APPLIED BIOCHEMISTRY AND BIOTECHNOLOGY 9, 143–152 (1984)

Interaction of Spermine, Spermidine, and Putrescine with Heparin Bound to Sepharose Beads

B. TADOLINI* and L. CABRINI

Istituto di Chimica Biologica, Università di Bologna, Via Irnerio, 48, 40126 Bologna, Italy

Received July 28, 1983; Accepted September 11, 1983

ABSTRACT

The binding of the polyamines spermine and spermidine, and of their precursor, putrescine, to heparin–sepharose have been investigated to provide the basic data for further application of this resin in the study of polyamine biological function.

(1) Spermine binds heparin–sepharose in a cooperative phase, the empirical index of cooperativity is two, and the apparent binding constant is $1.41 \times 10^6\ M^{-1}$.

(2) Spermidine binds heparin–sepharose in a cooperative and strong noncooperative phase. In the cooperative phase, the index of cooperativity is two and the apparent binding constant is $1.56 \times 10^6\ M^{-1}$. In the strong noncooperative phase, the apparent binding constant is $2 \times 10^5\ M^{-1}$.

(3) Putrescine binds heparin–sepharose in a strong noncooperative and a weak noncooperative phase. In the strong noncooperative phase the apparent binding constant is $2.6 \times 10^5\ M^{-1}$. In the weak noncooperative phase, the apparent binding constant is $3.2 \times 10^3\ M^{-1}$.

Index Entries: Heparin–sepharose, polyamine binding to; spermine, heparin–sepharose binding to; spermidine, heparin–sepharose binding to; putrescine, heparin–sepharose binding to; sepharose, heparin bound to.

*Author to whom all correspondence and reprint requests should be addressed.

0273-2289/84/0400-0143$02.00

INTRODUCTION

The interaction of polyamines with heparin is long since known (*1,2*), but is certainly a secondary matter when compared to the interaction between the polyamines and other polyanionic macromolecules, such as nucleic acids, ribosomes, and biological membranes (*3–5*).

Such interactions have sometimes proved negative, as in the study of the effect of polyamines on nucleic acid metabolism where heparin added to counteract RNAse reaction interfered with the polyamine under test (B. Barbiroli and B. Tadolini, unpublished observations). In some cases, however, this interaction has proved a useful tool.

We exploited this interaction to sequester polyamines, thus minimizing the aggregation of subcellular organelles that normally occurs in homogenate of tissues rich in these amines (*6*). The release of polyamines caused aggregation by heparin has been confirmed in human placenta (*7*). Free heparin, however, when added to a homogenate diffuses into the nucleus where it disrupts the chromatin structure (*8–10*). We used heparin bound to sepharose beads to achieve the same results without encountering similar difficulties (*11*). Apparently the immobilization of heparin on sepharose beads did not significantly affect its binding characteristics toward polyamines. On the other hand, heparin bound to sepharose beads gives undoubted advantages, such as the possibility of removing unreacted heparin–sepharose and polyamine/heparin–sepharose complexes, from the sample by low speed centrifugation (*11*).

For these reasons, we expect a wide application of heparin–sepharose as a tool in the study of polyamines biochemistry. We have thus further investigated the binding of polyamine/heparin–sepharose complexes by binding studies carried out with labeled polyamine. These experiments have provided the basic data for the use of heparin–sepharose in the study of spermine–phospholipid vesicles interaction and for the use of heparin–sepharose column to sequester polyamines from different biological material (12).

MATERIALS AND METHODS

Spermine tetrahydrochloride, spermidine trihydrochloride, and putrescine dihydrochloride were purchased from Sigma Chemical Company (St. Louis, MO, USA). ^{14}C-spermine tetrahydrochloride (122 mCi/mmol), ^{14}C-spermidine trihydrochloride (115 mCi/mmol) and ^{3}H-putrescine dihydrochloride (19 Ci/mmol) were obtained from the Radiochemical Centre (Amersham, Buck, England). Heparin–sepharose Cl-6B was purchased from Pharmacia Fine Chemical (Uppsala, Sweden). The concentration of anionic sites on the immobilized polymer was determined by spectrophotometric titration (*13,14*) using toluidine blue as the

indicator. BTS-450 was purchased from Beckman Instruments, Inc. (Irvine, CA, USA). All other reagents were reagent grade.

The binding of labeled polyamine to heparin–sepharose was measured by incubating heparin–sepharose (about 90 nmol anionic sites) with various concentrations of labeled polyamine in 5 m*M* MOPS (morpholinopropanesulfonic acid) buffer (pH 7) for 1 h at room temperature. The incubation mixture was then layered on 3 mL of 1.5*M* sucrose in the same buffer and centrifuged at 3000*g* for 5 min.

The labeled polyamine/heparin–sepharose complex was collected at the bottom of the tube and the radioactivity content of the pellet was determined, after digestion with 0.5 mL BTS-450, by scintillation counting. Adsorption of polyamines to glassware is a well-known phenomenon (*15*) and we took the maximum care to minimize and quantify it. We used siliconized glassware and, at the lowest concentrations of polyamines tested, less than 1% of the polyamine adsorbed both to the test tube and to an amount of sepharose CL-6B comparable to the sepharose component of the heparin–sepharose resin. At the same concentration, generally, the amount of labeled polyamine specifically bound to heparin–sepharose was 80% of the labeled polyamine in the reaction mixture. Thus is our experimental system the aspecific adsorption is less than the experimental error.

The concentration dependence of the binding of polyamines to heparin–sepharose is generally rather complex. To extract meaningful information from such data the methods described by Scatchard et al. (*16*) have been used. These methods were modified and extended to treat the special circumstances encountered in the present study according to the methods of data analysis given by Schreier and Schrimmel (*17*). Errors in the association constants are estimated to be within ±50%

RESULTS

Spermine Binding to Heparin–Sepharose

Figure 1 (a) gives a Scatchard plot for the binding of spermine to heparin–sepharose. The Scatchard plot shows a markedly nonlinear curve characterized by a well-pronounced maximum. A maximum in the Scatchard plot is characteristic of a system showing positive cooperativity.

The abscissa intercept gives an estimate of the total number of binding sites on heparin–sepharose, which is about 20 nmol spermine bound. The amount of heparin–sepharose in the assay mixture is about 90 nmol ionic sites. Thus a spermine molecule would bind a maximum of 90/20 = 4.5 consecutive anionic sites on the heparin lattice. This is only a rough estimate of the stoichiometry of the complex since heparin may be

considered a polymer without discrete binding sites. Thus the total number of anionic sites occupied by spermine is not simply the ratio of the number of anionic sites in the heparin molecule divided by the number of molecules of spermine bound. In fact, at a low degree of saturation, each spermine molecule will be presented with a large number of possible binding sites, each of which is independent and equivalent. However, as the degree of saturation increases, some of the residual binding sites are found in linear arrays not large enough to form a binding site (*18*). In Fig. 1 (b) is reported the Hill treatment of the data (*19*). The slope of the plot is the Hill coefficient, which is two, and gives a measure of the degree of interaction. The apparent value of the binding constant is the inverse of the half saturating ligand concentration (0.71 μM) and it amounts to $1.41 \times 10^6\ M^{-1}$.

Spermidine Binding to Heparin–Sepharose

Figure 2a shows the Scatchard plot of spermidine binding to heparin–sepharose. It clearly indicates a asymptotic bell-shaped character. This case is encountered when the macromolecule has one class of interacting sites together with one or more classes of independent sites.

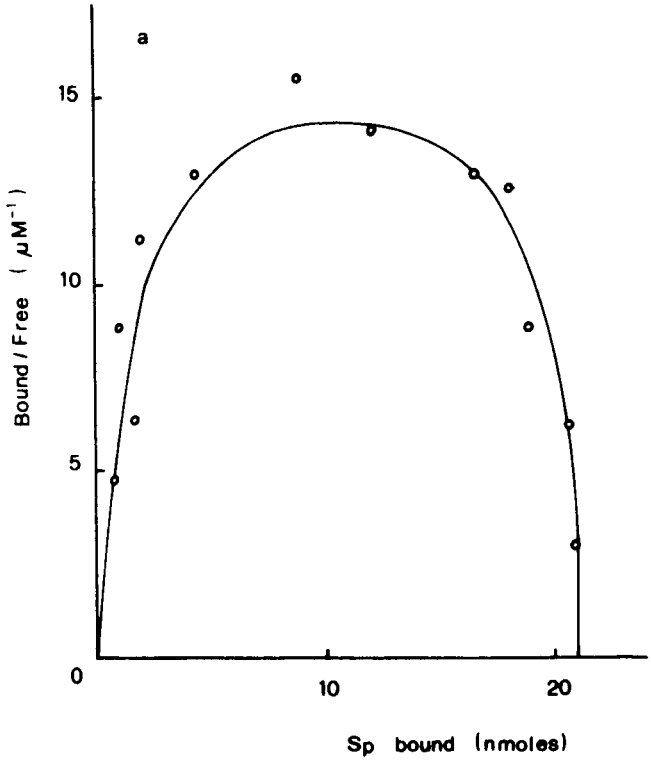

Fig. 1. Binding of spermine to heparin–sepharose: (a) Scatchard plot of the binding data. The points are experimental data obtained as described in the Materials and Methods section.

Figures 2b and 2c give plots of the cooperative phase and independent site phase, respectively, of the Scatchard plot on the left. These data were obtained by decomposing all the available data of the plot shown in Fig. 2 (a). Nine interacting sites are found with an apparent binding constant of $1.56 \times 10^6\ M^{-1}$ and an interaction coefficient of 2. Thirty strong, non-interacting sites are also present with an apparent binding constant of $2 \times 10^5\ M^{-1}$. The apparent site heterogeneity that is experimentally observed as a deviation in the Scatchard plots at a high degree of saturation is accounted by the curvation of the plot expected for the homogenous lattice (*18*). To completely titrate heparin–sepharose, spermidine should cover three consecutive anionic sites of heparin in the interacting sites, two anionic sites in the strong non-interacting sites.

Putrescine Binding to Heparin–Sepharose

Figure 3 shows the Scatchard plot of putrescine binding to heparin–sepharose. The binding isotherm is described by a binding site heterogeneity mechanism and does not arise solely as a consequence of the overlap of potential ligand sites (*18*). Decomposing the data, sixteen strong non-interacting sites are present with an apparent binding constant of $2.6 \times 10^5\ M^{-1}$.

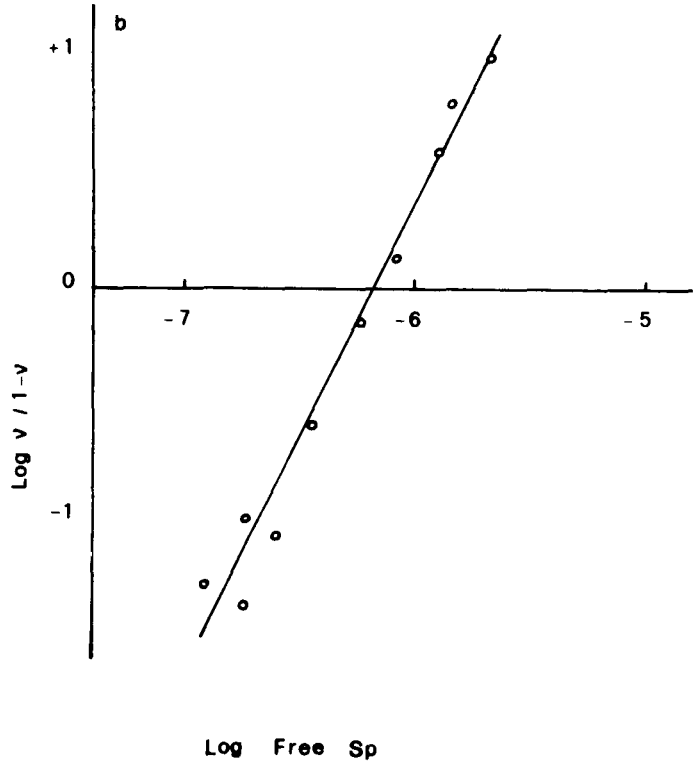

Fig. 1. (b) A Hill plot of the same data of Fig. 1a.

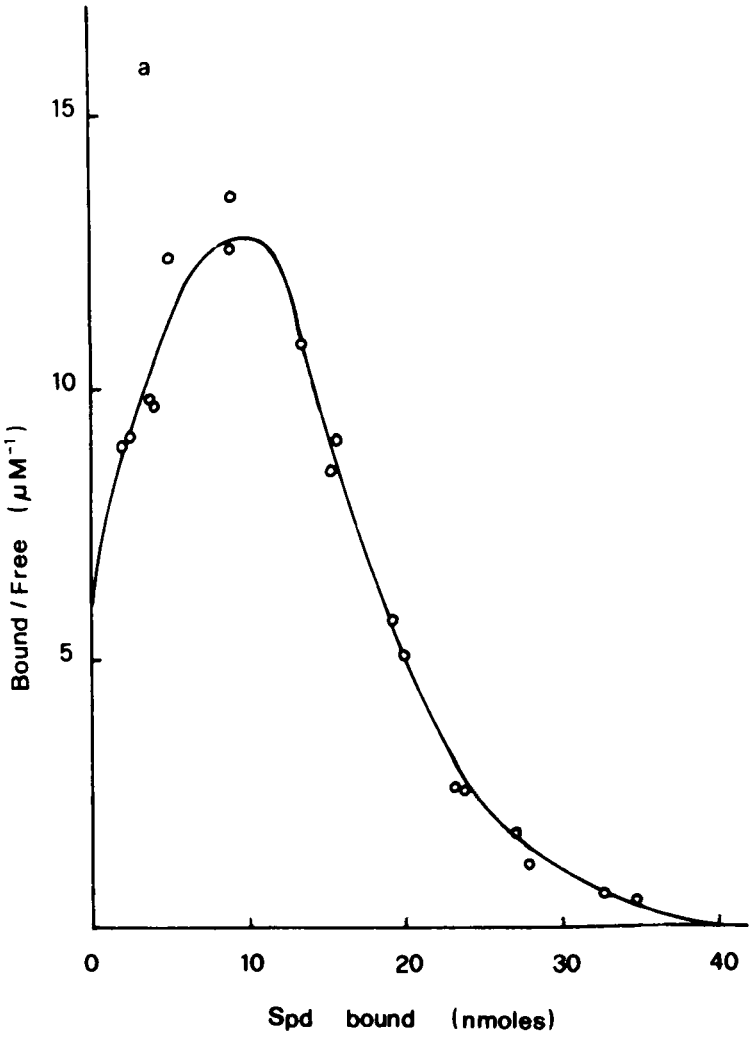

Fig. 2. Binding of spermidine to heparin–sepharose: (a) Scatchard plot of the binding data. The points are experimental data obtained as described in the Materials and Methods section.

For the weak independent binding sites, we estimate that the number of binding sites is about sixty with an apparent binding constant of $3.2 \times 10^3\ M^{-1}$. The homogenous heparin lattice would be completely saturated if putrescine covered two anionic sites in the strong non-interacting sites and one anionic site in the weak non-interacting sites.

DISCUSSION

Major objectives of our study were to assess modes of interaction between polyamines and heparin–sepharose and to determine formation constant for the polyamine/heparin–sepharose complexes. Interaction between polyamines and heparin–sepharose are variable and multiple.

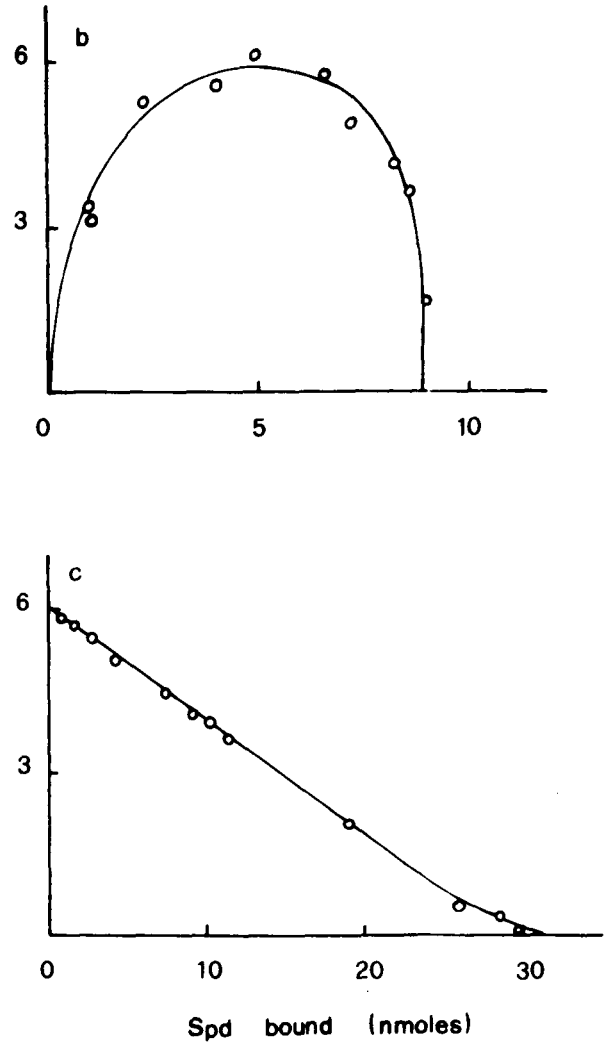

Fig. 2. (b) Scatchard plot of the cooperative phase of the total Scatchard plot given in Fig. 2(a). The curve is calculated as described in the text; (c) Scatchard plot of the independent sites phase of the total Scatchard plot given in Fig. 2(a). The curve is calculated as described in the text.

Reasonable evidence is established for the existence of three general classes of binding sites/mechanisms. These three classes are: strong interacting, strong independent, and weak independent binding mechanisms. The cooperative mechanism is achieved only by spermine and spermidine, with an apparent association constant of $1.41 \times 10^6\ M^{-1}$ and $1.56 \times 10^6\ M^{-1}$, respectively. Spermidine, however, is less efficient than spermine in recognizing and/or generating cooperative sites. Apparently whatever distribution of anionic charges on heparin allows spermine to bind in a cooperative way while spermidine has only less than 50% of spermine possibilities of contracting cooperative binding. This difference is evident at very low ionic strength, while in the presence of

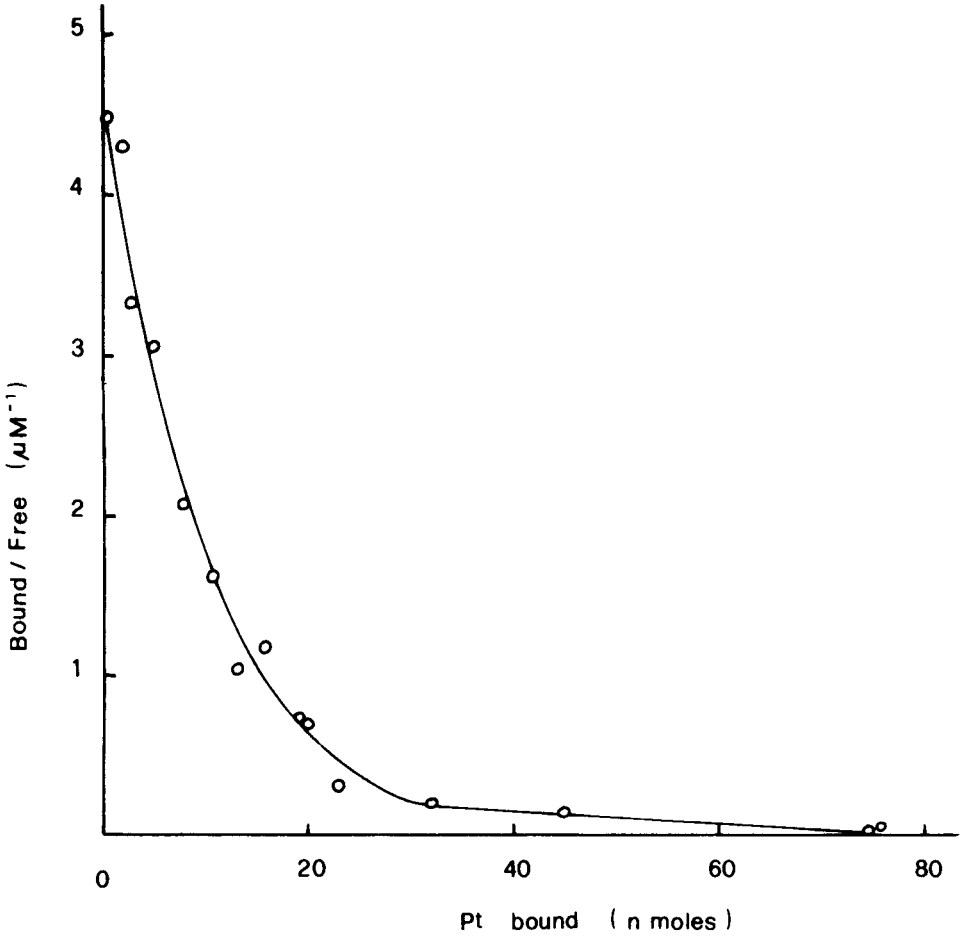

Fig. 3. Binding of putrescine to heparin–sepharose. Scatchard plot of the binding data. The points are experimental data obtained as described in the Materials and Methods section.

increasing concentrations of NaCl (> 20 m*M*), spermine has a binding pattern similar to spermidine (data not shown). NaCl probably binds some anionic sites of heparin lowering the number of the binding sites that interact cooperatively with the whole four charges of spermine. Spermine in this ionic conditions interact with these sites with three charges like spermidine in a strong non-interacting mode. The apparent association constant for this binding mechanism is $2 \times 10^5\ M^{-1}$.

Putrescine interact with heparin–sepharose with a similar binding mechanism (apparent association constant, $2.6 \times 10^5\ M^{-1}$), but the number of favorable arrays of charges is very low (16 binding sites). The total titration of heparin molecule is accomplished by putrescine by a weak interacting mechanism with an apparent association constant of $3.2 \times 10^3\ M^{-1}$. The key to the polyamines binding to heparin–sepharose may be in the fact that they are polyalkyl amines that contain no bulky subtituent or rigid bonds. Such molecules have a considerable amount of configurational flexibility and thus can generate

many different spatial distributions for their charged groups. This property enables them to adapt their configuration to complement and neutralize a wide variety of arrangements of anionic charges generated by a polyelectrolyte such as heparin.

This probably is the basis of the high capacity of heparin–sepharose for polyamine. For the same reason the data on polyamine binding to heparin–sepharose do not completely fit the characteristic of a lattice binding system since a different polyamine configuration exposing a lower number of charges is generated. This binds a linear array of anionic charges of the heparin molecule that is not large enough to form a stronger binding site.

In their complex, the experiments reported give a molecular basis and a quantification of the binding of polyamines to heparin–sepharose. Heparin–sepharose proved to have a high affinity for polyamines and also a very high capacity. For these reasons, heparin–sepharose should find wide application to study the complexation of polyamines with naturally occurring polyanionic compounds (i.e., phospholipid vesicles), and to remove the polyamines from different biological materials (i.e., serum, DNA, subcellular organelles, and so on). Both these types of application of heparin–sepharose in the study of polyamines biochemistry are presently under investigation.

ACKNOWLEDGMENTS

This work was supported by grants from Italian CNR. We are grateful to Professor A. M. Sechi and Professor C. A. Rossi for their interest in this research and for providing all facilities.

REFERENCES

1. Mager, J. (1959), *Biochim. Biophys. Acta* **36,** 529.
2. Kobayashi, Y. (1962), *Arch. Biochim. Biophys.* **96,** 20.
3. Cohen, S. S. (1971), *Introduction to the Polyamines,* Prentice-Hall, Englewood Cliffs, New Jersey.
4. Bachrach, U. (1973), *Function of Naturally Occurring Polyamines.* Academic Press, New York.
5. Russell, D. H. (ed.) (1973), *Polyamines in Normal and Neoplastic Growth,* Raven Press, New York.
6. Tadolini, B. (1980), *Biochem. Biophys. Res. Commun.* **92,** 598.
7. Pelkonen, O., and Pasanen, M. (1981), *Biochem. Pharmacol.* **30,** 3254.
8. Arnold, E. A., Yawn, D. H., Brown, D. G., Wyllie, R. C., and Coffey, D. S. (1972), *J. Cell Biol.* **53,** 737.
9. Cook, R. T., and Aikawa, M. (1973), *Exp. Cell Res.* **78,** 257.
10. Smith, M. R., and Cook, R. T. (1977), *Biochem. Biophys. Res. Commun.* **74,** 1475.
11. Tadolini, B., and Cabrini, L. (1983), *Appl. Biochem. Biotechnol,* **8,** 203.
12. Tadolini, B., Cabrini, L., and Sechi, A. M. (1984), *Appl. Biochem. Biotechnol.* **9,** 153.

13. Stone, A. L., and Bradley, D. F. (1967), *Biochim. Biophys. Acta* **148,** 172.
14. Gianazza, E., and Righetti, P. G. (1978), *Biochim. Biophys. Acta* **540,** 357.
15. Ackermann, D., and Wasmuth, W. (1939), *Hoppe-Seyl. Z.* **259,** 28.
16. Scatchard, G., Coleman, J. S., and Shen, A. L. (1957), *J. Amer. Chem. Soc.* **79,** 12.
17. Schreier, A. A., and Schimmel, R. R. (1974), *J. Mol. Biol.* **86,** 601.
18. McGhee, J. D., and von Hippel, P. H. (1974), *J. Mol. Biol.* **86,** 469.
19. Hill, A. V. (1910), *J. Physiol. (London),* 90 iv.