Interaction of Basic Compounds with Coated Vesicles†

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ABSTRACT: The effect of poly- and dibasic amines, including chloroquine and quinacrine, on the dissociation of coated vesicles at pH 7.4 in 0.01 M 2-(*N*-morpholino)ethanesulfonic acid has been evaluated by light scattering and sucrose gradient centrifugation. The degree of inhibition of dissociation by the polybases is proportional to the number of amine groups in each compound. However, very little difference in effectiveness was found in a series of dibasic compounds, $NH_2(CH_2)_{2-5}NH_2$. Chloroquine and quinacrine contain dibasic aliphatic chains as well as aromatic ring systems. These two antimalarials are more effective in inhibiting dissociation of coated vesicles than the dibasic aliphatic amines. The ring systems therefore appear to be contributing, independently, to the free energy of stabilization of the coat structure of coated vesicles. It is suggested that the interaction of poly- or dibasic compounds with clathrin or coated vesicles could influence the turnover of ligands in receptor-mediated endocytosis.

Coated pits and coated vesicles (CVs)[1] are the vehicles whereby many important macromolecules (i.e., lipoproteins, glycoproteins, hormones, and viruses) are endocytosed by different cells (Goldstein et al., 1979). Membrane lipids may also be translocated into the cytoplasm by this mechanism (Heuser & Reese, 1973). The function of the coat in the endocytotic process still remains to be elucidated. The shedding of the coat appears to be a very early and preliminary step before fusion of the vesicle (endosome) with cellular target organelles. It has been shown that uncoated vesicles (UVs) fuse much faster than CVs with lysosomes (Alstiel et al., 1983).

It is well-known that CVs dissociate to UVs and coat proteins (mostly clathrin and light chains) when the pH is raised above ~7 (Pearse, 1975; Woodward & Roth, 1978; Keen et al., 1979; Schook et al., 1979; Pretorius et al., 1981). Reassociation to form the coat structure also occurs readily when the pH is reduced below ~7 (Woodward & Roth, 1979; Nandi et al., 1982b). The major coat protein, clathrin, will also self-associate in the absence of UVs to form the characteristic polygonal coat structure ("baskets") when the pH is reduced below ~7 (Pearse, 1976; Schook et al., 1979; Woodward & Roth, 1979; Nandi et al., 1980, 1981; van Jaarsveld et al., 1981). The mechanism of the self-assembly of clathrin in both CV and basket formation is quite similar with respect to most factors except that of ionic strength. The formation of coat structure is completely inhibited by about 0.2 M NaCl in the absence of UVs whereas in the presence of UVs it is largely unaffected (Nandi et al., 1982b). It has been shown that a protein attached to UVs is necessary for the binding of clathrin(Unanue et al., 1981). This protein may provide the necessary stabilization so that coat formation can occur in the presence of salt.

We have shown that several types of basic compounds enhance the rate of self-association of clathrin to form baskets (Nandi et al., 1981). Some of these compounds could modify the equilibrium between clathrin and plasma membrane in the cell in order to control coated pit formation. We have now evaluated the effects of certain types of basic substances on the stability of CVs to dissociation at pH 7.4. Various basic compounds have been shown to inhibit internalization of numerous substances without affecting their binding to the membrane receptors (Haigler et al., 1980a; Salisbury et al., 1980; FitzGerald et al., 1980). Other basic compounds appear to inhibit the acidification of endosomes (UVs), a step needed to dissociate ligand and receptor in most receptor-mediated endocytic pathways (Tycko & Maxfield, 1982; Dickson et al., 1983; Berg & Tolleshaug, 1980).

Materials and Methods

Materials

2-(*N*-Morpholino)ethanesulfonic acid (Mes), putrescine, cadavarine, spermidine, spermine, the dibasic amines, quinacrine, and chloroquine were obtained from Sigma. Tris was obtained from Bethesda Research Laboratory and 1,6-diphenyl-1,3,5-hexatriene from Aldrich.

Methods

Preparation of CVs. Bovine brains were used for CV preparations. They were processed by a procedure previously described by us (Nandi et al., 1982a) which is a modification of the one introduced by Pearse (1975). CVs were normally stored as pellets at 4 °C when prepared. They are stable in this form for several weeks. As needed, pellets were dissolved in 0.10 M Mes, pH 6.5, 0.5 mM $MgCl_2$, 1 mM EGTA, and 3 mM azide and dialyzed overnight against 0.01 M Mes, pH 6.5. Solutions were then clarified by centrifugation at 10000 rpm for 5 min at 4 °C. The sucrose gradient pattern of a typcial preparation is illustrated in Figure 1. The Trp fluorescence curve is displaced slightly to lower fractions compared to that obtained by DPH fluorescence. The small difference in the two curves, after normalization, represents the contamination by baskets (Nandi et al., 1982a). The relative amount of baskets varies a little from preparation to preparation.

Preparation of Baskets. The baskets were prepared from 8 S clathrin as discussed elsewhere (Irace et al., 1982). They were polymerized at pH 6.0 and brought to pH 6.5 before addition of inhibitor. Native clathrin (8 S) was prepared as described previously (Irace et al., 1982).

Sucrose Gradient Centrifugation. CV solutions were sedimented in an SW40 rotor for 110 min at 27000 rpm at 20 °C in a Beckman Model L2-65 centrifuge. Linear gradients were formed by mixing equal volumes of 10% and 30% sucrose

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[1] Abbreviations: CVs, coated vesicles; UVs, uncoated vesicles; Mes, 2-(*N*-morpholino)ethanesulfonic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; Tris, tris(hydroxymethyl)aminomethane; Trp, tryptophan; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

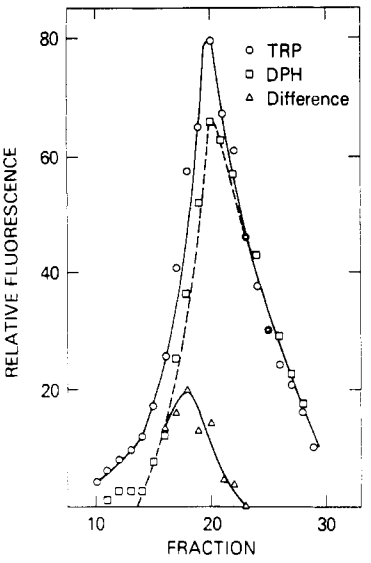

FIGURE 1: Sucrose gradient pattern of native CVs at pH 6.5 in 0.10 M Mes. Trp fluorescence and DPH fluorescence, respectively, represent the distribution of protein and phospholipid in the gradient fractions. The difference peak represents the relative amount of baskets present in the preparation. The latter curve was obtained by normalizing the two curves so that the descending limbs were superimposed.

solutions (w/w) in the same buffer used for the CV solutions, which included inhibitor when it was present in the reaction mixture. The gradient fractions were obtained by pumping solution from the bottom of the centrifuge tube through a long needle lowered through the gradient (Nandi et al., 1982a). Fractions were diluted to 1 mL with distilled water before being read.

Protein and Phospholipid Analysis. We have used Trp emission (280 → 330 nm) and DPH emission (366 → 430 nm) as a measure, respectively, of protein and phospholipid concentration (London & Feigenson, 1978; Nandi et al., 1982a). In the presence of certain inhibitors which absorb strongly, it was not possible to use fluorescence intensities as a measure of concentration. In some cases, we were able to make light scattering measurements. This analysis locates the position of the vesicles in the gradient. Dissociated clathrin (i.e., 8 S) contributes very little to the scatter, compared with either CVs or UVs. A Perkin-Elmer MPF-3 fluorometer was used for fluorescence measurements.

Light Scatter Measurements. A Phoenix-Brice light scattering photometer was used to measure the extent of dissociation of CVs and their inhibition by various types of inhibitors. Most changes in light scatter were over by the time the first measurement was recorded, usually ~1 min after the change in pH. The scatter was measured at 45° to the incident beam in a cylindrical cell at 23 °C (Nandi et al., 1982b). Solutions were irradiated at 436 nm with an H85A3 mercury lamp and a blue filter.

A Perkin-Elmer MPF-3 fluorometer was used to measure the light scatter of fractions emerging from sucrose gradients. The fractions containing 400 μL of solution were diluted with 600 μL of distilled water. Both monochromators were set at the same wavelength where there was little or no absorption by the compounds being tested.

Inhibition of CV Dissociation. The different inhibitors (at pH 6.5, 0.01 M Mes) were added to stock solutions of CVs (at pH 6.5, 0.01 M Mes) without significantly changing the volume. Very little or no change in light scatter was observed with time after the addition of inhibitor. The pH of the solution was then increased from 6.5 to 7.4 by the addition,

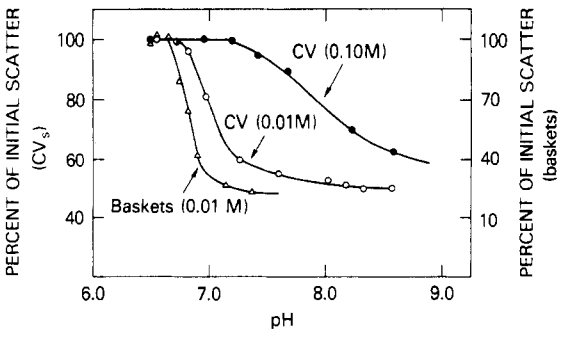

FIGURE 2: pH dependence of the dissociation of CVs in 0.01 and 0.10 M Mes and of baskets in 0.01 M Mes. Solutions containing 0.01 M Mes were titrated with small amounts of 0.20 M Tris buffer of varying pH (7.2–9.0). In 0.10 M Mes, 1.0–2.0 M Tris of varying pH (7.2–9.0) was used. The dilution at the end of each titration was less than ~5%. It should be noted that the decrease in scatter of the baskets (right scale) is greater than that of CVs (left scale).

with magnetic stirring, of about 50 μL of 0.2 M Tris buffer, pH 8.0, to 1 mL of solution. The concentration of Mes buffer was kept at 0.01 M to reduce the amount of Tris buffer needed for the pH adjustment to 7.4. Normally, most or all of the fall in light scatter occurred before the first observation (~ 0.5–1 min) after the pH was increased to 7.4. We have obtained "inhibition curves" of CV dissociation by plotting the loss of initial scatter between pH 6.5 and 7.4 against the concentration of inhibitor (in logarithmic units). In the absence of inhibitor, the scatter usually fell about 50 ± 5%. In the presence of excess inhibitor, as revealed by a plateau in the inhibition curve, a loss in scatter of 10-15% still persisted. The incomplete inhibition suggests that a small fraction was not responsive to inhibitor, presumably due to the fact that the CV preparations were not completely homogeneous.

Inhibition of Basket Dissociation. The procedure with clathrin baskets was essentially the same as with CVs. One important difference was the much greater decrease in scatter observed with the baskets compared with CVs. It should be noted that even with an excess of inhibitor (in the plateau region) a small loss in scatter, i.e., ~15%, was observed. In this respect, the behavior of the baskets to inhibitors was similar to that of the CVs.

Results

pH Dependence of the Dissociation of CVs and Baskets. The dissociation of CVs into UVs and clathrin at pH 8.5 is accompanied by a significant loss of light scatter. The reaction is reversible, and most of the loss of light scatter is recovered by reducing the pH below ~7 (Nandi et al., 1982b). We have used the loss of scatter to follow the degree of CV dissociation. The effect of pH is shown in Figure 2. The dissociation in 0.01 M Mes is essentially complete by pH 7.4. The dissociation curve is displaced, however, to much higher pH values in stronger buffers, i.e., 0.10 M Mes. In this case, dissociation only approaches completion near pH ~8.5 with relatively little change occurring at pH 7.4. Clathrin prepared at lower pH values in 0.10 M Mes will clearly have reduced yields. The extent of inhibition of CV dissociation can readily be measured from the smaller loss of light scatter at a pH where the dissociation is normally complete.

The coat protein, clathrin, is readily polymerized to baskets at pH values below ~7. This reaction is readily reversed by raising the pH (van Jaarsveld et al., 1981). We have now measured the extent of basket dissociation with pH in 0.01 M Mes. It can be seen in Figure 2 that baskets are only slightly less stable than CVs to alkaline dissociation in 0.01 M Mes.

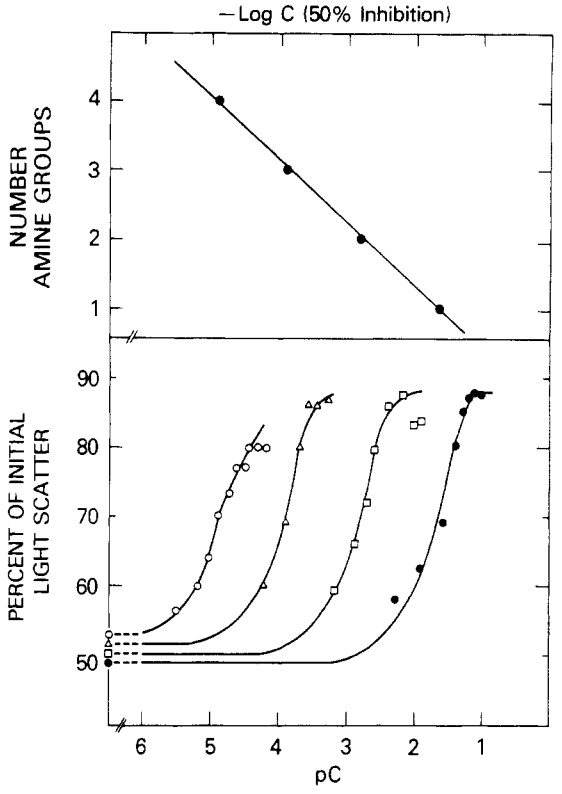

FIGURE 3: (Lower half) Loss of scatter of CVs at pH 7.4 in 0.01 M Mes vs. the concentration of inhibitor (pC = −log C) for a series of polybasic amines. The inhibitor was added to a solution of CVs at pH 6.5 in 0.01 M Mes. Solutions were then titrated to pH 7.4 with a small volume of 0.20 M Tris at pH 8.0 [spermine (○), spermidine (△), putrescine (□), and propylamine (●)]. These curves are referred to as inhibition curves of CV dissociation at pH 7.4 in 0.01 M Mes. (Upper half) Dependence of pC vs. the number of amine groups. The pC value used was that for a 30% decrease in scatter, which corresponds to about 50% dissociation in all four cases.

Inhibition of CV Dissociation. (*A*) *Polybasic Amines.* When the pH of CVs is raised from 6.5 to 7.4 in 0.01 M Mes, extensive dissociation into UVs and clathrin occurs, as analyzed by sucrose gradients (vide infra; Figure 6, lower left half). The protein was distributed in two peaks; the major one was in fraction 5 and contained the coat proteins (i.e., clathrin, light chains, and about half of the protein in the M_r 100000–110000 range); the minor one was in fraction 11 and represents the proteins that remain associated with the UVs (Nandi et al., 1982b). The minor protein peak occurs in the same fraction as the phospholipid peak, i.e., fraction 11. This peak corresponds to the UVs, which also contain most of the M_r 50000–55000 proteins and about half of the M_r 100000–110000 proteins present in the original CVs (Nossal et al., 1983). In the presence of 30 μM spermine, the dissociation of CVs at pH 7.4 is strongly inhibited, and the protein and phospholipid peaks in sucrose gradients are near the positions of native CVs.

An inhibition curve was obtained as a function of spermine concentration by measuring the loss of light scatter between pH 6.5 and 7.4. The relative loss in scatter is shown in Figure 3 (lower half). A similar series of inhibition experiments were performed with several other basic compounds analogous to spermine but containing shorter chains with fewer amine groups. The inhibition curves resemble that of spermine except that they are shifted to considerably higher concentrations. The curves for spermidine, putrescine, and propylamine are displaced, respectively, to higher concentrations relative to that of spermine (Figure 3, lower half). The concentrations giving $I_{7.4}/I_{6.5}$ values of 0.7 (~50% inhibition) have been plotted in

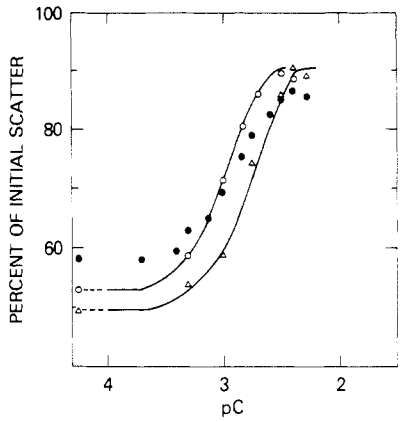

FIGURE 4: Loss of scatter of CVs at pH 7.4 in 0.01 M Mes vs. the concentration of inhibitor for a series of dibasic amines [ethylenediamine (△), diaminopropane (○), and cadavarine (●)]. Only the points are shown for cadavarine to facilitate the readability of the figure.

Figure 3 (upper half) against the number of amine groups. A linear relation with a slope of 1.1 is obtained. Since the concentration of the basic ccompounds needed to stabilize CVs increases with decreasing number of $(CH_2)_3NH_2$ units, the amine moieties are probably the strongly interacting group.

(*B*) *Dibasic Amines.* In the above series of basic compounds, the principal differecnes were in the size of the chain and in the number of amine groups. Since the amine groups were separated by at least three methylene residues in the polybasic compounds, it is not apparent whether there is a contributing effect of the methylene groups to that of the basic groups to the inhibiting activity. A series of dibasic amines were selected, therefore, where the two amine groups are separated by an increasing number of methylene groups, i.e., $NH_2(CH_2)_{2-5}NH_2$. The ethylenediamine curve was very close to that of putrescine (see Figure 3). As can be seen in Figure 4, cadavarine, diaminopropane, and ethylenediamine were about equally effective in inhibiting CV dissociation. The total separation of the inhibition curves for the four dibasic compounds was only about 0.2 log units, and they showed no trend with size.

(*C*) *Quinacrine and Chloroquine.* These two compounds differ by one benzene ring in their aromatic ring systems in addition to the chlorine atom. Their alkyl side chain contains two amine groups separated by four carbon atoms which, therefore, resembles the dibasic compounds discussed above. It has been reported that quinacrine and chloroquine, respectively, can inhibit the internalization of epidermal growth factor into normal rat kidney cells (Haigler et al., 1980b) and of *Pseudomonas* toxin into mouse fibroblasts (Fitzgerald et al., 1980). We have shown that these two antimalarial compounds enhance the rate of polymerization of clathrin into baskets. Since they interact with clathrin, their effect on CV stability was also evaluated.

Chloroquine is somewhat less effective than quinacrine in inhibiting CV dissociation (Figure 5). Both are much more inhibitory than any of the dibasic amines shown in Figure 4, suggesting that the aromatic ring structures contribute independently to the affinity by hydrophobic interactions. The sucrose gradient pattern of this preparation of CVs at pH 7.4 in 0.01 M Mes is shown in Figure 6 (lower left half). The separation of the protein and phospholipid components is clearly evident. The sucrose gradient pattern obtained in the presence of 3.6×10^{-4} M chloroquine is also illustrated in Figure 6 (upper right half). The inhibition appears to be complete although a small amount of aggregation of CVs is

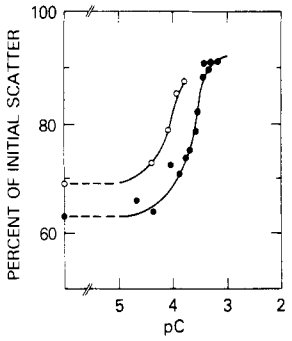

FIGURE 5: Loss of scatter of CVs at pH 7.4 in 0.01 M Mes vs. the concentration of quinacrine (○) and chloroquine (●). $pC = -\log C$.

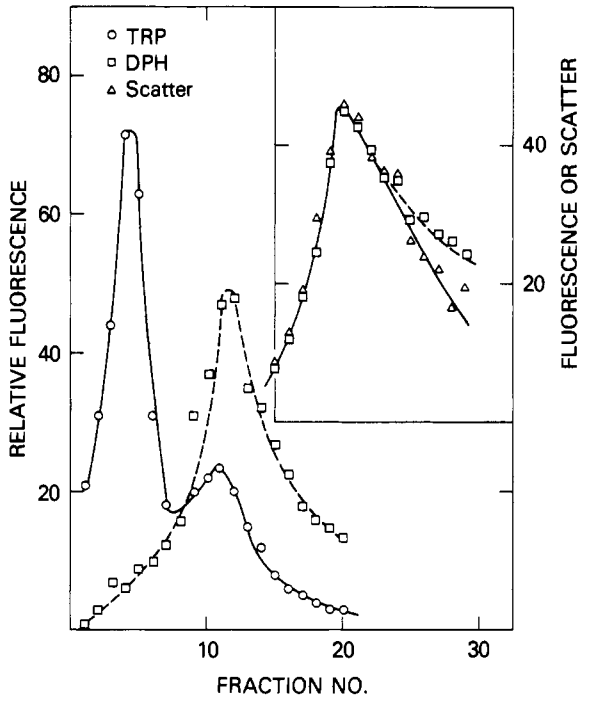

FIGURE 6: Sucrose gradient pattern of the CVs at pH 7.4 in 0.01 M Mes in the presence (upper right half) and absence (lower left half) of 3.6×10^{-4} M chloroquine. The scatter (△, upper right) measures the distribution of particles, either CVs or UVs. The position of the scatter points represents the usual position of CVs. By diluting the quinacrine and hence reducing its absorption, we were able to measure the light scatter of the gradient fractions (after adding 0.60 mL to a 0.40-mL sucrose fraction; see Methods) and locate the position of the vesicles. The gradients were therefore analyzed by Trp fluorescence for protein and by light scatter for particle size.

seen since the curves extend to higher fractions than was observed with native CVs (see Figure 1). Due to the absorption of chloroquine interfering with tryptophan excitation, light scatter was used to measure the particle size distribution of the CVs.

Quinacrine is more effective than chloroquine in inhibiting CV dissociation at pH 7.4. This presumably results from a separate contribution to the free energy of stabilization by the extra benzene ring of quinacrine compared to chloroquine. The quinacrine inhibition curve (Figure 5) does not show a plateau but decreases at concentrations above 2×10^{-4} M (not shown) due to interference with the light scatter assay (in the reaction vessel) by the strong absorption of the inhibitor.

Inhibition of Basket Dissociation. Spermine and Spermidine. The influence of the vesicle on the stability of the coat structure in CVs could be evaluated by comparing the effects of the polybasic compounds on the inhibition of dissociation of CVs and baskets. The inhibition curves of the two most

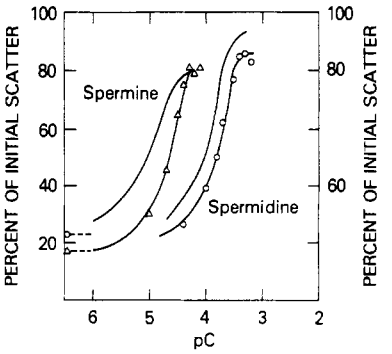

FIGURE 7: Loss of scatter of baskets at pH 7.4 in 0.01 M Mes vs. the concentration of spermine (△) and spermidine (○). The curves without points, to the left of those with points, represent the curves obtained with CVs (see Figure 3) under identical conditions for each polybasic amine.

effective inhibitors, spermine and spermidine, on basket dissociation at pH 7.4, 0.01 M Mes, are shown in Figure 7. We have also shown the corresponding curves for CVs in Figure 7 by the lines without points. It can be seen that the two curves for CVs occur, respectively, at slightly lower concentrations of inhibitor in each case. The difference represents the stabilization provided by the UVs. This difference, i.e., ~0.2–0.3 log C unit, is similar to the difference observed in the curves of the pH dependence of dissociatin in 0.01 M Mes reported in Figure 2.

Discussion

The coated pit regions of membranes are the loci for the internalization of numerous substances by membrane receptors. The recycling of receptors between the plasma membrane, coated pits, and endosomes has been shown to require only several minutes (Bridges et al., 1982; Brown et al., 1983; Ciechanover et al., 1983). The delivery of the endocytosed ligand to its target organelle is equally fast. The role of clathrin in the formation and processing of coated pits (and CVs) remains unclear.

We have explored further the interaction of CVs with certain basic compounds, some of which are known to inhibit receptor-mediated endocytosis. We have shown that the dissociation of CVs and their reassociation are rapid reactions occurring in the neutral pH region (Nandi et al., 1982b). It is known that the binding of small molecules to one protein of an equilibrium reaction can shift the equilibrium of the reaction (Levitzki & Schlessinger, 1974; Steiner, 1974; Ingham et al., 1975a,b). In the case of CVs, the binding of a basic compound could alter the equilibrium (or kinetics) so that, for instance, association can take place in a pH region where dissociation normally occurs. In this way, the binding of this compound could associate clathrin at a pH value where it is normally dissociated. We have shown that Ca^{2+} can induce the reassociation of UVs and clathrin even at pH values as high as 8.5 (Nandi et al., 1982b). We have now investigated certain basic compounds which enhance the rate of clathrin self-association to form baskets. These compounds also stabilize CVs so that they are stable at pH values they would ordinarily dissociate.

Their relative effectiveness in inhibiting dissociation of CVs has been found to depend on the number of amine groups in a series of polybasic amines. The differences in the concentrations of these compounds needed to produce the same degree of stabilization are so large (~1.1 log unit ≈ 1550 cal) that the important influence of the amine group is clear.

We also evaluated the effect of increasing separation of the two amine groups in a series of dibasic amines. The distance

of separation had little or no effect on their relative efficiencies of inhibiting CV dissociation at pH 7.4. This is a somewhat surprising result, since these substances would be expected to bind to two *fixed* acidic sites on clathrin. This result would suggest that there may be several acidic sites on clathrin at slightly different distances from a primary site which offers equally strong binding and stabilization. This is also implicit in the polybasic amine series where the increasing effectiveness depends on the number of basic groups in the compound. Presumably, the number of occupied binding sites on clathrin increases with the number of amine groups in the polybasic series.

It is likely that spermine and spermidine bind to the same groups in CVs as in baskets since they are almost equally effective in inhibiting dissociation. The small difference may be due to interactions between clathrin and a protein present in UVs which modifies the binding properties of clathrin (Unanue et al., 1981). The polymerization of clathrin in the presence and absence of UVs has been compared since the structure of the coat is very similar in CVs and baskets (Pearse, 1975; Crowther & Pearse, 1981). It has been shown that the dependence of the rate of polymerization of clathrin to form either CVs or baskets is very similar with respect to protein concentration and very dissimilar with respect to ionic strength (Van Jaarsveld et al., 1981; Nandi et al., 1982b). It is evident that the two reactions are similar with respect to their interactions with spermine and spermidine. Presumably they bind *strongly* only with clathrin and not with other components present in CVs. They would not be expected to be incorporated into the lipid bilayed of CVs due to their high charge.

The relative amounts of quinacrine and chloroquine needed to inhibit CV dissociation are comparable to those needed to enhance the rate of clathrin self-association (van Jaarsveld et al., 1982). These results also suggest that quinacrine and chloroquine stabilize CVs by interacting with clathrin.

Basic substances inhibit the proton pump necessary for the separation of many receptors from their specific ligands in the endosome (Gonzalez-Noriega et al., 1980; Berg & Tolleshaug, 1980; Maxfield, 1982). The inhibition of internalization of various ligands by other basic compounds could depend on their inhibition of receptor recycling either by failure of the proton pump or by interference in the recycling of clathrin. The pathway of clathrin in receptor-mediated endocytosis still awaits clarification. It is evident, however, that the binding of basic compounds to clathrin in CVs could interfere with their function in endocytosis.

Registry No. Spermine, 71-44-3; spermidine, 124-20-9; putrescine, 110-60-1; propylamine, 107-10-8; cadaverine, 462-94-2; diaminopropane, 26545-55-1; ethylenediamine, 107-15-3; chloroquine, 54-05-7; quinacrine, 83-89-6.

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