Biochemical Pharmacology, Vol. 31, No. 5, pp. 793–798, 1982.
Printed in Great Britain.

0006-2952/82/050793–06 $03.00/0
Pergamon Press Ltd.

EFFECTS OF SEVERAL ANTIMALARIALS AND PHENOTHIAZINE COMPOUNDS ON THE FORMATION OF COAT STRUCTURE FROM CLATHRIN

PIETER P. VAN JAARSVELD, ROLAND E. LIPPOLDT, PRADIP K. NANDI and HAROLD EDELHOCH*

Clinical Endocrinology Branch, National Insititute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, MD 20205, U.S.A.

(*Received* 1 *April* 1981; *accepted* 30 *July* 1981)

Abstract—We have evaluated the effects of two phenothiazine and several antimalarial drugs on the rates of polymerization of 8S clathrin molecules to 300S coat structures. Most of the drugs investigated have been shown in other studies to inhibit receptor-mediated endocytosis through the coated pit regions of plasma membranes. The two types of drugs were found to accelerate the polymerization rate without having much effect on the size distribution of the polymer species. The activities of the drugs appear to depend on a dibasic moiety and a large, hydrophobic aromatic ring in their structures.

Information concerning the mechanism of receptor-mediated endocytosis has accumulated rapidly in the last few years. A major pathway of this internalization process is through specific regions in the plasma membrane referred to as coated pits [1–5]. These regions are readily identified by electron microscopy due to their characteristic appearance in the plasma membrane [6, 7]. It has been shown that the protein responsible for the coat structure of coated pits is clathrin [8–10]. In fact, clathrin can form closely related, if not identical, polyhedral coats in the absence of an interior membrane [10–15].

The internalization process depends on the functioning of the metabolic machinery of the cell since receptor binding of ligand, but not endocytosis, occurs at 5° [3, 5]. It has been shown that certain compounds which inhibit various enzymes (i.e. transglutaminase [16] and phospholipase A_2 [17] or bind to proteins [calmodulin] [18] reduce or prevent endocytosis. Thus, compounds may impair one or more of the steps involved in endocytosis, i.e. clustering of receptor–ligand complexes, recruitment of clathrin to form coated pits, or the pinching off of coated pits to form coated vesicles or receptosomes [1, 19]. The latter process may involve a reorganization of the clathrin in coated pits from hexagonal into pentagonal polyhedra [6, 7]. In this case, the conformational state of clathrin may place an important role in the endocytotic process.

We have shown that the clathrin protomer, with a sedimentation constant of 8S, can be polymerized into coat structure, i.e. baskets, with average sedimentation constants of 150S and 300S [14, 15]. The electron microscopic structure of these two particles closely resembles the coat structure of coated vesicles [9]. In addition, it is possible to follow the rate of this polymerization process by turbidimetric procedures, i.e. light scatter or transmission [20]. Using this methodology, we have evaluated the effects of two pharmacologically active groups of drugs (antimalarials and phenothiazines) on the rate of formation of coat structures, i.e. baskets, from clathrin protomer molecules. We selected these drugs because they have been shown to inhibit endocytosis of various ligands by the coated pit regions of the plasma membrane of different cells.

MATERIALS AND METHODS

Preparation of clathrin. Bovine brains were used for preparing clathrin by a procedure described previously [14] which is a modification of the method of Schook *et al.* [12]. The 8S protomer of clathrin is obtained by extracting a crude vesicle preparation with 0.05 M Tris (pH 8.0), followed by ammonium sulfate precipitation (30%) and gel chromatography on Sepharose 4B-Cl in 0.02 M Tris, 0.25 M NaCl, 5 mM NaN_3 (pH 8.0). Three distinct peaks are obtained in the gel chromatographic separation. The major band (peak II) contains 8S clathrin. This peak was analyzed routinely by sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis [15], to check the uniformity of the polypeptide composition of the 8S clathrin preparation. In all cases, about 80–90% of the total protein had a molecular weight of clathrin, i.e. 175,000. Most investigators have used preparations of clathrin with the same protein components. Since the rate of clathrin polymerization is very sensitive to salt concentration [20], we routinely dialyzed all fractions against 0.1 M ammonium acetate (pH 8.0), except as otherwise stated, for 3 hr before experiments were performed. Clathrin preparations were then centrifuged at 30,000 *g* for 30 min to eliminate any aggregates. The ammonium acetate was a convenient salt to use since the pH of the clathrin solutions could be reduced from 8 to 6.0–6.9 by addition of a small volume of buffer.

Measurement of polymerization rates. To follow the rate of polymerization of clathrin, we used light

* Author to whom all correspondence should be addressed.

scatter measurements as described previously [20]. A Brice Phoenix Universal light scatter photometer equipped with a blue filter (435 nm) was used, and the measurements were made at 45°. One ml solutions of clathrin (~ 0.3–0.6 mg/ml) were used in a polished cylindrical cell obtained from Precision Cell, Inc., Hicksville, N.Y. Small volumes (1–100 μl) of buffer or salt solutions were added to the clathrin solution and mixed rapidly (~ 7 sec) by a magnetic stirring bar in the cell. The temperature of measurement was 23 ± 1°.

Sucrose density gradient centrifugation. A Beckman model L2-65 ultracentrifuge was used for sucrose density gradient centrifugation. All centrifugations were performed with the SW27 rotor at a temperature setting of 5°. Linear gradients were formed by mixing equal volumes of 10 and 30% sucrose solutions (w/w) in the same buffer used for polymerizing clathrin. One ml solutions of polymerized clathrin were layered on top of the gradients. After centrifugation, the gradients were fractionated by pumping 1 ml fractions from the bottom of the tubes with the aid of a needle lowered through the gradient. An LKB peristaltic pump set at 2 ml/min was used for this purpose. The protein concentration was monitored by measuring tryptophan fluorescence in a Perkin Elmer MPF-3 fluorometer. Samples were excited at 280 nm, and the emission was monitored at 340 nm.

Electron microscopy. Electron microscopical examination of the morphology of clathrin baskets was performed by Dr. B. Kramarsky (Electronucleonics Biological Research, Rockville, MD). Negative staining with 1% uranyl acetate was performed.

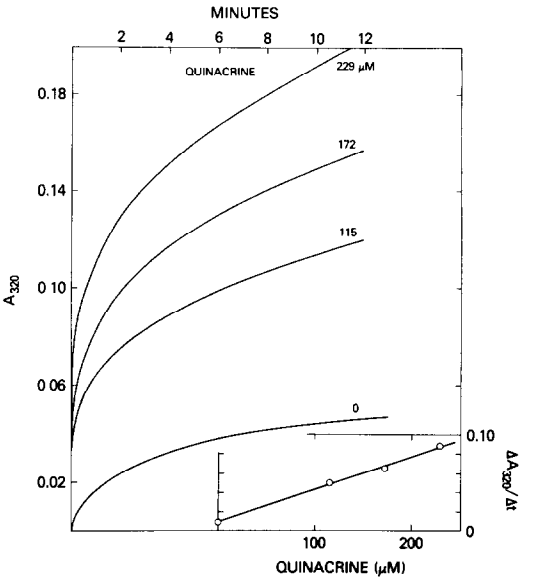

Fig. 1. Effect of quinacrine on the rate of polymerization of clathrin as measured by the change in absorbance at 320 nm. One ml solutions of 0.7 mg/ml clathrin in 0.1 M ammonium acetate (pH 7.5) were placed in the sample and reference cells. Small volumes of a 24 mM quinacrine stock solution (pH 7.5) were then added to each solution. The polymerization was started by addition of 50 μl of 1 M MES buffer (pH 6.45) to the solution in the sample compartment, which lowered the pH to 6.65. An equal volume of 1 M MES (pH 7.5) buffer was added to the reference cell. The insert in the lower right part of the figure shows the increase in initial rates (expressed as the increase in absorbance after 0.5 min) as a function of quinacrine concentration.

RESULTS

Antimalarials. We have evaluated the effects of one acridine and several quinoline compounds, i.e. chloroquinine, quinine, quinidine and 8-OH quinoline, in enhancing the rate of clathrin polymerization. Quinacrine proved to be the most effective of these compounds. The increase in rate produced by micromolar amounts of quinacrine is illustrated in Fig. 1. A linear dependence of rate on quinacrine concentration was observed if the increase in absorbance between either 0 and 0.5 min (or 0 and 1 min) was plotted against drug concentration (Fig. 1, lower half).

Of the quinoline group, quinine was the next most effective drug tested. However, millimolar quantities of quinine were needed to produce rates comparable to those of quinacrine. A linear dependence of initial rate against quinine concentration was also observed by a similar plot of the kinetic data (Fig. 2). When quinidine, the optical isomer of quinine, was compared with quinine, both compounds had the same effectiveness in increasing the rate of clathrin polymerization.

Chloroquine was found to be less active than quinine on a molar basis. With this compound, the procedure of adjusting the pH to start the polymerization was modified because of its limited solubility at alkaline pH values. In this case, the polymerization was initiated without drug by adding MES [2-(*N*-morpholino)ethanesulfonic acid] buffer (pH 6.25) to a pH 8.0 solution of clathrin to produce a pH of 6.44. After the initial increase in light scatter had subsided, i.e. 4 min, a small volume of chloroquinine solution in MES buffer at pH 6.44 was added to the clathrin solution (Fig. 3). A second increase in light scatter largely reflected the influence of chloroquine. A plot of the initial rates between 4 and 4.5 min (i.e. between 0 and 0.5 min after drug addition) with chloroquine concentration showed slight sigmoidal curvature (not shown).

In another series of experiments, chloroquine was compared with quinine by using the same clathrin preparation and the identical experimental conditions (pH 6.49). It was observed that chloroquine was less active than quinine in stimulating the rate of clathrin polymerization. When the nonpolar moiety of chloroquine, i.e. 8-OH quinoline, was evaluated at the same pH, the rate of clathrin polymerization was unaffected* (Fig. 4).

An increase in rate of polymerization could occur

* To ensure complete solubility, a 5% solution of dimethylsulfoxide (DMSO) was used as a solvent for the 8-OH quinoline experiments. We have found little or no difference in either the rate of polymerization or in the sedimentation behavior of clathrin baskets between water and 5% DMSO solution.

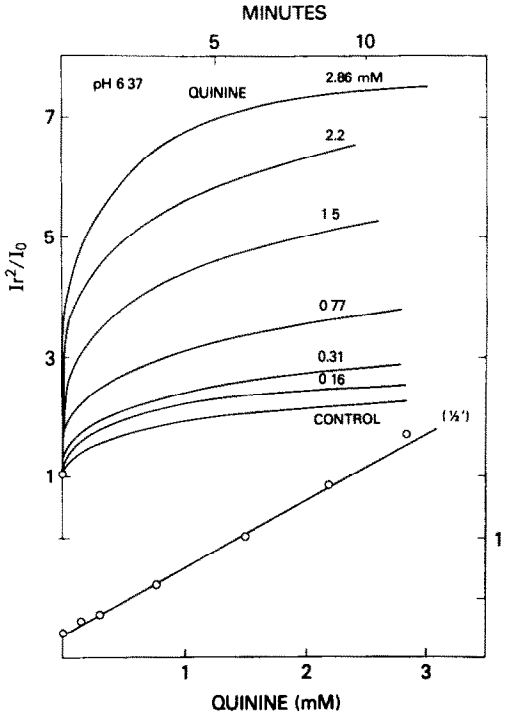

Fig. 2. Effect of quinine on the rate of polymerization of clathrin as measured by light scatter at 45°. Small volumes of a 33 mM stock solution of quinine (pH 7.0) were added to a 1 ml solution of clathrin (0.4 mg/ml) in 0.1 M ammonium acetate (pH 8.0). The polymerization was started by lowering the pH to 6.37 by the addition of 50 μl of 1 M MES. At the bottom of the figure, initial rates (the change in light scatter value between 0 and 0.5 min of polymerization) are shown as a function of quinine concentration.

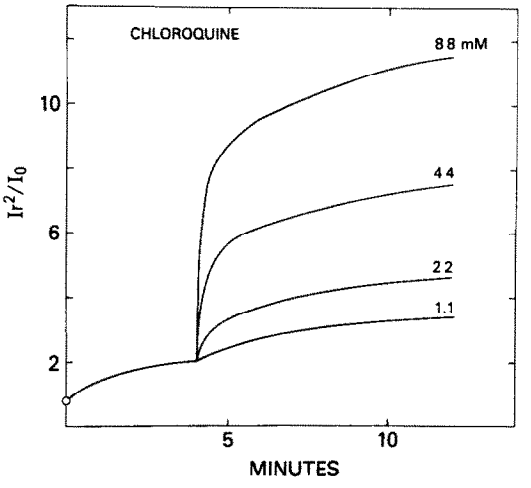

Fig. 3. Effect of chloroquine on the rate of polymerization of clathrin. Due to the low stability of chloroquine at slightly alkaline pH values, the polymerization of 1 ml cathrin solutions (0.3 mg/ml in 0.1 M ammonium acetate, pH 8.0) was first started by the addition of 50 μl of 1 M MES to lower the pH to 6.44. Small volumes of chloroquine from a 48 mM stock solution (pH 6.44) were then added after 4 min of polymerization, to observe the effect of chloroquine on the rate of increase in light scatter at 45°.

either by the drug affecting the rate constants of the normal pathway of polymerization or by modifying the pathway. In the latter event, one might expect a change in the size or size distribution in the population of basket molecules. The distribution of sedimentation coefficients of baskets formed at pH 6.8 has been published elsewhere [15]. We have used a different procedure to observe a change in the size distribution since the absorption of unbound quinine in the centrifuge cell severely reduces our ability to analyze the absorption profile of the polymerized baskets; therefore, band sedimentation in a sucrose gradient was used instead of boundary centrifugation.

The polymerization was carried out at pH 6.50 in the presence of 1.4 mM quinine. After 2 hr at 23° the solution was allowed to reach equilibrium by incubating further for 48 hr at 4°. With quinine present during polymerization, there were only minor changes in the sucrose gradient profile compared to the control (Fig. 5). The principal boundary of ~300S clathrin baskets did not change significantly (Fig. 5). However, there was a small decrease in the 150S peak and a small amount of a new boundary appeared which sedimented faster than 300S. The fluorescence of 8S clathrin, which remains near the top of the density gradient, was strongly quenched by the presence of unbound quinine. When the same experiment was performed with 2.8 mM quinine, similar results were obtained, only there was a greater loss of 150S and a larger amount of clathrin sedimenting faster than 300S. Even at this high level of quinine (i.e. 2.8 mM), the 300S molecular species was the major peak (>70%). When the same sucrose gradient experiments were performed with quinacrine (150 μM), similar results were found, i.e. the principal band sedimented as 300S.

Phenothiazines. Two phenothiazine drugs, i.e. trifluoperazine and chlorpromazine, were also found to be effective in enhancing the rate of clathrin

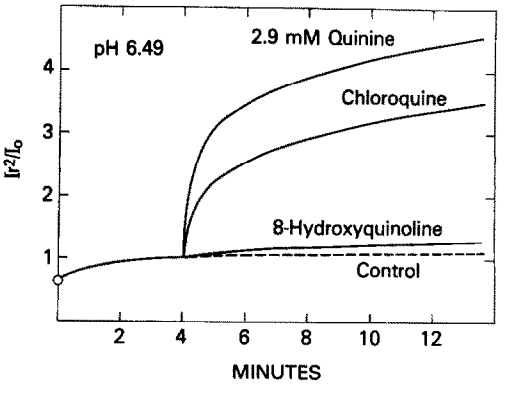

Fig. 4. Effect of 2.9 mM solutions of quinine, chloroquine and 8-OH quinoline on the rate of polymerization of clathrin. One ml clathrin solutions (0.3 mg/ml in 0.1 M ammonium acetate, pH 8.0) were first acidified to pH 6.49 by addition of 50 μl of 1 M MES buffer. After 4 min, 100 μl of a 33 mM stock solutions of the drug, dissolved in 50% DMSO–0.25 M MES (pH 6.49), was added.

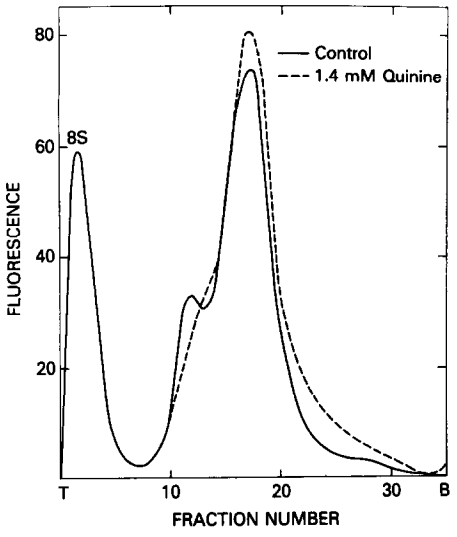

Fig. 5. Sucrose gradient centrifugation analysis of clathrin polymerized in the absence (solid line) and presence (dashed line) of 1.4 mM quinine. One ml solutions of clathrin (0.7 mg/ml in 0.1 M ammonium acetate) were polymerized at pH 6.50. The polymerization continued for 2 hr at 23° followed by 48 hr at 4°. The baskets were separated from unpolymerized 8S protomer by centrifugation on 35 ml of a linear 10–30% sucrose gradient. Centrifugation was for 85 min at 24,000 rpm in the SW27 rotor of a Spinco centrifuge. The gradients were made in the same buffer as was used for polymerization. T and B indicate the top and bottom of gradient.

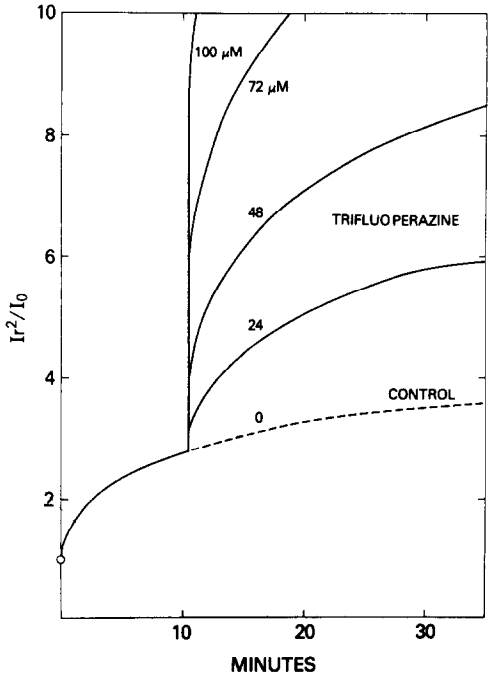

Fig. 6. Effect of trifluoperazine on the polymerization of clathrin as measured by light scatter at 45°. One ml solutions of clathrin (0.47 mg/ml in 0.1 M ammonium acetate) were first acidified to pH 6.38 by addition of 50 μl of 1 M MES buffer. Small volumes of a 25 mM stock solution of trifluoperazine at pH 6.38 were added after 10 min of polymerization without the drug.

polymerization. The reaction was initiated in the same way as chloroquinine since the phenothiazines also have little solubility above neutral pH.

Trifluoperazine was the most effective compound we studied in increasing the rate of clathrin polymerization (Fig. 6). Larger amounts of chlorpromazine, compared to trifluoperazine, were needed to produce equivalent increases in rate (Fig. 7). In both cases, polymerization was initiated about 10 min before the drug was added. Examination of the rates of polymerization clearly indicates a sigmoidal dependence of initial rates on chlorpromazine concentration since 33 μM was almost without any effect, whereas 100 μM showed a signficant increase in initial rate and 133 μM an even larger effect. The data with trifluoperazine can be similarly understood, although the sigmoidicity is somewhat smaller with this drug. A plot of the initial rates against drug concentration for both phenothiazine drugs is seen in Fig. 8. The initial rate values obtained with chlorpromazine and trifluoperazine are clearly less precise than those obtained with quinicrine and quinine since the method of initiating the reaction with the latter compounds is more direct. In any case, there appear to be drugs that show a linear dependence on initial rates and others that exhibit a sigmoidal dependence.

Sucrose gradient analysis of clathrin baskets formed at pH 6.3 in the presence of 310 μM chlorpromazine or 47 μM trifluoperazine gave sedimentation patterns similar to those observed with quinine and quinacrine; the principal boundary was at the position of 300S baskets, with shoulders on both the ascending (~150S) and descending (>300S) limbs of the tryptophan fluorescence profile. We have observed that two other substances that accelerate

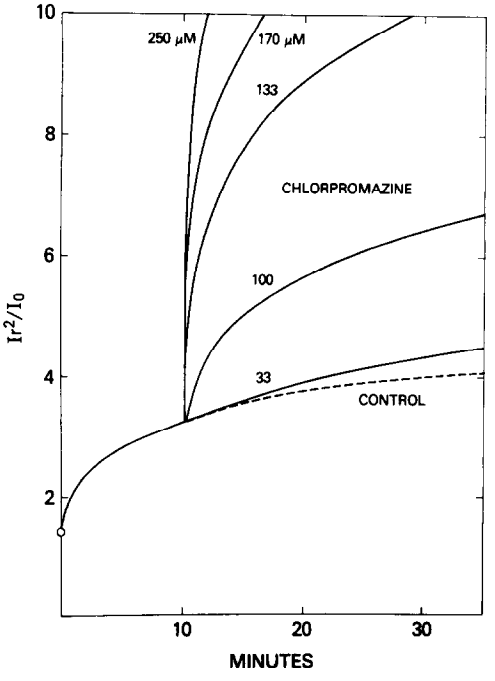

Fig. 7. Effect of chlorpromazine on the rate of polymerization of clathrin. The experimental details were similar to those in the legend of Fig. 6.

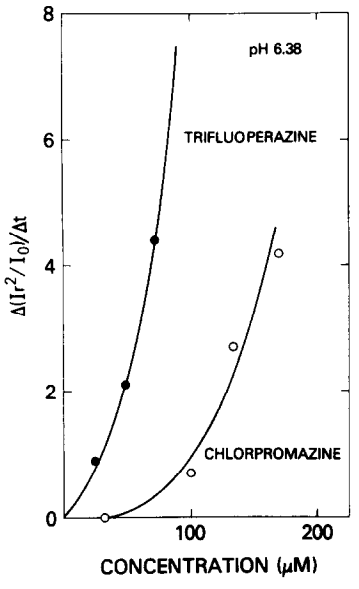

Fig. 8. Increase in initial rate of polymerization of clathrin as a function of trifluoperazine (●) and chlorpromazine (○) concentrations. The initial rate was measured as the change in light scatter between 0 and 0.5 min after addition of the drug (see Figs. 6 and 7 respectively).

polymerization of clathrin, calcium and dansyl cadavarine, also do not significantly alter the size distribution of the baskets [21].

DISCUSSION

We have evaluated the behaviors of three antimalarial and two phenothiazine drugs towards one of the several processes known to be involved in receptor-mediated endocytosis by the coated pit regions of plasma membranes. Four of the drugs have been shown to inhibit endocytosis of several different proteins into various types of cells: quinacrine and chlorpromazine affect epidermal growth factor endocytosis in normal rat kidney cells [17], chloroquin blocks Pseudomonas toxin internalization into mouse fibroblasts [22], and trifluoperazine prevents IgM endocytosis by cultured human lymphoblastoid cells [18]. Of these four drugs, only chlorpromazine significantly reduces the amount of ligand binding to receptor [17].

We have shown that both antimalarial and phenothiazene drugs accelerate the rate of formation of clathrin baskets from protomer molecules. Trifluoperazine was the most, and chloroquine the least, effective accelerator. It has been postulated by Salisbury *et al.* [18] that trifluoperazine prevents the recruitment of clathrin to the membrane opposite ligand–receptor complexes and thereby precludes the formation of the coated pit regions needed for endocytosis. It is possible that the recruitment of clathrin to the potential coated pit regions of membranes can be prevented by its polymerization in the cytoplasm by the drugs tested. It has been reported by most investigators that their coated vesicle preparations contain an important percentage of baskets. The binding of drug to clathrin may also shift the equilibrium in favor of basket formation and thereby inhibit the formation of coated pits in the plasma membrane. These drugs could also inhibit endocytosis by binding to clathrin in a coated pit region and changing its interaction with the membrane, thereby altering the processing whereby ligands or receptor–ligand complexes are internalized. Heuser [7] has presented electron micrographs of coated pits and vesicles which suggest that coated vesicles are pinched off from coated pits by changes in the structure of the clathrin basketwork that forms the coat structure of the coated pits.

Analysis of the rate of clathrin polymerization shows that the data do not fit either a first- or a second-order process, but require a much more complicated process [20]. The effects of the various drugs on the initial rates of polymerization also indicate further complexities, at least with chlorpromazine and trifluoperazine. The latter exhibit a higher than first-order dependence on drug concentration. A first-order dependence, however, was observed with quinacrine and quinine. The specific meaning of this difference in order of reaction is unclear since the drug data cannot be analyzed more rigorously until the polymerization kinetics in the absence of drugs is better understood. In fact, the method we have used in defining the rate is empirical since an exact analysis of the kinetic data does not exist. However, the first-order dependence obtained with quinacrine and quinine suggests that the method of analysis of the rate data offers a meaningful way of analyzing the dependence on drug concentration. In this respect, a linear dependence of initial rates on Ca^{2+} and Mn^{2+} concentrations has also been obtained by the same procedure as used here [21]. The strong sigmoidal behavior observed with trifluoperazine and chlorpromazine implies a different mechanism of activation, one involving either cooperativity between drugs bound to different sites on a single molecule or a greater affinity of the drug for the basket molecule than for the promotor molecule [23].

Although the order of reaction appears to vary, the products of polymerization remain much the same with the various drugs. Sucrose gradient analyses reveal that the 300S species is the major product when clathrin baskets are formed in the presence of quinacrine, quinine, trifluoperazine or chlorpromazine. The effect of the various drugs, therefore, is to increase the rate of polymerization but not the nature of the product.

In another report [21], we showed that spermine and dansyl cadavarine, which are basic substances, also enhance the rate of clathrin polymerization. The two types of drugs studied presently are substances which resemble spermine and dansyl cadavarine in two respects. All the drugs contain both a dibasic and a hydrophobic moiety. The dibasic group consists (except for dansyl cadavarine) of two nitrogen atoms situated either at the ends of a 4-carbon chain (chloroquine and quinacrine) or a 3-carbon chain (chlorpromazine and trifluoperazine). Quinine has either 3 or 5 carbon atoms between its two nitrogen atoms, depending on whether one counts across the benzene ring or along its side. All five drugs, of course, also have a large hydrophobic, aromatic

group. Thus, a common molecular basis exists among these drugs which can account for their binding to a single protein, i.e. clathrin.

Spermine fulfills the above requirements if the ends of the molecule, i.e. $—(CH_2)_3NH_2$, are considered as hydrophobic substituents. This is a reasonable assumption since the $(CH_2)_4NH_2$ side chain of lysine has been shown to be moderately hydrophobic [24]. Dansyl cadavarine clearly contains both structural features except that there are 5 carbon atoms between the two nitrogens of the cadavaryl moiety. Since cadavarine does not enhance the rate of clathrin polymerization, the hydrophobic dansyl group presumably provides the extra free energy necessary for efficient binding.

Thus, both the antimalarial and phenothiazine drugs could bind to a common site on clathrin through their diabasic structures, and the function of the hydrophobic ring structure would be related either to permeability factors or to an increase in the free energy of binding to clathrin. This possiblity could also explain the effects of spermine and dansyl cadavarine in increasing the rate of clathrin polymerization.

REFERENCES

1. J. L. Goldstein, R. G. W. Anderson and M. S. Brown, *Nature, Lond.* **279**, 679 (1979).
2. J. Schlessinger, Y. Schechter, M. C. Willingham and I. Pastan, *Proc. natn. Acad. Sci. U.S.A.* **75**, 2659 (1978).
3. F. R. Maxfield, J. Schlessinger, Y. Shechter, I. Pastan and M. C. Willingham, *Cell* **14**, 805 (1978).
4. P. Gorden, J-L. Carpentier, S. Cohen and L. Orci, *Proc. natn. Acad. Sci. U.S.A.* **75**, 5025 (1978).
5. D. A. Wall, G. Wilson and S. L. Hubbard, *Cell* **21**, 79 (1980).
6. T. Kanaseki and K. Kadota, *J. Cell Biol.* **42**, 202 (1969).
7. J. Heuser, *J. Cell Biol.* **84**, 560 (1980).
8. B. M. F. Pearse, *J. molec. Biol.* **97**, 93 (1975).
9. B. M. F. Pearse, *Proc. natn. Acad. Sci. U.S.A.* **73**, 1255 (1976).
10. B. M. F. Pearse, *J. molec. Biol.* **126**, 803 (1978).
11. M. P. Woodward and T. F. Roth, *Proc. natn. Acad. Sci. U.S.A.* **75**, 4394 (1978).
12. W. Schook, S. Puszkin, W. Bloom, C. Ures and S. Kochwa, *Proc. natn. Acad. Sci. U.S.A.* **76**, 116 (1979).
13. J. H. Keen, M. C. Willingham and I. H. Pastan, *Cell* **16**, 303 (1979).
14. P. K. Nandi, H. T. Pretorius, R. E. Lippoldt, M. L. Johnson and H. Edelhoch, *Biochemistry* **19**, 5917 (1980).
15. H. T. Pretorius, P. K. Nandi, R. E. Lippoldt, M. L. Johnson, J. H. Keen, I. Pastan and H. Edelhoch, *Biochemistry* **20**, 2777 (1981).
16. P. J. A. Davies, D. R. Davies, A. Levitzki, R. Maxfield, P. Milhaud, M. C. Willingham and I. Pastan, *Nature, Lond.* **23**, 162 (1980).
17. H. T. Haigler, M. C. Willingham and I. Pastan, *Biochem. biophys. Res. Commun.* **94**, 630 (1980).
18. J. L. Salisbury, J. S. Condeelis and P. Satir, *J. Cell Biol.* **87**, 132 (1980).
19. M. Willingham and I. Pastan, *Cell* **21**, 67 (1980).
20. P. P. Van Jaarsveld, P. K. Nandi, R. E. Lippoldt, H. Saroff and H. Edelhoch, *Biochemistry* **20**, 4129 (1981).
21. P. K. Nandi, P. P. Van Jaarsveld, R. E. Lippoldt and H. Edelhoch, *Biochemistry* **20**, 6706 (1981).
22. D. FitzGerald, R. E. Morris and C. B. Saelinger, *Cell* **21**, 867 (1980).
23. C. Friden, *Rev. Biochem.* **40**, 653 (1971).
24. D. D. Jones, *J. theor. Biol.* **50**, 167 (1975).