since in the absence of UVs, a lot more clathrin would react with $AP_{180}$ to form baskets. On the other hand, if actually there were less baskets formed in the presence of UVs than in the absence of UVs, then the difference curve would become somewhat broader, resembling the CV curve in Figure 6B (closed circles). At any rate, the formation of CVs is clearly demonstrated in this result. That CVs are actually formed is also confirmed by electron microscopy, which indicated a mixture of baskets and CVs (Figure 7B). Baskets formed from clathrin and $AP_{180}$ are also shown in Figure 7A for comparison. It therefore appears that $AP_{180}$ by itself in the absence (or a large depletion) of 110–100–50–47-kDa proteins can polymerize the clathrin coat on the stripped vesicles.

## Discussion

We presented here a relatively simpler purification procedure for the recently reported clathrin assembly protein $AP_{180}$ and also presented here a way of identifying it on SDS gels. Despite previous confusion, the mobilities of clathrin heavy chains and $AP_{180}$ are seen as significantly different when the gels are run over longer periods of time after the dye (gel front) entered the electrode buffer. A typical experimental condition is a 5–15% gradient gel, with electrophoresis at 10 mA for 12–15 h followed by 20–30 mA for the next 3–4 h. The purified protein has a molecular weight of 115 000 as determined by sedimentation equilibrium analysis either in 0.01 M Tris-HCl, pH 8.0, or in 0.5 M Tris-HCl, pH 8.0. This confirms the earlier report that the mobility of $AP_{180}$ on SDS gels is anomalous. $AP_{180}$ has a sedimentation coefficient of 3.50 ± 0.05 S and appears to be fairly asymmetric with a frictional ratio of 2.43. Circular dichroism data show that it has 30% helical structure, 14% β-structure, 27% β-sheets, and the rest random peptides. A purified 100-kDa protein reported by us previously has an almost identical helical structure though the implications of this interesting similarity are not clear. A low extinction coefficient indicates that there are very few tryptophans and tyrosines. Both the 110–100–50–47-kDa group of proteins and the new $AP_{180}$ enhance the kinetics of the polymerization reaction significantly. Like the 100-kDa protein that appeared to react with clathrin in stoichiometric amounts to induce polymerization (Prasad et al., 1986), the new $AP_{180}$ also reacts in stoichiometric amounts, with approximately one molecule of $AP_{180}$ reacting with one clathrin triskelion to polymerize into baskets. The baskets formed from $AP_{180}$ are relatively less densely packed, sedimenting with an extremely sharp boundary in the analytical ultracentrifuge with

[3] This method of looking at the tryptophan difference curve is found clearly justified when a similar experiment was done using a 0.01 M Tris-HCl, pH 8.5, extract of CVs and 0.01 M Tris-HCl (pH 8.5)-extracted AN-labeled UVs. In the absence of UVs, a small amount of clathrin polymerized into baskets. On the other hand, when UVs were present, a large amount of clathrin polymerized to give baskets and CVs. The difference tryptophan fluorescence of the two curves was exactly superimposable on the AN fluorescence of CVs. Electron microscopy confirmed the formation of a large number of CVs.

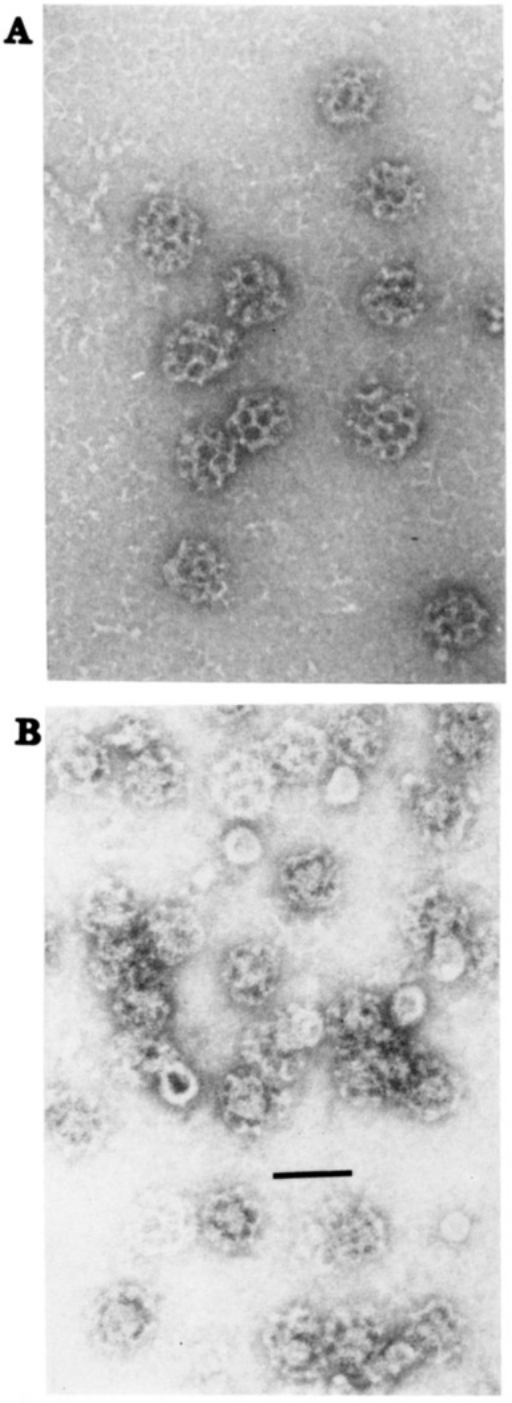


FIGURE 7: Electron micrograph of polymerized baskets (A) from clathrin and $AP_{180}$ and re-formed CVs (B) from clathrin, $AP_{180}$, and UVs in 0.1 M Mes–NaOH, pH 6.5. The bar is 100 nm.

a sedimentation coefficient of 130 S.

The $AP_{180}$ resembling the other group of assembly proteins, 110–100–50–47 kDa, is also involved in binding of clathrin to the stripped vesicles. This result coupled with the information that different assembly proteins make a different variety of basket structures (Pearse & Robinson, 1986; Ahle & Ungewickell, 1986; Keen, 1987) might imply that each group of assembly proteins is associated with a different population of coated vesicles in the cell. Robinson (1987) has shown that different 100-kDa proteins are associated with golgi and plasma membrane regions in the cell by immunofluorescence studies. On a similar note, Pearse (1985) has shown that mannose 6-phosphate receptor reacts with 100-kDa proteins but transferrin receptor does not. Studies along these lines might shed more information on such speculations.

## ACKNOWLEDGMENTS

We thank Dr. Jan Wolff for guidance and advice throughout the course of this work.

## REFERENCES

Ahle, S., & Ungewickell, E. (1986) *EMBO J. 5*, 3143.
Brown, M. S., Anderson, R. G. W., & Goldstein, J. L. (1983) *Cell (Cambridge, Mass.) 32*, 663.
Goldstein, J. L., Brown, M. S., Anderson, R. G. W., Russell, D. W., & Schneider, W. J. (1985) *Annu. Rev. Cell Biol. 1*, 1.
Heuser, J. E., & Reese, T. S. (1973) *J. Cell Biol. 57*, 315.
Keen, J. H. (1985) in *Endocytosis* (Pastan, I., & Willingham, M. C., Eds.) p 85, Plenum, New York.
Keen, J. H. (1987) *J. Cell Biol. 105*, 1989.
Keen, J. H., Willingham, M. C., & Pastan, I. H. (1979) *Cell (Cambridge, Mass.) 16*, 303.
Kirchhausen, T., & Harrison, S. C. (1981) *Cell (Cambridge, Mass.) 23*, 755.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Manfredi, J. J., & Bazari, W. L. (1987) *J. Biol. Chem. 25*, 12182.
Nandi, P. K., Irace, G., Van Jaarsveld, P. P., Lippoldt, R. E., & Edelhoch, H. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5881.
Pearse, B. M. F. (1975) *J. Mol. Biol. 97*, 93.
Pearse, B. M. F. (1985) *EMBO J. 4*, 2457.
Pearse, B. M. F., & Robinson, M. S. (1984) *EMBO J. 3*, 1951.
Pfeffer, S. R., Drubin, D. G., & Kelly, R. B. (1983) *J. Cell Biol. 97*, 40.
Prasad, K., Alfsen, A., Lippoldt, R. E., Nandi, P. K., & Edelhoch, H. (1984) *Arch. Biochem. Biophys. 235*, 403.
Prasad, K., Lippoldt, R. E., & Edelhoch, H. (1985) *Biochemistry 24*, 6421.
Prasad, K., Yora, T., Yano, O., Lippoldt, R. E., Edelhoch, H., & Saroff, H. (1986) *Biochemistry 25*, 6942.
Pretorius, H. T., Nandi, P. K., Lippoldt, R. E., Johnson, M. L., Keen, J. H., Pastan, I., & Edelhoch, H. (1981) *Biochemistry 20*, 2777.
Provencher, S. W. (1982a) *Comput. Phys. Commun. 27*, 213, 229.
Provencher, S. W. (1982b) EMBL Technical Report DA05, European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany.
Schook, W., Puszkin, S., Bloom, W., Ures, C., & Kochwa, S. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 116.
Ungewickell, E., & Branton, D. (1981) *Nature (London) 289*, 420.
Woodward, M. P., & Roth, T. F. (1979) *J. Supramol. Struct. 11*, 237.
Zaremba, S., & Keen, J. H. (1983) *J. Cell Biol. 97*, 1339.