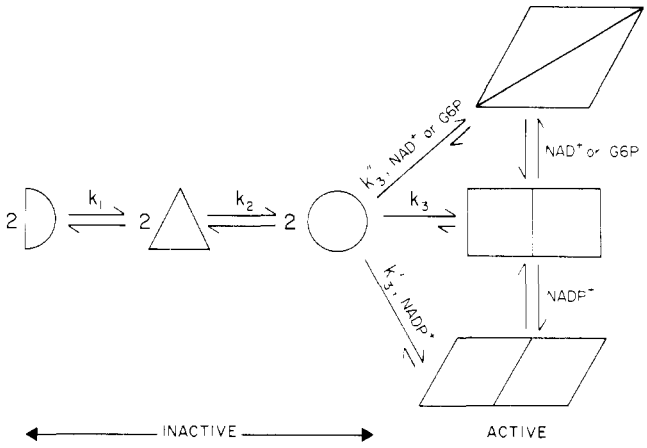

FIGURE 7: Model for reconstitution of urea-denatured glucose-6-phosphate dehydrogenase. The completely unfolded, urea-denatured monomers are shown at the extreme left. For rate constants see Table II.

25 °C. A comparison between the rate constants shows $k_2 > k_3' > k_3'' > k_3$.

Our results imply that in vivo assembly of *L. mesenteroides* glucose-6-phosphate dehydrogenase is not dependent on the presence of specific ligands but that the concentrations of glucose 6-phosphate, NAD^+, and $NADP^+$ in the cell may influence the rate of this process.

References

Bartholmes, P., & Jaenicke, R. (1978) *Eur. J. Biochem. 87*, 563–567.
Bell, J. E. (1981) in *Spectroscopy in Biochemistry*, Vol. 1, pp 155–194, CRC Co., Cleveland, OH.
Cornish-Bowden, A., & Eisenthal, R. (1978) *Biochim. Biophys. Acta 523*, 268–272.
Creighton, T. E. (1978) *Prog. Biophys. Mol. Biol. 33*, 231–297.
Deal, W. C., Jr. (1969) *Biochemistry 8*, 2795–2805.
Eisenthal, R., & Cornish-Bowden, A. (1974) *Biochem. J. 139*, 715–720.
Gabriel, O. (1971) *Methods Enzymol. 22*, 565–578.
Grossman, S. H., Pyle, J., & Steiner, R. J. (1981) *Biochemistry 20*, 6122–6128.
Haghighi, B., & Levy, H. R. (1982) *Biochemistry* (second paper of three in this issue).
Haghighi, B., Flynn, T. G., & Levy, H. R. (1982) *Biochemistry* (first paper of three in this issue).
Hedrick, J. L., & Smith, A. J. (1968) *Arch. Biochem. Biophys. 126*, 155–164.
Ishaque, A., Milhausen, M., & Levy, H. R. (1974) *Biochem. Biophys. Res. Commun. 59*, 894–901.
Jaenicke, R. (1978) *Naturwissenschaften 65*, 569–577.
Jaenicke, R., Rudolph, R., & Heider, I. (1979) *Biochemistry 18*, 1217–1223.
Kawaguchi, A., & Bloch, K. (1974) *J. Biol. Chem. 249*, 5793–5800.
Krebs, H., Rudolph, R., & Jaenicke, R. (1979) *Eur. J. Biochem. 100*, 359–364.
Milhausen, M. (1974) Ph.D. Dissertation, Syracuse University.
Muhlrad, A., Hegyi, G., & Horanvi, M. (1969) *Biochim. Biophys. Acta 181*, 184–190.
Olive, C., & Levy, H. R. (1971) *J. Biol. Chem. 246*, 2043–2046.
Rudolph, R., & Jaenicke, R. (1976) *Eur. J. Biochem. 63*, 409–417.
Rudolph, R., Gerschitz, J., & Jaenicke, R. (1978) *Eur. J. Biochem. 87*, 601–606.
Teipel, J., & Koshland, D. E., Jr. (1971a) *Biochemistry 10*, 792–798.
Teipel, J., & Koshland, D. E., Jr. (1971b) *Biochemistry 10*, 798–805.
Wetlaufer, D. B., & Ristow, S. (1973) *Annu. Rev. Biochem. 42*, 135–158.
Wood, D. C., Jurgensen, S. R., Geesin, J. C., & Harrison, J. H. (1981) *J. Biol. Chem. 256*, 2377–2382.
Zamenhof, S. (1957) *Methods Enzymol. 3*, 696–704.

Reversibility of Coated Vesicle Dissociation†

P. K. Nandi, K. Prasad, R. E. Lippoldt, A. Alfsen, and H. Edelhoch*

ABSTRACT: The dissociation of the coated vesicles to clathrin and uncoated vesicles and their reassociation have been studied under various conditions. The extent of reassociation is pH dependent and increases slightly with increasing concentrations of the components. Unlike the self-association of clathrin which is strongly salt dependent, the reassociation of clathrin and uncoated vesicles is practically independent of salt concentration. The coated vesicle gradually loses its coat with increasing pH, and the dissociation process is not an all or none reaction. Ca^{2+} inhibits dissociation of the coated vesicles and enhances the reassociation of clathrin and uncoated vesicles. Our results show that, although many conditions result in reassociation of protein and lipid vesicle, few conditions result in vesicles of both the same size and composition as native coated vesicles.

Coated pits and vesicles are involved in receptor-mediated endocytosis (Goldstein et al., 1979; Ockleford & Whyte, 1977; Pearse, 1980), secretion of glycoproteins (Rothman et al., 1980), and membrane exchange (Heuser & Reese, 1973). Clathrin is readily dissociated from coated vesicles (CV)[1] by various methods which are mild enough not to cause denaturation, i.e., elevated pH, 2 M urea, and 0.50 M Tris (Pearse, 1975; Shook et al., 1979; Keen et al., 1979; Woodward &

† From the Clinical Endocrinology Branch, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20205. *Received June 4, 1982.*

[1] Abbreviations: CV, coated vesicle(s); Mes, 2-(*N*-morpholino)-ethanesulfonic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate.

Roth, 1979; Pretorius et al., 1981). Clathrin self-associates with increasing rate as the pH is reduced from 7.0 to 6.0 to give coat structures, i.e., baskets, which closely resemble the coat in CV (Pearse, 1976; Schook et al., 1979; Keen et al., 1979; Woodward & Roth, 1979; Nandi et al., 1980; Van Jaarsveld et al., 1981). A tryptic digest of clathrin of M_r 110 000 has been shown capable of self-association to give a coat type of structure (Schmid et al., 1982).

Unanue et al. (1981) have measured the binding of clathrin to stripped or uncoated vesicles and calculated an affinity constant of 2×10^9. They also reported from electron micrographs that a considerable percentage of the stripped vesicles recovered their coats.

In this report, we have evaluated the effects of various conditions both on the dissociation of CV to clathrin and uncoated vesicles and on their reassociation. We have used sucrose gradient centrifugation instead of moving boundary centrifugation since the protein and phospholipid compostions can be measured in each sedimenting species in the former but not in the latter technique. This former technique permits a quantitative accounting of the protein and phospholipid in both the dissociated and reassociated states of CV. We have found that, although many conditions result in reassociation of protein and lipid to form vesicles of the appropriate size, few conditions result in vesicles of both the same size and composition as native CV.

Materials and Methods

Chemicals. Ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid (EDTA) and 2-(*N*-morpholino)ethanesulfonic acid (Mes) were obtained from Sigma. Sodium chloride, sodium mono- and dihydrogen phosphate, sodium azide, and calcium and magnesium chloride were certified grade reagents from Fisher Scientific Co. Analytical grade urea was from Bethesda Research Laboratory, and gold-label deuterium oxide and 1,6-diphenyl-1,3,5-hexatriene (DPH) were from Aldrich Chemicals.

Preparation of Coated Vesicles. Bovine brains were homogenized in Mes buffer (0.10 M Mes, 1 mM EGTA, 0.5 mM $MgCl_2$, and 3 mM NaN_3) pH 6.5, following the procedure of Pearse (1975). The crude coated vesicle pellet obtained from a 100000*g* centrifugation was dispersed in 0.10 M Mes buffer, pH 6.5, and centrifuged at low (20000*g*) and high (100000*g*) speeds. The new coated vesicle pellet was purified in a 8% sucrose, D_2O–H_2O gradient following the procedure of Nandi et al. (1982). The coated vesicle pellet was resuspended in the Mes buffer and centrifuged at 20000*g* for 10 min to clarify the solution. For kinetic experiments, the supernatant was dialyzed against 0.01 M Mes, pH 6.5, and used for further manipulation. Coated vesicle preparations were stored at 5 °C as pellets from the sucrose, D_2O–H_2O gradient and were stable in this condition for several weeks.

Dissociation and Reassociation of Coated Vesicles. To change experimental conditions for dissociation, we dialyzed the 0.01 M Mes, pH 6.5, solution of coated vesicles for 4 h against the appropriate buffer at 23 °C. It was always ascertained that equilibration was reached with the new buffer solution. The contents of the dialysis bag were stirred for 10 s every 2 min. A similar procedure, i.e., dialysis, was usd to adjust the conditions to reassociate the dissociated coated vesicles. When dialysis was not used to change the buffer and pH, a statement will be made in the text concerning the manner of changing conditions.

Sucrose Gradient Centrifugation. Prior to placement of the coated vesicle solutions on the sucrose gradient, DPH was added (in a small volume of tetrahydrofuran) to give a concentration of 1×10^{-6} M. These solutions were allowed to incubate for 2 h before they were placed on gradients. Solutions were sedimented with a 10–30% linear sucrose gradient in an SW40 rotor, at 27 000 rpm for 110 min at 20 °C, in a Beckman Model L2-65 B ultracentrifuge. The fractions from the sucrose gradient were collected from the bottom of the tube by using a peristaltic pump.

Fluorescence Analysis. Phospholipid was analyzed by the fluorescence intensity of DPH (λ_{ex} = 366 nm; λ_{em} = 430 nm). Protein was determined from the intrinsic tryptophan fluorescence (λ_{ex} = 280 nm; λ_{em} = 340 nm).

Kinetics of Dissociation and Reassociation. The rate of dissociation of coated vesicles or of reassociation of products was measured by monitoring the change in light scatter at 45° to the incident beam. A polished cylindrical cell (diameter = 10 mm) from Precision Cell, Inc., was used in a Brice Phoenix Universal light scattering photometer which was modified by C. N. Wood. A blue filter was used to isolate the 436-nm wavelength.

The relation between turbidity and light scatter is given in a previous paper (Van Jaarsveld et al., 1981). The quantity that is measured is the Rayleigh ratio, i.e., Ir^2/I_0. Solutions were centrifuged at 10 000 rpm for 10 min before being pipetted into the light scattering cell. A small magnetic stirrer was placed in the cell for rapid mixing. pH or other adjustments could be made while the cell was in the instrument, since it was equipped for stirring. Dissociation of coated vesicles was accomplished by adding 50 μL of 0.20 M Tris, pH 8.55, to 1 mL solution of 0.01 M Mes at pH 6.5. The decrease in light scatter affords a measure of the degree of dissociation. A similar procedure was used to decrease the pH of the solution when reassociation was being studied. The increase in light scatter revealed the extent of reassociation.

Protein Concentration. Clathrin concentration was measured by its absorption at 280 nm where $E^{1\%}_{280nm}$ = 10.9. The concentration of protein in coated vesicles was determined by diluting in concentrated guanidinium chloride solutions to eliminate their turbidity and measuring their absorption at 280 nm. The same extinction coefficient was used as for clathrin.

Results

In order to measure the composition of sedimenting species in solutions of native CV and dissociated and reassociated CV, we have used an extrinsic membrane probe, i.e., DPH, to measure phospholipid and the intrinsic tryptophan fluorescence to measure protein. A symmetrical distribution of sedimenting particles with a constant ratio of protein to phospholipid was observed to the sucrose gradient boundary with native CV (Figure 1). This constant ratio indicates that neither clathrin baskets nor uncoated vesicles are present in measurable amounts since these two particles have different sedimentation rates and phospholipid and protein compositions from those of CV.

Effects of pH. (*1*) *Light Scatter*: *Kinetics.* (*a*) *Dissociation.* The rate of dissociation of CV and of reassociation of uncoated vesicles and clathrin was evaluated by following the intensity of light scattering. When the pH of a solution of CV was increased from 6.5 to 8.5 with Tris, the scattering intensity fell about 50% in a few seconds and then remained constant. The change in scatter was complete before the first measurement was made, which took about 10 s after the addition of Tris buffer to raise the pH.

(*b*) *Reassociation.* After about 10 min at pH 8.5, the pH was reduced to values between 7.11 and 6.02 by adding small volumes of Mes buffer (1 M) directly to the scattering cell while the solution was stirred. In all cases, there was a rapid

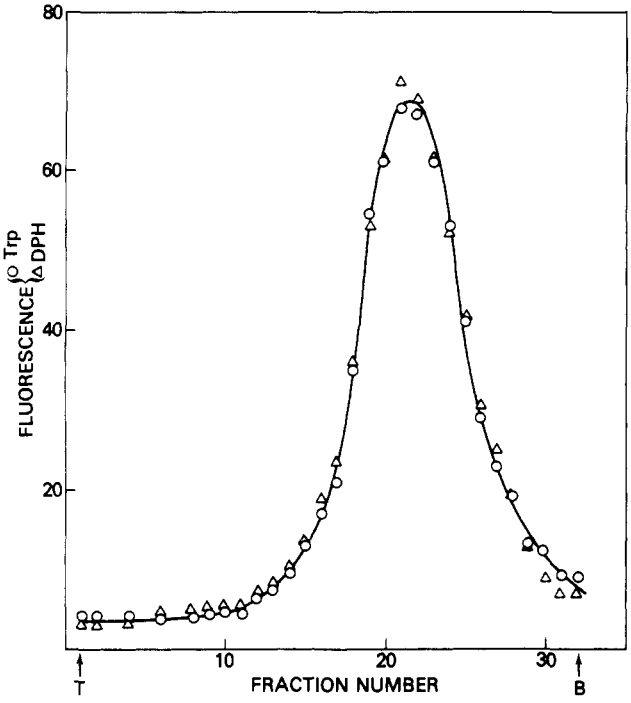

FIGURE 1: Fluorescence analysis of coated vesicles after sucrose gradient centrifugation (10–30%) at 27 000 rpm for 110 min at 20 °C. (○) Trp; (△) DPH. T is the top and B is the bottom of the gradient.

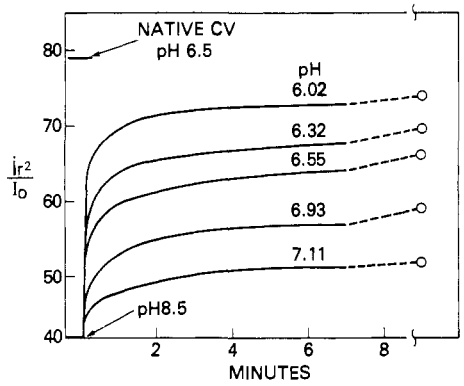

FIGURE 2: Changes in light scatter with pH. Native coated vesicles (0.25 mg/mL) at pH 6.5, 0.01 M Mes were brought to pH 8.5 by addition of 50 μL of 0.20 M Tris, pH 8.55, to 1 mL of solution. The light scatter fell from 78 to 40 units. This fall is a measure of the degree of dissociation of coated vesicles. Solutions were then brought to lower pH values by addition of 50 μL of 1 M Mes (adjusted to different pH values). The rates of increase in light scatter were measured continuously for 7 min and then after 12 h (○). Final solutions contained 0.01 M Tris and about 0.05 M Mes. The temperature was 23 °C. The increase in light scatter is a measure of the degree of reassociation of uncoated vesicles and clathrin.

increase in scatter which reached about 80–90% of its final value in 2–4 min (Figure 2). The light scatter recovered from 25 to 80% of the initial intensity between pH 7.11 and 6.02, respectively. Thus, the extent of reassociation of clathrin with uncoated vesicles is seen to be pH dependent between pH 7 and 6. We have shown that a similar pH dependency occurs in the self-assembly of clathrin to form baskets (Van Jaarsveld et al., 1981).

We have evaluated the effect of concentration on the rate of reassociation at pH 6.5, after dissociating at pH 8.5. The rates of reassociation are shown in Figure 3 in solutions containing between 0.044 and 0.44 mg/mL protein. As can be seen in Figure 3, the rate and extent of reassociation increased only slightly with increasing concentrations. This small de-

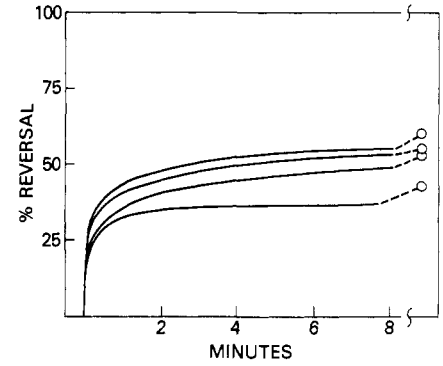

FIGURE 3: Effect of concentration of coated vesicles on the rate and extent of reassociation. The pH of native coated vesicles was increased from 6.5 to 8.5 with Tris with a 50% loss in light scatter. The pH was then returned to 6.5 with Mes. At pH 8.5, coated vesicles are dissociated into uncoated vesicles and clathrin. At pH 6.5, reassociation occurs. The extent of reassociation was calculated from the degree of recovery of the loss in light scatter at pH 8.5. Final recovery values of light scatter were obtained after 12 h. Final solutions contained 0.05 M Mes–0.01 M Tris. Coated vesicle concentrations were 0.44, 0.22, 0.090, and 0.044 mg/mL proceeding from the upper to the lower curve.

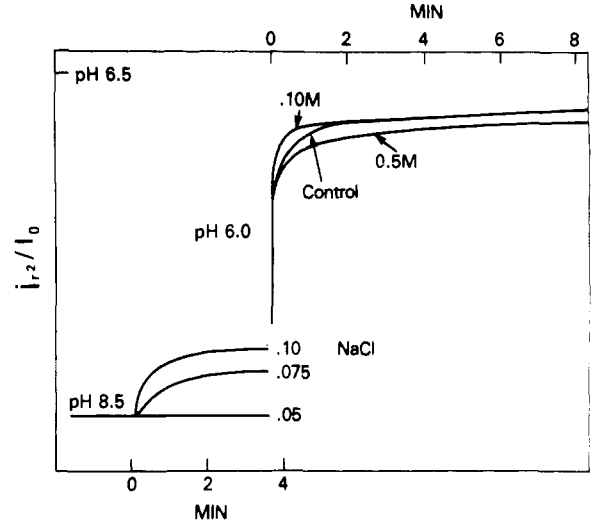

FIGURE 4: Effect of NaCl on the rate and extent of reassociation. The pH of native coated vesicles was increased from 6.5 to 8.5 with Tris with a 50% loss in light scatter. NaCl was added at pH 8.5. A small increase in light scatter occurred which was finished in 3–4 min. After 4 min, the pH was reduced to 6.0 with Mes. A much faster and larger increase in light scatter occurred. At pH 6.0, most of the initial light scatter was recovered.

pendence on concentration resembles the data obtained for the self-association of clathrin to form baskets (Van Jaarsveld et al., 1981).

The influence of salt, i.e., NaCl, on the reassociation reaction was also investigated since the polymerization of clathrin to form baskets is strongly inhibited by NaCl. In the absence of added NaCl, a very rapid increase in light scatter occurred when the pH was reduced to 6.0 with Mes buffer (Figure 4). Addition of 0.10 or 0.50 M NaCl had little effect on the rate of reassociation at pH 6.0 or at pH 6.3 or 6.5. There is a small increase in light scatter when NaCl is added at pH 8.5. The increase in light scatter upon reducing the pH is not significantly affected by the concentration of NaCl. There is, therefore, a very important difference between clathrin self-association and binding to uncoated vesicles with respect to the effect of NaCl, i.e., ionic strength.

(*2*) *Sucrose Gradient Centrifugation*: *Equilibrium*. (*a*) *Dissociation*. CV are prepared at pH 6.5, 0.10 M Mes, where

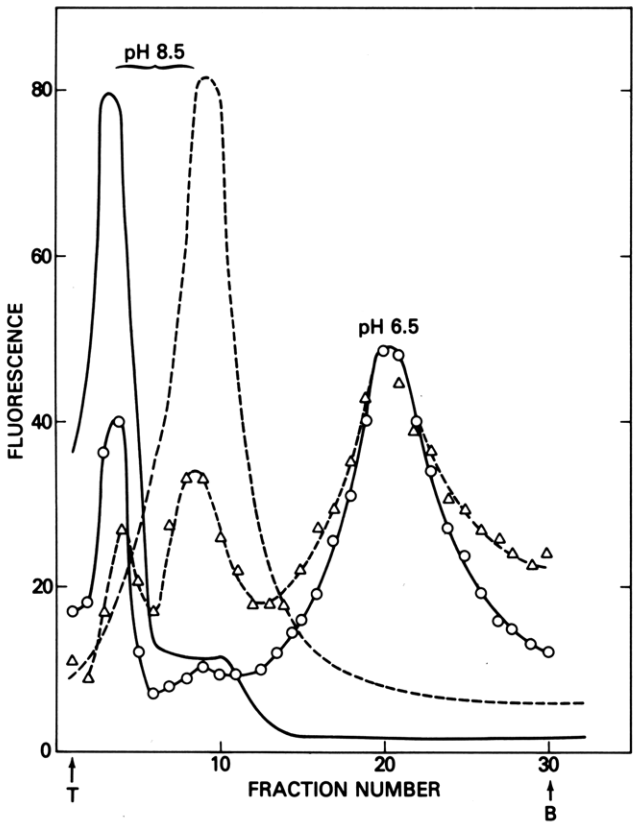

FIGURE 5: Sucrose gradient centrifugation (10–30%) of coated vesicles dissociated at pH 8.5, 0.10 M Tris (curves without symbols) and reassociation of dissociated coated vesicles at pH 6.5, 0.10 M Mes (curves with symbols). (○) Trp fluorescence; (△) DPH fluorescence. Centrifugation was at 27 000 rpm for 110 min at 20 °C.

they are quite stable. In order to evaluate the effect of pH on the stability of CV, we increased the pH to 7.5, 8.0, and 8.5, in 0.10 M Tris (by dialysis at 23 °C for 4 h). Only two sedimenting bands are observed at pH 8.5 (Figure 5), which are centered at fractions 3 and 9. The slower sedimenting band contains clathrin (and other coat proteins) and is largely devoid of phospholipid. The faster sedimenting band contains most of the phospholipid and about 10% of the protein. We will refer to this phospholipid species as uncoated vesicles.

A $NaDodSO_4$ gel of uncoated vesicles resembled that reported by Unanue et al. (1981) after clathrin was stripped off from CV in 10 mM Tris, pH 8.5. However, they report that 70% of the M_r 180 000 band is removed whereas we find that 95% is depleted. Evidently, 0.10 M Tris is more effective in stripping clathrin from CV than is the more dilute buffer (vide infra). Moreover, we find that the remaining M_r 180 000 band is not clathrin but other proteins of this size (P. K. Nandi, K. Prasad, R. E. Lippoldt, A. Alfsen, and H. Edelhoch, unpublished experiments).

At pH 7.5 (Figure 6) and 8.0, three sedimenting bands are present, all containing both protein and phospholipid. The position of the fastest sedimenting band is at fraction 18 at pH 7.5 and at fraction 15 at pH 8.0. The positions of these boundaries occur at earlier fractions than with CV which is at fraction 22. The gradual displacement of this phospholipid boundary with pH suggests that the vesicle gradually loses its coat. The peak of the phospholipid boundary of the uncoated vesicle is at fraction 9 at pH 8.5 (Figure 5). Only a small percent of the total protein remains associated with this vesicle which has lost its coat. This regular shift of CV particles to slower sedimentation values indicates that the coat does not dissociate in an all or none reaction since the latter effect would

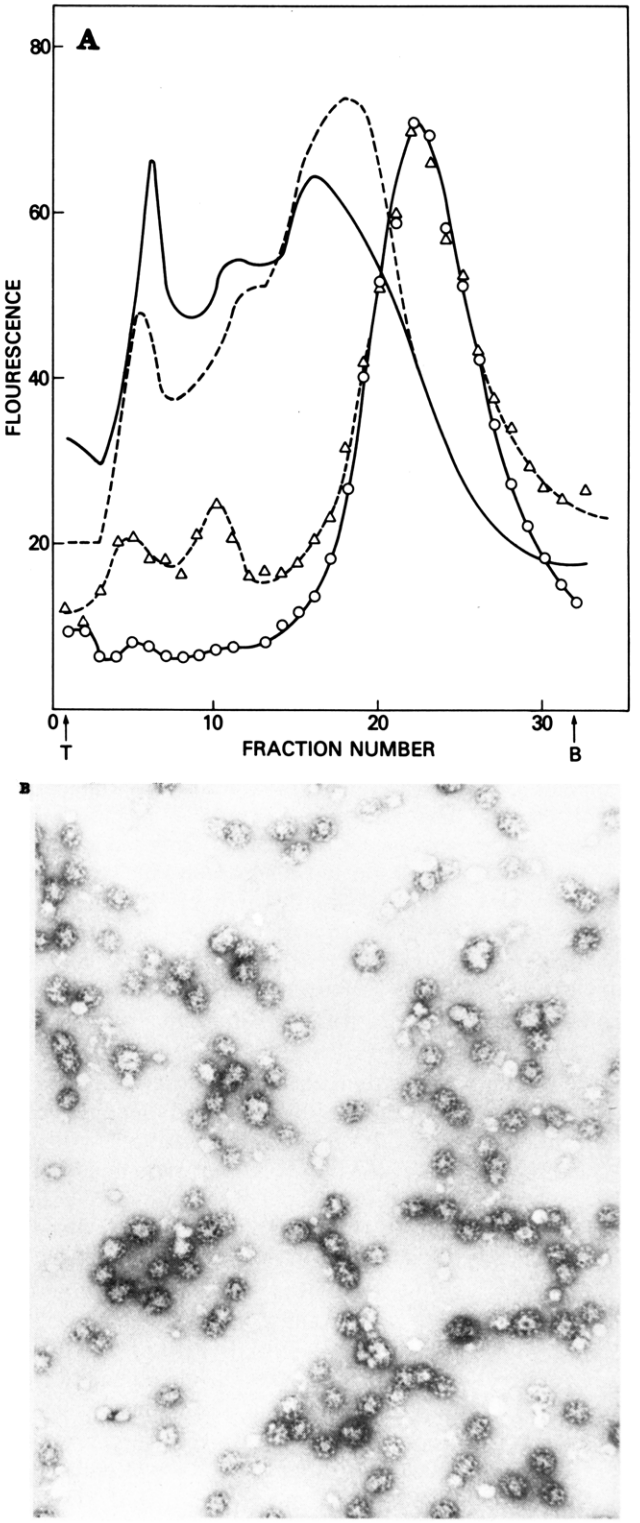

FIGURE 6: (A) Sucrose gradient centrifugation (10–30%) of coated vesicles dissociated at pH 7.5, 0.1 M Tris (curves without symbols) and reassociation of dissociated coated vesicles at pH 6.5, 0.1 M Mes (curves with symbols). (○) Trp fluorescence; (△) DPH fluorescence. Centrifugation was at 27 000 rpm for 110 min at 20 °C. (B) Electron micrographs of reassociated coated vesicles. Numerous uncoated vesicles are also seen which appear to have the same size as the vesicles in coated vesicles. Coated vesicles were dissociated by dialysis in 0.1 M Tris, pH 8.5, and reassociated by dialysis in 0.1 M Mes, pH 6.5.

produce a reduction in the height of the CV boundary at fraction 22 instead of the gradual displacement.

At pH 8.0, there were still three sedimenting bands. However, the two faster sedimenting bands were reduced in intensity while the slowest one was correspondingly increased.

Very little difference in sedimenting boundaries was observed when the CV was dialyzed against pH 8.0, 0.1 M Tris for 24 h at 4 °C instead of for 4 h at 23 °C.

The influence of Tris concentration on the dissociation of CV at pH 8.5 was also assessed. Only two, slow sedimenting boundaries were found at concentrations between 0.01 and 0.10 M Tris. The protein and phospholipid boundaries in either 0.01 or 0.05 M Tris, centered at fractions 5 and 11–12, sedimented faster than the two boundaries in 0.10 M Tris by two fractions. There was also slightly more protein in the phospholipid boundary at the two lower Tris concentrations than in 0.10 M Tris, which may account for their greater sedimentation rates.

(*b*) *Reassociation.* The three CV solutions, dissociated at pHs of 7.5, 8.0, and 8.5, were dialyzed against the standard buffer, i.e., pH 6.5, 0.10 M Mes, for 4 h. In the reversal from pH 7.5, most of the protein and phospholipid appeared as a single, symmetrical boundary at the position in the sucrose gradient of native CV, i.e., fraction 22 (Figure 6A). The ratio of protein to phospholipid was constant in the upper half of the boundary but diverged somewhat at both extremities. Only small amounts of both components remained at their former positions, indicating extensive recombination of components. Electron micrography showed a large percentage of coated vesicles as well as numerous uncoated vesicles (Figure 6B).

Reversal of the pH 8.0 solution to pH 6.5 resulted in a sedimentation pattern similar to that observed in the pH 7.5 to 6.5 reversal. However, the ratio of protein to phospholipid was not constant anywhere in the boundary centered at fraction 21. Thus, the composition and consequently the structure of native CV were not recovered although most of the dissociated species recombined to form vesicles similar in size to that of the native CV. It should be pointed out that some native CV may be present but their constant ratio may be obscured by the variable ratios of other particles sedimenting with the same or similar velocities.

The sedimentation pattern obtained after reversal from pH 8.5 to 6.5 (Figure 5) superficially resembled that observed in the reversal from pH 7.5 to 6.5. However, the extent of recombination was significantly less, and, more importantly, the ratio of protein to phospholipid of the re-formed vesicles (centered at fraction 20–21) was even more variable than that observed in the pH 8.0 to 6.5 reversal.

It is evident that increasing the pH results in greater dissociation of CV into protein and phospholipid components. However, the higher the pH, the less the recovery of CV with the parameters of the native species. The amount of phospholipid and protein reassociating is also reduced.

The effects of phosphate buffer have been compared with those of Tris on the dissociation of CV and the recombination of the products. CV solutions at pH 6.5, 0.10 M Mes were dialyzed against pH 6.5, 0.033 M phosphate buffer for 4 h at 23 °C. The sedimentation pattern resembled that observed at the same pH in 0.10 M Mes. Increasing the pH from 6.5 to either 7.5 or 8.0 in 0.033 M phosphate resulted in three sedimenting boundaries similar to those observed in 0.10 M Tris at the same pH. When the pH 7.5 solution was returned to pH 6.5, about 60–70% of the protein and phospholipid re-formed a faster sedimenting boundary with a peak at fraction 22. At pH 8.0, the extent of reversal was somewhat less, about 50%. The ratio of protein to phospholipid was constant across the upper half of the boundary at pH 7.5 but varied in the pH 8.0 experiment. Thus, except for some minor differences, the pH is the important variable in determining the extent of reversibility and homogeneity of re-formed CV.

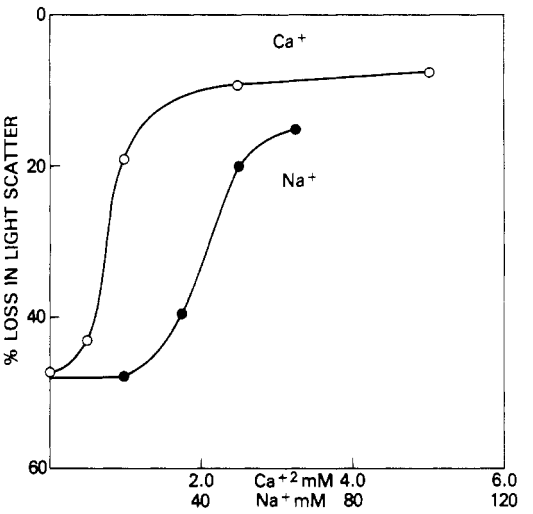

FIGURE 7: Inhibition of dissociation of coated vesicles at pH 8.5, 0.01 M Tris by Ca^{2+} and Na^{+}. Various concentrations of either $CaCl_2$ or NaCl were added to the coated vesicles at pH 6.5, 0.01 M Mes, before titration with Tris to pH 8.5. The percentage of the loss of light scatter was obtained by comparing the values of light scattering of the solution of coated vesicles in the presence of either Ca^{2+} or Na^{+} at pH 8.5 with the value of the light scatter in the absence of these cations at pH 8.5. The temperature was 23 °C.

Effect of Ca^{2+}. (*1*) *Inhibition of Dissociation at pH 8.5.* We have found that the extent of loss in light scatter of CV solutions when brought to pH 8.5 with Tris (0.01 M) was strongly affected by Ca^{2+}. In the absence of Ca^{2+}, a 47% decrease in light scatter occurred. Almost complete inhibition of loss of light scatter and consequently of dissociation occurred at 2.5 mM Ca^{2+}. The dependence on Ca^{2+} concentration was very strong since almost no effect on the loss in light scatter was observed at 1 mM Ca^{2+}. If the percent loss in scatter is plotted against the Ca^{2+} concentration, a sigmoidal curve is obtained (Figure 7). In all experiments, the loss in light scatter occurred within 10–20 s.

NaCl was also evaluated in order to see if the effect of Ca^{2+} concentration was specific or only one of ionic strength. NaCl was found to inhibit the loss in light scatter when the pH of CV solutions was increased to 8.5. No time dependence of loss in light scatter was observed in the NaCl solutions. However, about 60 times as much Na^{+} was needed to produce the same degree of inhibition as Ca^{2+} (Figure 7). It is apparent that Ca^{2+} has a very large specific effect on the stability of CV since complete dissociation would otherwise occur at pH 8.5.

(*2*) *Reassociation at pH 8.5.* In the absence of a kinetic barrier, Ca^{2+} should reassociate uncoated vesicles and clathrin at pH 8.5, 0.01 M Tris. As seen in Figure 8, the reaction is 80–90% complete after 2–4 min at all Ca^{2+} concentrations. The extent of reaction is strongly dependent on Ca^{2+} concentration. When the Ca^{2+} concentration is plotted against the extent of reassociation, a sigmoidal curve is obtained (Figure 8). The quantity of Ca^{2+} needed for reassociation is similar to that needed to prevent dissociation (Figure 7).

The effect of NaCl was also investigated to determine the importance of the ionic strength in the reassociation reaction. About 40–50 times as much NaCl was needed to increase the light scatter as compared with $CaCl_2$, and recovery was only about half as great (Figure 8). Moreover, above about ~0.1 M NaCl, the reassociation was strongly inhibited.

The effect of Ca^{2+} concentration on the reassociation of uncoated vesicles with clathrin has also been evaluated as a function of pH and Tris concentration by sucrose gradient

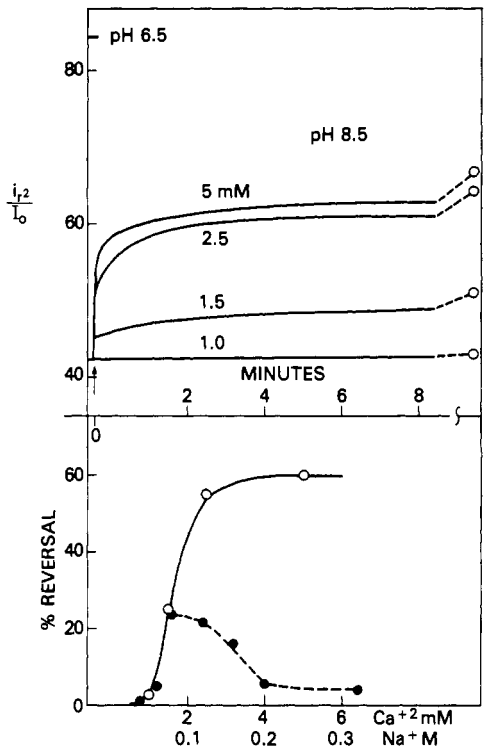

FIGURE 8: (Upper panel) pH 6.5 solutions of coated vesicles were brought to pH 8.5 with Tris buffer. The light scatter decreased from 85 to 42 units. Various concentrations of Ca^{2+} (1–5 mM) were then added at pH 8.5, and the increase in light scatter was measured continuously for 8.5 min and then after 12 h (○). (Lower panel) Percent of recovery of the loss of light scatter (see upper panel) at pH 8.5, 0.01 M Tris as a function of Ca^{2+} concentration (○). Similar experiments were performed with Na^+ at pH 8.5, and their effects on the recovery of the loss of light scatter are also shown (●).

analysis. Addition of 5 mM Ca^{2+} to a solution of CV dissociated at pH 8.5, 0.10 M Tris had no effect on the reassociation of protein and phospholipid but displaced each boundary to slightly higher fractions, i.e., from 3 to 5 and from 9 to 11, respectively (not shown). When 5 mM Ca^{2+} was added to a CV solution dissociated at pH 7.5 in 0.10 M Tris, most of the protein and phospholipid formed a peak which was centered at fraction 22 (Figure 9). The ratio of protein to phospholipid is constant for fractions above the peak but deviates strongly below the peak. The discrepancy is due to a protein shoulder centered near fraction 16 which is not associated with a phospholipid peak. The position of this shoulder corresponds to the 150S basket (Irace et al., 1982). The presence of baskets alters the ratio of protein to phospholipid on the ascending side of the main boundary (Figure 9). It is apparent that at pH 7.5, 0.10 M Tris the rates of CV and basket formation from their components are comparable since both are formed. It has been reported that Ca^{2+} enhances the rate of clathrin self-association (Van Jaarsveld et al., 1981). Mg^{2+} has been shown to inhibit the dissociation of baskets and to enhance the self-association of clathrin to form baskets (Woodward & Roth, 1979).

Similar results were obtained after addition of 5 mM $CaCl_2$ to CV solutions dissociated at pH 8.0, 0.10 M Tris. At this pH, the extent of reassociation was less since about 40% of the slow sedimenting protein and phospholipid components did not reassociate compared to 20% at pH 7.5.

We have studied the influence of Tris buffer on the reassociation of protein and phospholipid at pH 8.5 in 5 mM Ca^{2+} since no reaction occurred in 0.10 M Tris. In 0.05 M Tris, pH 8.5, about 50% reassociation occurred whereas in 0.01 M

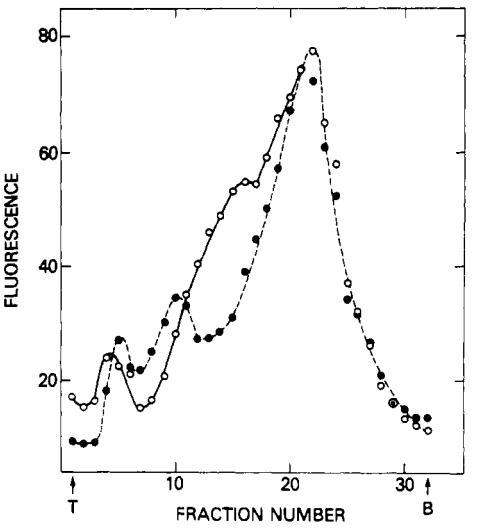

FIGURE 9: Reassociation by 5 mM Ca^{2+} of coated vesicles dissociated at pH 7.5, 0.10 M Tris (see Figure 6 for details of dissociation). Calcium was added to pH 7.5, 0.10 M Tris solution. (○) Trp fluorescence; (●) DPH fluorescence. Centrifugation was at 27 000 rpm for 110 min at 20 °C.

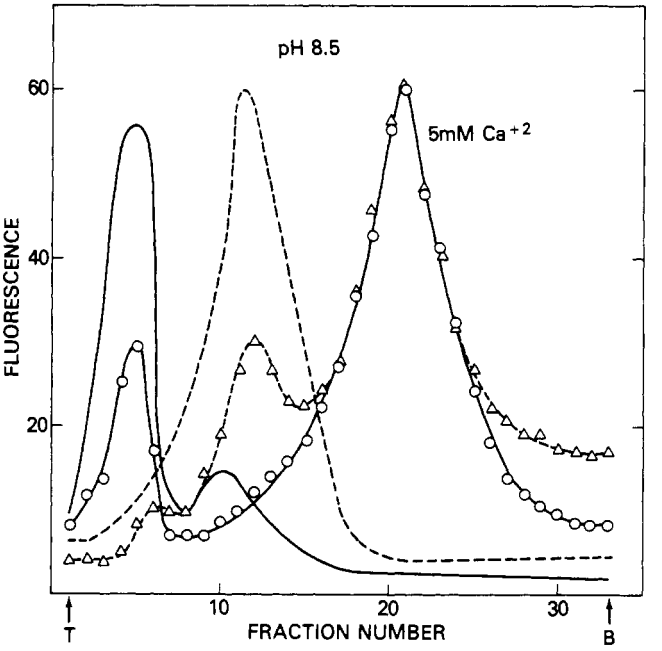

FIGURE 10: Dissociation of coated vesicles at pH 8.5, 0.01 M Tris (curves without symbols) and reassociation by 5 mM Ca^{2+} at pH 8.5, 0.01 M Tris (curves with symbols). (○) Trp fluorescence; (△) DPH fluorescence. Centrifugation on a sucrose gradient (10–30%) was at 27 000 rpm for 110 min at 20 °C.

Tris, pH 8.5, the reaction was even more complete (Figure 10). In both solutions, the ratio of protein to phospholipid was constant across the upper portion of the re-formed CV boundary, suggesting that the native CV structure was recovered. There was no evidence of a protein shoulder, i.e., basket formation, as was observed at pH 7.5 and 8.0 in 0.10 M Tris. When 5 mM Mg^{2+} was added to solutions of CV dissociated at pH 8.5 in 0.01 M Tris, only about 25% of the protein and phospholipid reassociated. Mg^{2+} is also less effective than Ca^{2+} in enhancing the rate of self-association of clathrin (Nandi et al., 1981).

Effect of Urea. Urea at a concentration of 2 M has been used in the preparation of clathrin from CV. Urea dissociates clathrin without the need to increase the pH from where it is prepared, i.e., 6.5. Under these conditions, the CV is almost

completely dissociated. However, the phospholipid appears in two slow-sedimenting bands of about equal intensities with peaks at fractions 5 and 9–10. In most other conditions where dissociation is extensive, only the faster phospholipid peak was observed. The protein, however, shows the normal type of distribution. The major protein peak at fraction 4 contains most of the protein and is not associated with the slower sedimenting phospholipid peak at fraction 5.

Dialysis of the 2 M urea solution at pH 6.5 to remove the urea resulted in about 50% reversal to a boundary with a peak at fraction 23. The ratio of protein to lipid was approximately constant in the descending but not in the ascending part of the boundary.

Discussion

There are numerous reports on the release of clathrin from CV in the form of an 8S protomer and of its self-association. Little or no attention has been paid to assessing either the rates or the extents of the reaction. Unanue et al. (1981) have shown that clathrin recombines with the stripped or uncoated vesicle to form CV. There has been no description, however, of the composition of the re-formed vesicles. Most investigators have used the appearance of baskets or CV in the electron microscope as a criterion of reassociation. Using fluorometry and light scattering, we have assessed the influence of a number of variables on the dissociation of CV and the reassociation of clathrin and uncoated vesicles.

When the pH of CV is increased to 8.5 in 0.10 M Tris, the rate of dissociation is too fast to measure by light scattering, i.e., less than several seconds. Examination of this solution by sucrose gradient sedimentation revealed two sedimenting boundaries, one of protein (containing coat molecules) and one of phospholipid (containing the vesicles and membrane proteins). When dissociation occurred at lower pH values, i.e., 7.5 and 8.0 with 0.10 M Tris, the degree of dissociation decreased. The extent of reversal, however, was greater with decreasing pH. This result is understandable if fewer of the surface proteins are removed. With increasing pH, the surface may be disrupted, and the interactions necessary for recovery of the coat structure may not be available. It should be stressed that the binding of clathrin does not necessarily reproduce the native coat structure. The latter may be formed only when other surface proteins are present which are required to interact with clathrin. The binding can only be judged reversible if the sedimentation rate, composition, and morphology of the re-formed vesicles are the same as those of the native vesicle.

It is of interest to compare the interactions of clathrin when it self-associates with its recombination with uncoated vesicles to form its typical, polygonal structure (Crowther et al., 1976; Crowther & Pearse, 1981). The two reactions resemble each other fairly closely although there are some important differences between them. The kinetics of both reactions are very similar since they approach zero order in concentration (Van Jaarsveld et al., 1981). They also occur in the same pH range, i.e., 7–6, and the extent of reaction increases importantly with acidity. Both the self-association and binding are strongly enhanced by millimolar concentrations of Ca^{2+}. All of these effects clearly indicate that clathrin–clathrin interactions control the rate and extent of reaction. There is, however, one factor which affects the two reactions differently and suggests a role for other proteins forming part of the uncoated vesicle surface. This factor is the influence of ionic strength. Basket formation is strongly inhibited by ammonium acetate or NaCl (Van Jaarsveld et al., 1981). The reassociation of clathrin with the uncoated vesicle, however, is not affected by NaCl. This difference in behavior can be understood if an interaction occurs between an uncoated vesicle and clathrin which is not sensitive to NaCl. These additional interactions in CV would also make CV more stable to dissociation than baskets.

The influence of Ca^{2+} on the reassociation is of particular interest since in the presence of millimolar concentrations the vesicle can be coated at physiological pH values. In the absence of Ca^{2+}, somewhat lower pH values are needed. Thus, the coat can be either removed or replaced by regulating the Ca^{2+} concentration. The coat needs to be eliminated prior to vesicle–lysosome fusion (Pastan & Willingham, 1981; Pearse, 1980). Thus, the clathrin coat may stabilize CV and inhibit fusion with cellular organelles which are not the appropriate repository site of the CV in a particular tissue. Altstiel et al. (1982) found that coated vesicles stripped of their coat fuse more easily with lysosomes.

References

Altstiel, L. D., Giedd, K., & Branton, D. (1982) *Biophys. J. Abstr. (2)*, 27.

Crowther, R. A., & Pearse, B. M. F. (1981) *J. Cell Biol. 91*, 790–797.

Crowther, R. A., Finch, J. T., & Pearse, B. M. F. (1976) *J. Mol. Biol. 103*, 785–798.

Goldstein, J. L., Anderson, R. G. W., & Brown, M. (1979) *Nature (London) 279*, 679–685.

Heuser, J. E., & Reese, T. S. (1973) *J. Cell Biol. 57*, 315–344.

Irace, G., Lippoldt, R. E., Edelhoch, H., & Nandi, P. K. (1982) *Biochemistry* (in press).

Keen, J. H., Willingham, M. C., & Pastan, I. (1979) *Cell (Cambridge, Mass.) 16*, 303–312.

Nandi, P. K., Pretorius, H. T., Lippoldt, R. E., Johnson, M. L., & Edelhoch, H. (1980) *Biochemistry 19*, 5917–5921.

Nandi, P. K., Van Jaarsveld, P. P., Lippoldt, R. E., & Edelhoch, H. (1981) *Biochemistry 20*, 6706–6710.

Nandi, P. K., Irace, G., Van Jaarsveld, P. P., Lippoldt, R. E., & Edelhoch, H. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5581–5885.

Ockelford, C. D., & Whyte, A. (1977) *J. Cell Sci. 25*, 293–312.

Pastan, I. H., & Willingham, M. C. (1981) *Science (Washington, D.C.) 214*, 504–509.

Pearse, B. M. F. (1975) *J. Mol. Biol. 97*, 93–98.

Pearse, B. M. F. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1255–1259.

Pearse, B. M. F. (1980) *Trends Biochem. Sci. (Pers. Ed.) 5*, 131–134.

Pretorius, H. T., Nandi, P. K., Lippoldt, R. E., Johnson, M. L., Keen, J. H., Pastan, I., & Edelhoch, H. (1981) *Biochemistry 20*, 2777–2783.

Rothman, J. E., Bursztyn-Pettegrew, H., & Fine, R. E. (1980) *J. Cell Biol. 86*, 162–171.

Schmid, S. L., Matsumoto, A. K., & Rothman, J. E. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 91–95.

Schook, W., Puszkin, S., Bloom, W., Ures, C., & Kochwa, S. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 116–120.

Unanue, E. R., Ungewickell, E., & Branton, D. (1981) *Cell (Cambridge, Mass.) 26*, 439–446.

Van Jaarsveld, P. P., Nandi, P. K., Lippoldt, R. E., Saroff, H., & Edelhoch, H. (1981) *Biochemistry 20*, 4129–4135.

Woodward, M. P., & Roth, T. F. (1979) *J. Supramol. Struct. 11*, 237–250.