Biochimica et Biophysica Acta, 427 (1976) 392–409

BBA 37314

THE SOLUTION BEHAVIOR OF THE BOVINE MYELIN BASIC PROTEIN IN THE PRESENCE OF ANIONIC LIGANDS
BINDING BEHAVIOR WITH THE RED COMPONENT OF TRYPAN BLUE AND SODIUM DODECYL SULFATE

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(Received July 7th, 1975)

SUMMARY

The interaction of the azo dye (2,3′-dimethyldiphenyl-7-azo-8-amino-1-napthol 3,6-disulfonic acid (TBR)) and sodium dodecyl sulfate with the bovine myelin basic protein has been studied using absorbance, circular dichroism and 220 MHz PMR spectroscopy. Additional analyses of the binding reaction were carried out using light scattering, ultracentrifugal and electrophoretic techniques. A procedure for preparing pure TBR was developed. A modified structure for this synthesized TBR has been suggested.

The mechanism of TBR binding to the myelin basic protein was found to be metachromatic. In addition, the interaction of TBR with the basic protein which gives rise to aggregation of the dye bound species was found to be analogous to the model proposed by Schwarz, G. and Seelig-Löffler, A. ((1975) Biochim. Biophys. Acta 379, 125–138) to explain the binding of acridine orange with poly(α-L-glutamic acid). PMR spectral analyses suggested that arginine residues provide the majority of primary sites of attachment on the basic protein for TBR.

The effect of sodium dodecyl sulfate binding with the bovine myelin basic protein was found to induce a minimal change in the conformation of the protein. The induction of only about 20% α helial structure could be demonstrated and the binding was reversed by raising the solution temperature to 73 °C.

The difference in the observed behavior of basic protein arising from TBR binding as opposed to the binding of sodium dodecyl sulfate is viewed as resulting from two different binding mechanisms. The binding behavior of TBR is primarily a consequence of charge-charge interaction while the binding effects of sodium dodecyl sulfate are a consequence of hydrophobic interaction. The sodium dodecyl sulfate binding acts as a shield which limits charge-charge interaction in the basic protein molecule thus preventing aggregate formation while TBR imposes no such restraints.

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INTRODUCTION

Studies of dye protein interactions have been used for many years to obtain information related to protein structure and function. The interaction of the dye trypan blue with myelin proteins was detected in the course of a histochemical study of myelin by Adams and Bayliss [1]. These investigators claimed that trypan blue exhibited a specificity towards the myelin basic protein and that the dye could be used as a marker for this protein under controlled staining conditions. The specificity of trypan blue for the myelin basic protein under conditions reported by Adams and Bayliss [1] was subsequently challenged by Dickerson and Aparicio [2]. Both groups of investigators appeared to be unaware of a study by Kelly [3, 4] that had shown commercial preparations of trypan blue to contain another dye species that was red. This red component was reported to selectively stain nuclei that were pycnotic, degenerating or no longer undergoing division. The affinity of such nuclei for this red dye was attributed to the nucleolar basic proteins. It appeared reasonable to us that the controversy of trypan dye specificity for the myelin basic protein might be amenable to resolution by a study of the binding behavior of purified myelin basic protein with the purified red component of trypan blue. An additional contribution to be derived from such a study would be the elucidation of some aspects of lipid binding to this protein.

The structure of trypan blue is known [5], but the structure of the red contaminant was not known when this study was initiated. Our study permitted an assignment of a structure, differing slightly from the one proposed by Dijkstra [6]. The structure for trypan blue (TBB) and the red component (TBR) are given in Fig. 1. Since the red component was found to give a more selective and tractable interaction with the basic protein, we have focused this study on the basic protein TBR system.

The interaction of sodium dodecyl sulfate with human myelin basic protein had been reported by Anthony and Moscarello [7]. Since sodium dodecyl sulfate has amphipathic properties with some similarity to the properties of TBR and therefore should exhibit similarities in its interaction with the bovine basic protein, we sought to further define this parallelism.

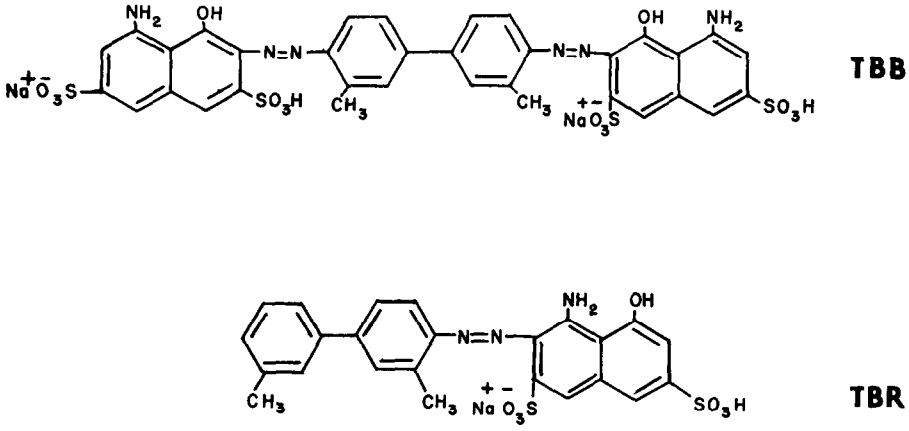

Fig. 1. Structural formulas for trypan blue red (TBR) and trypan blue blue form (TBB).

MATERIALS AND METHODS

Bovine myelin basic protein was prepared by the procedure reported previously [8].

Preparation and purification of TBR

This azo dye (2,3′-dimethyldiphenyl-7-azo-8-amino-1-napthol 3,6-disulfonic acid) was prepared by the method of Hartwell and Fieser [9] incorporating the following modifications. The H-acid (Eastman Organic) was purified by gel filtration on Sephadex G-10 using deairated distilled water for elution. The diazotization of *ortho*-tolidine (Fischer) with nitrous acid was carried out under conditions that permitted an excess of amine at all times. The control of free nitrous ion by amine was found to be preferable to the use of urea as a means of removing excess nitrous ion. The coupling reaction of H-acid and *othro*-tolidine was allowed to proceed in 85 percent ethanol at an apparent pH of 6.0. The reaction was monitored at periodic intervals by means of thin-layer chromatography on Silica gel G strips. The strips were developed in acetone/methanol/ethanol (2:2:1, v/v/v) and at the appearance of TBB, the reaction mixture was rapidly taken to dryness on a rotary evaporator. This procedure maximized the amount of TBR formed. Separation of TBR was achieved by extracting the dry residue repeatedly with the acetone/methanol/ethanol solvent and concentrating the extract. The concentrate was chromatographed on Sephadex LH-20, the TBR eluted with acetone/methanol/ethanol mixture and the uncoupled reactants which eluted first were discarded. The red-violet fraction was collected, the eluate concentrated, and the process repeated twice. The material, chromatographed three times, was taken to dryness on a rotary evaporator and the product obtained was dried at 60 °C in a vacuum oven. The TBB that was retained on the columns was eluted with methanol, taken to dryness, and dried in the same manner as with TBR.

TBR analysis for $C_{24}H_{20}N_3S_2O_6Na \cdot 4H_2O$

Calculated: C = 46.42, H = 4.14, N = 6.62

Found: C = 46.45, H = 4.35, N = 6.77

The λ_{max} at 550 has an E/I_{em} = 502 in water and E/I_{em} = 450 in 0.1 M NaOH.

Light scattering

Light scattering measurements were obtained with a Sofica model 42000 (701) instrument equipped with a Heath Co. digital multimeter model IM-102 connected in tandem with a Hewlett-Packard Mosely Autograf strip chart recorder.

Absorption of ultraviolet and visible light

The absorbance and difference spectra were obtained with a Beckman ACTA III spectrophotometer.

Circular dichroism

CD spectra were obtained with a Jasco ORD/CD-5 instrument modified to the Sproul SS-20 configuration.

Flow electrophoresis

Analysis of the TBR·basic protein complex by an electrophoretic procedure

was carried out using a Brinkman model FF continuous flow electrophoresis apparatus. Buffers were 0.04 M sodium acetate pH 4.8 for the running buffer and 0.1 M sodium acetate, pH 4.8, for the electrode vessels. Solutions of different TBR to protein ratios were subjected to electrophoresis. The fractions obtained were analyzed by the same procedures described in the following section on the analysis of dye·protein complexes prepared by titration of protein with dye.

Analyses of TBR bound to basic protein

Solutions of TBR and basic protein were prepared with 0.04 M acetate buffer, pH 4.8, such that each solution contained a known but different dye to protein ratio. The solutions were kept at room temperature for 2 h and then centrifiuged for 1 h at 35 000 $\times$ g. The supernatant was decanted from the pellet and analyzed for TBR and protein content. A measured aliquot of the supernatant was removed and a measured volume of 0.2 M NaOH was added to give a final solution that was 0.1 M in NaOH. This dissociated the soluble dye·protein complex and the absorbance at 550 nm was determined. The dye concentration was calculated from the absorbance at 550 nm using the extinction coefficient for the dye ($E^{1\%}_{1cm} = 450$). The dye solution obeyed Beer's law over the range of $2.5 \cdot 10^{-6}$ to $5.0 \cdot 10^{-3}$ g/100 ml. Protein concentrations were determined in the presence of TBR using the microbiuret procedure reported by Janatova et al. [10] calibrated with bovine myelin basic protein.

Sodium dodecyl sulfate-basic binding

Sodium dodecyl sulfate/basic protein solutions of desired sodium dodecyl sulfate to protein ratios for CD and PMR studies were prepared by mixing equal volumes of a known concentration of basic protein with sodium dodecyl sulfate in either 0.05 M (pH 4.8) acetate buffer or 0.05 M (pH 7.4) phosphate buffer.

220 MHz proton magnetic resonance

PMR spectra were obtained on a Varian Associates 220 MHz high resolution spectrometer. The polarizing field of 52 000 gauss was furnished by a superconducting solenoid. The temperature in the sample zone was maintained at $\pm$ 1 °C of the desired temperature by a stream of nitrogen.

RESULTS

Synthesis of TBR

The evidence in the literature and our thin-layer chromatographic analysis of commercial trypan blue indicated that the dye contained a number of impurities. Therefore, it was necessary to undertake the synthesis and purification of TBR from TBB.

The synthesis that was used and the method of purification that was devised yielded sufficient quantities of the purified dye for use in binding studies. The absorbance spectrum of our preparation of TBR is shown in Fig. 2. This spectrum agrees with the spectrum reported by Dijkstra [6] for a red component isolated from trypan blue. The elemental analysis, the conditions of synthesis, and NMR data are consistent with the structure suggested in Fig. 1. Our structure differs from that suggested by Dijkstra [6] in that the position of the NH_2 and OH functions have been reversed. This

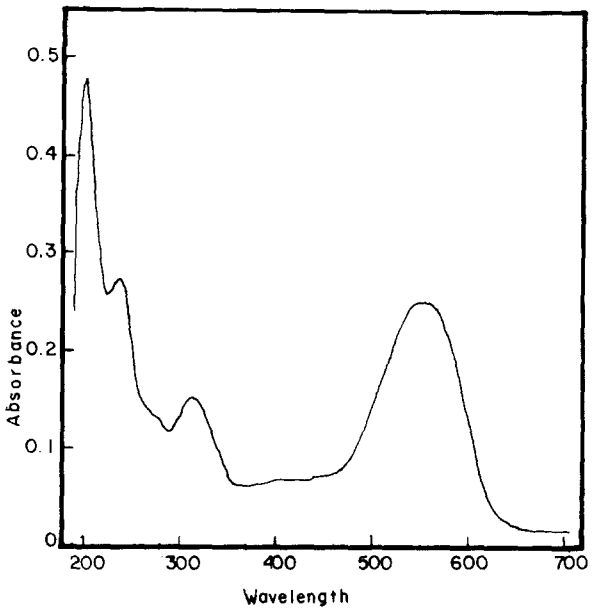

Fig. 2. The absorbance spectrum of TBR in water solution (concentration 0.0007%).

change was made on the basis of the known coupling reactivity of H-acid in the pH region of 5 to 7 [11]. Keeping the coupling reaction at 4 °C, pH 6, and allowing the reaction to proceed for 30 min favored TBR formation. If the reaction was run at room temperature, pH 9, for 4 h, the formation of TBB was favored.

TBR binding

The binding of TBR to basic protein was followed spectrophotometrically. The λ_{max} at 550 nm of the free dye is blue shifted to 505 nm when the dye is bound to protein (Fig. 3). Also, the binding to basic protein is accompanied by a small red shift of the free dye maximum at 313 nm to a maximum at 318 nm. The difference spectrum of TBR bound to basic protein is shown in Fig. 4. An isobestic point is present with curves 1 through 9. Curves 10 and 11, obtained from solutions having dye to protein ratios of 16.0/1 and 17.6/1 respectively, exhibit an incremental shift of the 565-nm λ_{max} toward a λ_{max} of 550 nm. Both curves diverge from the 521-nm isobestic point. The difference spectrum shows that only after reaching a TBR to protein ratio greater than 15 to 1 is there any evidence for unbound TBR. A more accurate assignment of the number of moles of TBR bound per mole of protein could not be made. TBR is firmly bound by dialysis and ultrafiltration membranes. Hence the use of these materials in experiments designed to elucidate the binding behavior of TBR to basic protein was not feasible.

The binding of TBR to the bovine basic protein was found to be accompanied by an extrinsic Cotton effect both in the visible and near ultraviolet regions of the circular dichroic spectrum. Fig. 5 shows the data resulting from the bovine basic protein in an equal molar concentration of TBR ($6 \cdot 10^{-5}$ M). The observed biphasic Cotton effect is centered about 497 nm in the visible region of the spectrum and a

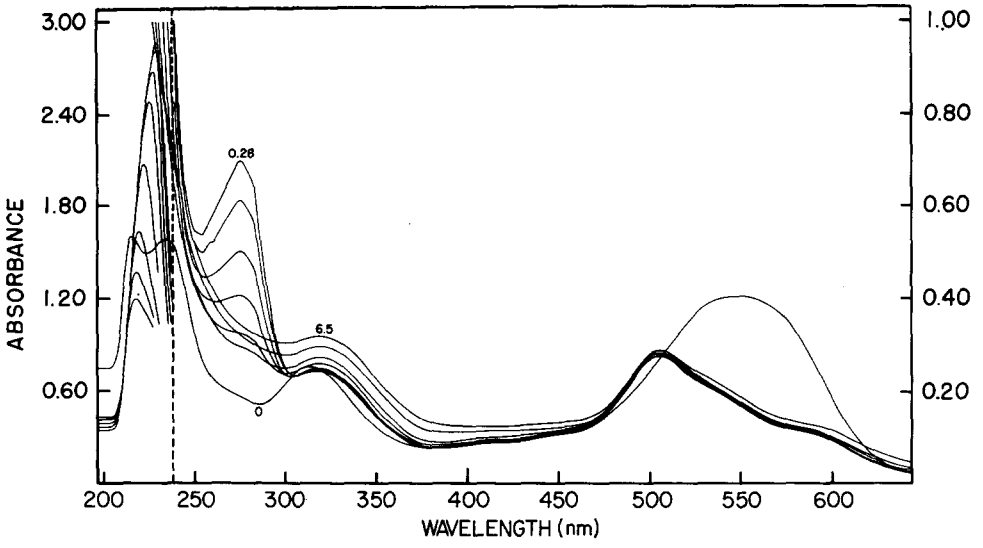

Fig. 3. The absorbance spectra after the addition of myelin basic protein to TBR. The TBR/protein molar ratios are 0, 0.28, 0135, 0.47, 0.70, 1.4, 2.7, 4.8 and 6.5. Acetate buffer, 0.05 M, pH 4.9.

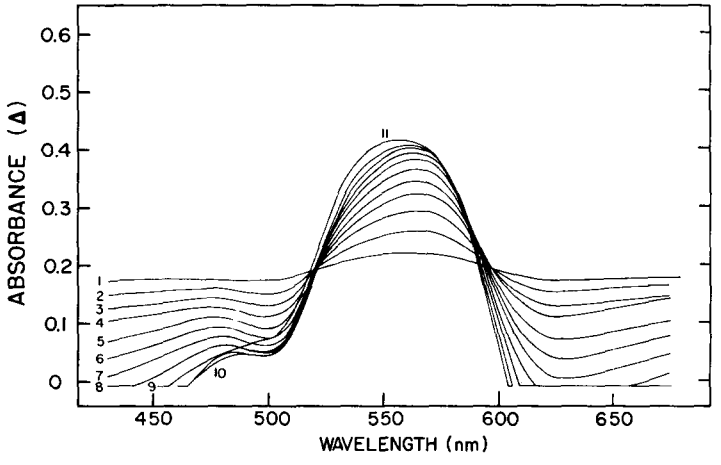

Fig. 4. Different spectra obtained by titrating a $5 \cdot 19^{-9}$ M myelin basic protein solution with 50 μl increments of an $8.1 \cdot 10^{-9}$ M TBR solution. Acetate buffer, 0.05 M, pH 4.8. Curves 1–11 contain the respective TBR/basic protein molar ratios: 1.6, 3.2, 4.8, 6.4, 8.0, 9.6, 11.2, 12.8, 14.4, 16 and 17.6.

weaker biphasic curve is centered about 315 nm in the near ultraviolet region. Neither TBR nor the bovine basic protein showed any optical activity in either of these regions. In the far ultraviolet region no evidence of a conformational change in the BP could be detected from CD spectra obtained over a range of TBR/BP ratios equivalent to that examined in Fig. 3. When CD data were obtained in the presence of trifluoroethanol or with the addition of 8 M urea, the observed Cotton effects were abolished:

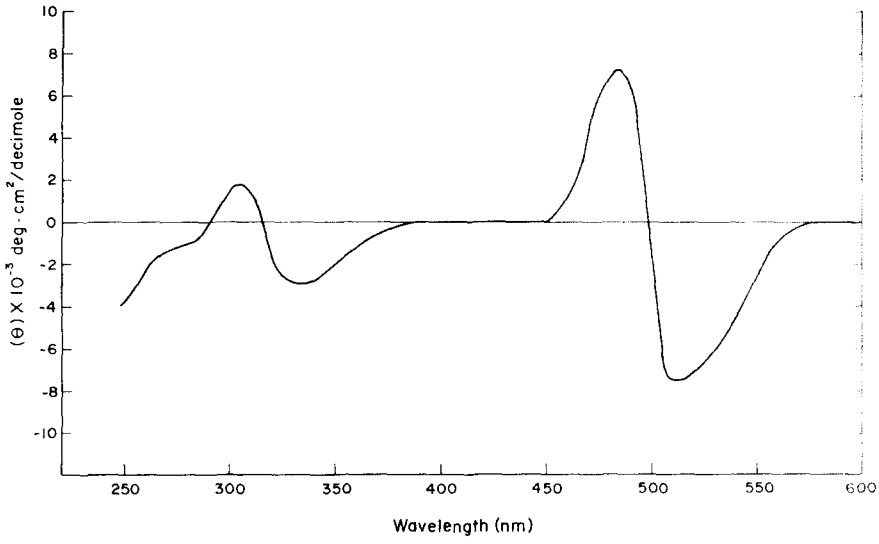

Fig. 5. The CD spectrum obtained from an equimolar concentration of TBR and the basic protein ($6 \cdot 10^{-5}$ M).

It was thus of interest to determine the composition of the TBR·basic protein complex that gave rise to this extrinsic optical activity. In an attempt to quantitate these binding forms, electrophoretic methods were used to study the binding of TBR to basic protein but were found to be complicated by a number of factors. In continuous flow electrophoresis experiments, extensive aggregation of the TBR·protein complex was observed. This effect was the result of the high concentrations of protein and dye required to offset the large dilution that occurred during the electrophoretic separation. Despite the difficulties encountered, it was possible to ultilize the flow electrophoresis system to achieve a partial separation of the various TBR·protein complexes and to analyze these complexes for their stoichiometry and extrinsic optical activity. In a typical experiment 44 fractions were separated and of these, fractions 1–35 contained traces of free TBR. Fractions 40–44 contained free basic protein. Fractions 36–39 contained the TBR·protein complexes. Analysis of these fractions yielded TBR to basic protein ratios of 0.121, 0.176, 0.280, and 0.290. The first three of these fractions exhibited extrinsic Cotton effects with a crossover at 505 nm (Fig. 6). This uniquely corresponded to the absorption maximum shift from 550 to 505 nm when the dye is presumably asymmetrically bound (Fig. 7). Fraction 39 showed a complex absorption spectrum that no longer contained the prominant 505 nm shift. The slight increase in absorbance beyond 600 nm is interpreted as indicative of the presence of aggregation effects. Coinciding with these effects is the loss of optical activity with this fraction. From the increase of the TBR/basic protein ratios as the fractions move toward the cathode, it appeared that aggregation effects were still evident since the binding of TBR by the basic protein would neutralize positive charges on the protein and migration of the fractions with high dye to protein ratios would be expected to behave in the reverse order to that observed.

Quantitative analyses of the dye protein complex that remained in solution

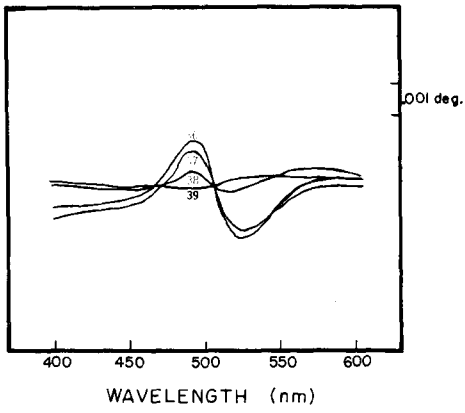

Fig. 6. The CD spectra in the visible region obtained from four fractions (36–39) of a TBR/basic protein mixture separated by flow electrophoresis. The TBR/basic protein molar ratios were determined to be 0.121, 0.176, 0.280 and 0.290, respectively.

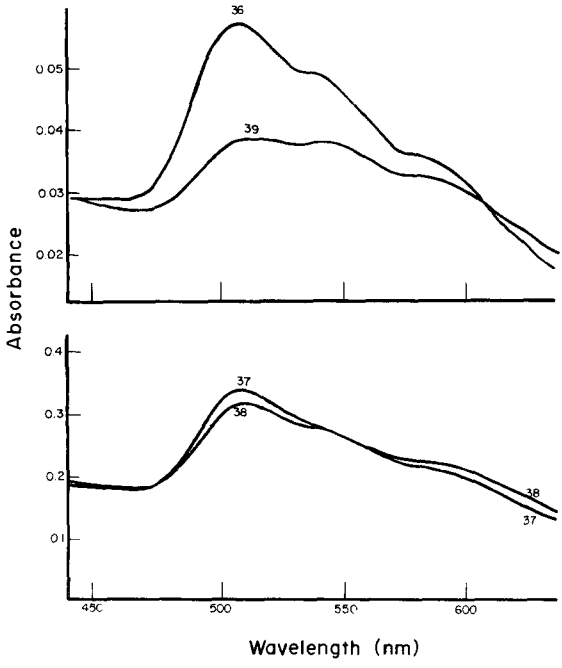

Fig. 7. The absorbance spectra of the TBR/basic protein fractions described in the legend to Fig. 6.

were obtained when basic protein was titrated with TBR and vice versa. The data are presented in Table I. The analysis was achieved in the following way: Solutions containing different TBR to protein ratios were prepared and subjected to centrifugation at 30 000 $\times$ g for 2 h. The supernatant was then analyzed for dye and protein content. The following symbolism is used to represent the various TBR and basic protein parameters: D_o is the initial dye concentration in solution; D_a, the dye concentration

TABLE I

TBR BASIC PROTEIN LOW SPEED CENTRIFUGATION DATA

o, initial concentration of the component in solution prior to centrifugation; a, concentration of the component in the complex; s, concentration of the component in solution after centrifugation.

D_o*	D_a*	D_s*	P_o*	P_a*	P_s*	D_o/P_o	D_a/P_a	D_s/P_s
1.51	1.40	0.113	2.06	0.148	1.91	0.733	9.46	0.059
1.51	1.45	0.063	0.74	0.120	0.624	2.04	12.1	0.101
1.51	1.46	0.050	0.42	0.162	0.257	3.60	9.01	0.195
0.96	0.88	0.078	1.38	0.109	1.27	0.696	8.07	0.061
1.91	1.82	0.094	1.38	0.161	1.22	1.38	11.3	0.077
2.86	2.76	0.100	1.38	0.180	1.20	2.07	15.3	0.083
5.69	5.56	0.134	1.38	0.520	0.855	4.12	10.7	0.157

* $\cdot 10^5$ M.

in the dye·protein aggregate; D_s, the dye concentration remaining in solution after dye·protein aggregate was removed by centrifugation; P_o, the initial protein concentration in solution, P_a, the protein concentration on the dye·protein aggregate; and P_s, the protein concentration remaining in solution after the dye·protein aggregate was removed by centrifugation. Analysis of these results revealed that more than 90% of the TBR was always utilized in formation of the insoluble complex, regardless of how much excess protein was present. To aid in elucidating this effect, light scattering data as a function of time and at an external angle of 90° were obtained from the same TBR to bovine basic protein molar ratios. Data were obtained under conditions such that at pH 4.8, the protein had a net charge of 30, at pH 9.0 the net charge was 14 and at pH 11.0 the protein net charge was 9. It has already been demonstrated that this protein tends to self associate when the charge is reduced at pH values ≥ 7 [8]. However the light scattering intensities that were observed when equal amounts of TBR ($1.8 \cdot 10^{-6}$ M) were added to the same protein concentrations at each of these pH values, yielded dramatic increases that were a minimum of 5-fold greater in intensity than the I_{90} intensity observed under the same conditions in the absence of TBR. Fig. 8 shows aggregate formation for the same TBR/bovine basic protein ratios (0.160) as a function of time at these three pH values. It is evident that a moderately slow increase in aggregate formation results when the net charge of the protein is 30 at pH 4.8 as opposed to the dramatic initial increase in aggregate formation and subsequent reduction in I_{90} intensity at the pH 9 and 11 values.

The 220 MHz PMR spectra of the dye·protein complex in DMSO-d_6 are shown in Figs. 9 and 10. A clearly discernable temperature effect at 53 °C when compared with data obtained at 5 °C was found. By comparing the calculated low and high field spectra obtained from the basic protein alone [8] with the temperature dependence of the TBR protein spectra, it was possible to determine the amino acid residues most perturbed by the bound TBR. In the high field region, a broadening of valine, leucine, isoleucine and threonine resonances is observed at 220, 270 and 320 Hz. A more dramatic broadening of the arginine resonances occurring at 360, 410 and 700 Hz is also seen. The other basic amino acids, lysine and histidine, appear to be perturbed to a much smaller extent. The perturbation of glutamate and aspar-

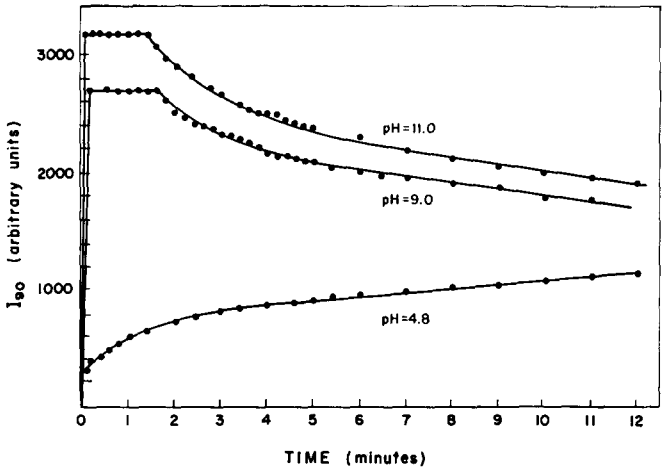

Fig. 8. Time dependent 90° light scattering obtained from the same TBR/basic protein molar ratio (0.16) at pH 4.8, 9 and 11. Protein concentration $5.4 \cdot 10^{-5}$ M, wavelength of the scattered light 436 nm, 0.1 *I* acetate buffer was used at pH 4.8, 0.1 *I* carbonate at pH 9 and 11.

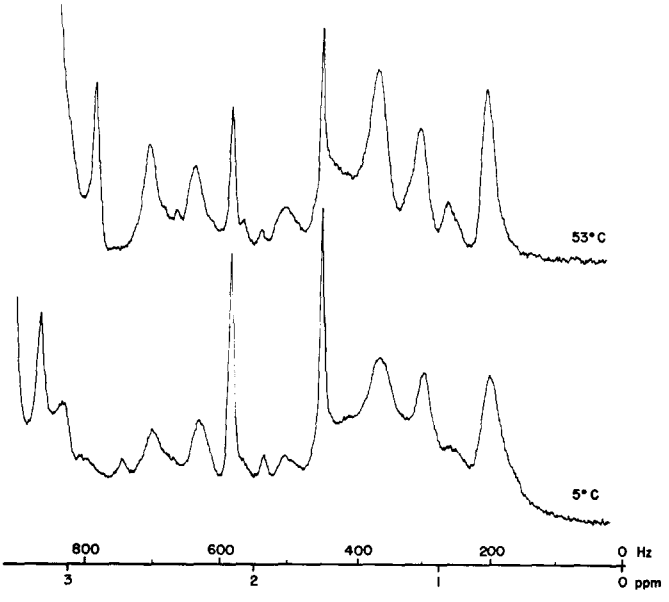

Fig. 9. 220 MHz PMR spectrum of TBR·basic protein in the high field region at 5 °C and 53 °C. The TBR/basic protein molar ratio is 1.6. Solvent is DMSO-d_6. The concentration of TBR is too low to observe proton resonances from the dye molecule itself.

tate residues is readily discernable. Aromatic amino acid residues are also affected by TBR binding. The tyrosine meta proton resonance at 1540 Hz is slightly broadened and the phenylalanine, tyrosine and histidine proton resonances in the region of 1600 Hz are extensively broadened.

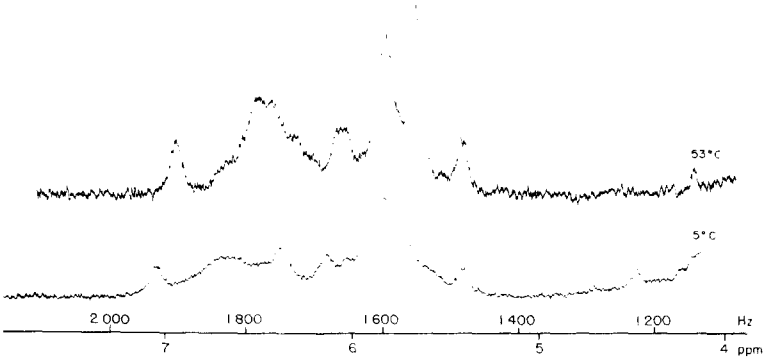

Fig. 10. 220 MHz PMR spectrum of TBR·basic protein in the low field region at 5 °C and 53 °C. The TBR/basic protein molar ratio is 1 6. Solvent is DMSO-d_6.

Sodium dodecyl sulfate binding to basic protein

The interaction of sodium dodecyl sulfate with myelin basic protein was investigated at pH 4.8 and 7.4. At a pH of 4.8, the maximum ratio of sodium dodecyl sulfate to basic protein that could be prepared was 20.4 to 1. At pH 7.4, the maximum sodium dodecyl sulfate to protein ratio was 6.4 to 1. The CD spectra obtained at pH 4.8 and 7.4 are presented in Figs. 11 and 12, respectively. The Cotton effect minima at both pH values are approximately the same but the intensitites differ appreciably. The

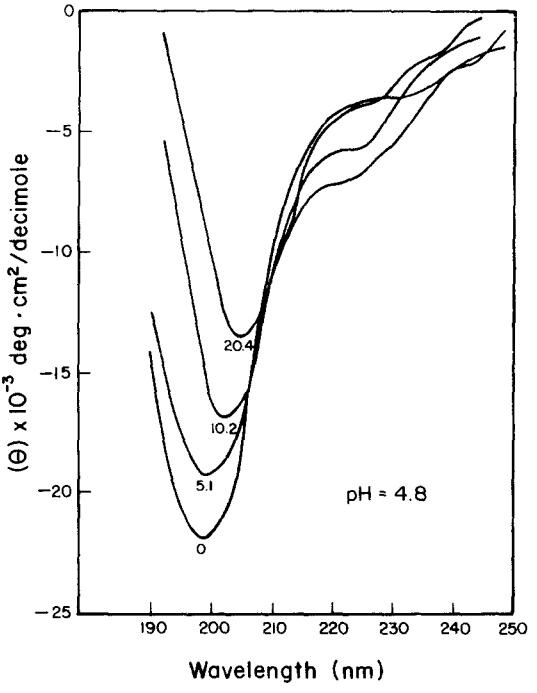

Fig. 11. The CD spectra of myelin basic protein as a function of sodium dodecyl sulfate concentration in acetate buffer, 0.05 M, pH 4.8. The maximum sodium dodecyl sulfate/basic protein molar ratio was 20.4 to 1.

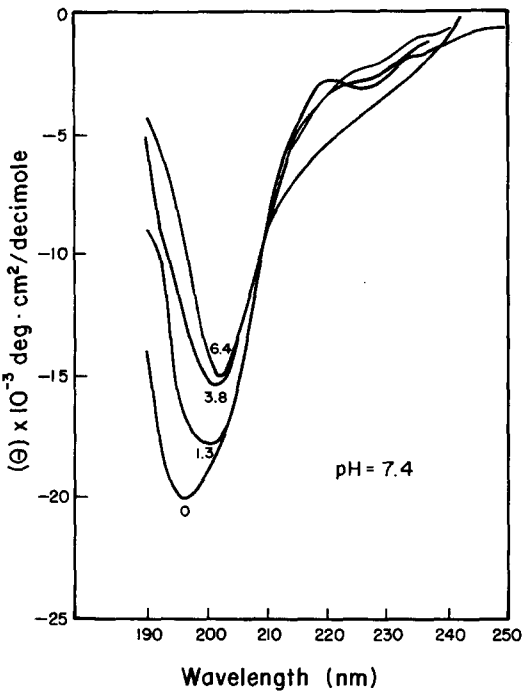

Fig. 12. The CD spectra of myelin basic protein as a function of sodium dodecyl sulfate (SDS) concentration in phosphate buffer 0.05 M, pH 7.4. The maximum sodium dodecyl sulfate/basic protein molar ratio was 6.4 to 1.

shape of the CD curves, the wavelength at which the major minimum appears, and the intensity in the 218–322 nm regions indicate that even in the presence of sodium dodecyl sulfate the helical content is extremely low and, at best, does not exceed 20% (at pH 4.8).

As a correlate to the CD data, the 220 MHz PMR spectra of the basic protein in the presence of varying concentrations of sodium dodecyl sulfate were obtained as a function of temperature and pD. Spectra obtained at a pD of 5 and sodium dodecyl sulfate to protein ratio up to 19.2 to 1 were similar to the spectra obtained at a pD of 7 and a sodium dodecyl sulfate to protein ratio up to 6.4 to 1. The spectra obtained at pD 5 are shown in Fig. 13. The increase in the sodium dodecyl sulfate to protein ratio results in a slight broadening of both the aliphatic and aromatic resonance lines. This line broadening is indicative of the introduction of some small degree of organized structure into the basic protein molecule. The resolution of most of the resonance lines at the highest sodium dodecyl sulfate to protein ratios provides additional support to this interpretation of the data.

The temperature depedence of the sodium dodecyl sulfate protein interaction over the region 31 °C to 73 °C at $pD_c = 7$ is presented in Fig. 14. The resonance lines are sharpened appreciably with increasing temperature, indicating the dissociation of the sodium dodecyl sulfate from the basic protein. The spectrum at 73 °C is almost identical to the spectrum of the protein obtained at room temperature in the absence of sodium dodecyl sulfate.

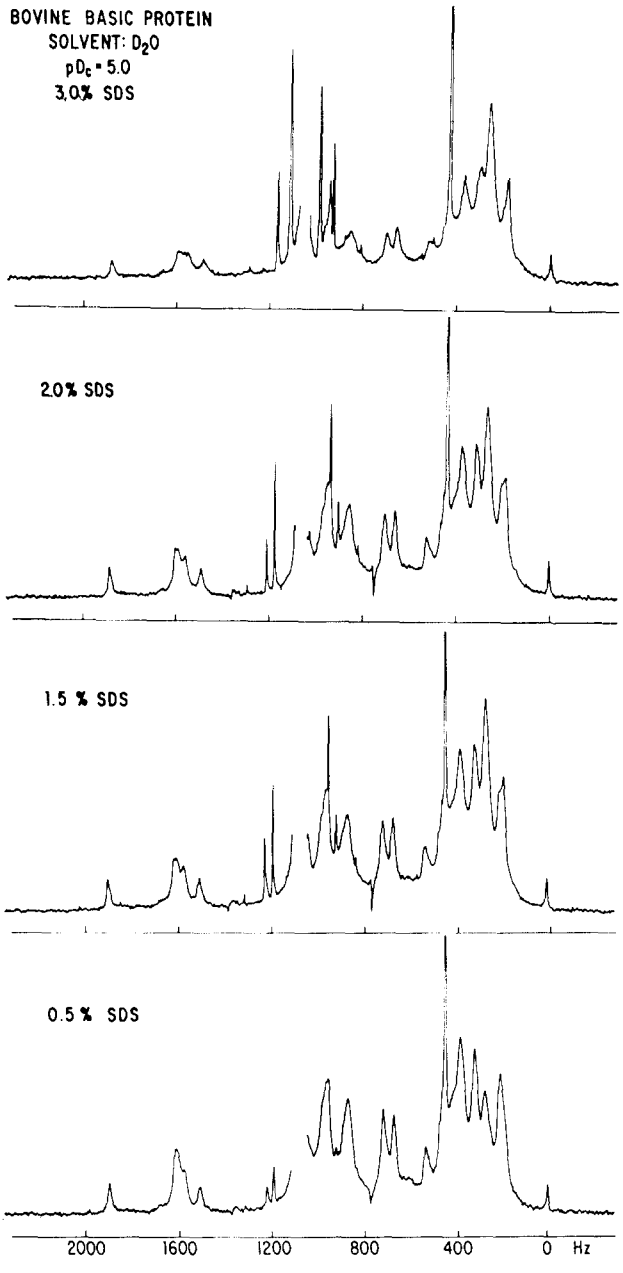

Fig. 13. 220 MHz PMR spectra of myelin basic protein as a function of added sodium dodecyl sulfate. The molar sodium dodecyl sulfate/basic protein ratios are 3.2, 9.6, 12.8 and 19.2, respectively.

DISCUSSION

The results of this investigation have revealed that previous studies of the binding of trypan blue to proteins should be reappraised to take account of the demon-

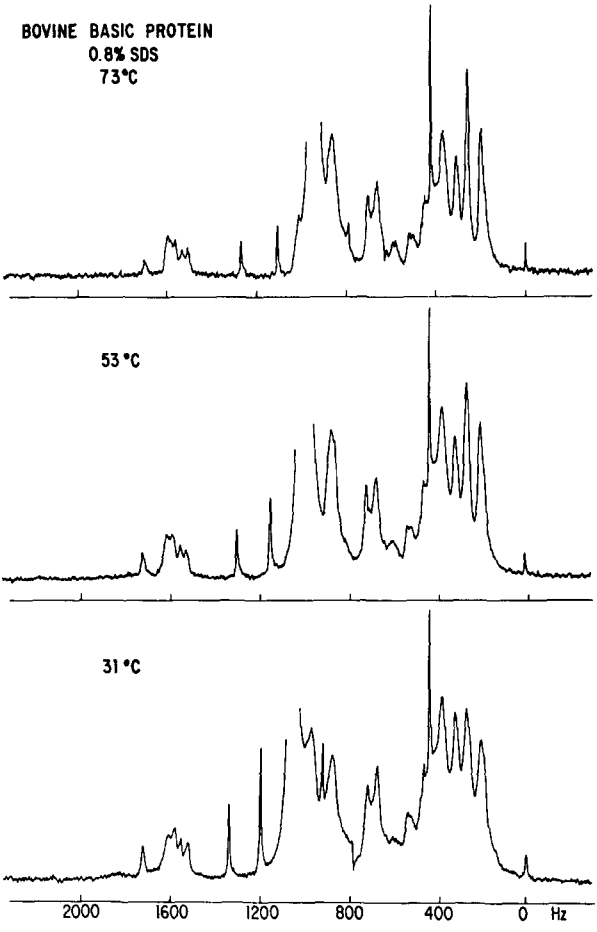

Fig. 14. 220 MHz PMR spectra of myelin basic protein (10% w/v) in the presence of sodium dodecyl sulfate (0.8% w/v) as a function of temperature. The molar sodium dodecyl sulfate/basic protein ratio is 5.1.

strated multicomponent composition of commercial preparations of this dye. We have presented a facile method of synthesis and purification for the red component found in trypan blue. The chemical, spectral and analytical data have permitted a structure assignment for TBR as shown in Fig. 1.

The binding study of TBR with the myelin basic protein can best be explained in terms of a metachromatic model of binding such as the Bradley-Wolf [12] model. The evidence in support of a metachromatic model for the present system is: (a) the aggregation of the dye·protein complex as the charge on the protein is neutralized by bound dye molecules; (b) a 45-nm blue shift in the visible region, typically characteristic of metachromatic dye binding [13], along with a 5-nm red shift in the near ultraviolet region; (c) the appearance of two extrinsic, biphasic Cotton effects (Fig. 5) in aqueous solutions of the dye·protein complex. (The visible region biphasic curve has a crossover at 497 nm and the near ultraviolet region curve has a crossover at 315 nm.)

These Cotton effects are abolished in the presence of trifluoroethanol or in 8 M urea. Abolition of the Cotton effects by these agents is accompanied by reversion of the 505 and 318 nm absorption maximums to 550 and 313 nm, respectively; (d) the binding of 95% of the dye molecules to only 7% to 48% of the protein molecules (Table I) as detected from the analysis of the supernatent from centrifuged solutions of mixed dye and protein.

The binding of TBR with the basic protein is analogous in many ways to that observed by Schwarz and Klose [14] in studies of the binding of proflavin with the linear polyanions poly(acrylic acid) and poly(α-L-glutamic acid). These workers have shown that a cooperative binding effect is caused by the aggregation of stacked dye along the poly-anion, which serves as a primary template supplying negative charges to neutralize the repulsive forces of the positively charged proflavin dye molecules. The large number of cationic sites on the basic protein, the anionic charge on TBR, and the absence of stacking interaction in the free dye itself (over the concentration range $4 \cdot 10^{-8} \cdot - 8 \cdot 10^{-5}$ M) appears to be an equivalent anionic dye/poly-cation analogy to the proflavin-poly-anion system.

Since more than 90% of the TBR was always utilized in the formation of the insoluble TBR·basic protein complex, a detailed and rigorous interpretation of the binding behavior is not feasible. However, the spectral and light scattering data in conjunction with the binding data allow the following assessment. The experimental results indicate that a more complicated process than just simple binding of TBR to the basic protein is occurring. The changes in D_a/P_a coupled with the loss of optical activity in the last cationic fraction isolated in the flow electrophoresis experiment may be indicative of association of the dye·protein complex at higher D_o/P_o ratios. Such an interpretation is support by the light scattering results which show increased rates of aggregation and size of aggregates for the same D_o/P_o ratios when the net charge on the basic protein is reduced at pH 9 and 11. A minimum of two modes of interaction appear to be operative in this system. The TBR binds metachromatically as indicated by the metachromatic shifts in the near ultraviolet and visible regions of the dye absorbance spectrum and the induced optical activity in the TBR chromophoric system. As more cationic sites on the protein are covered by bound dye species, the charge repulsion between the remaining charged groups on the protein molecule is reduced, allowing self association between dye bound protein species. Similar aggregation effects have recently been observed with polymer·dye complexes [15]. From studies with acridine orange and poly(α-L-glutamic acid), Schwarz and Seelig-Löffler have suggested that upon binding of acridine orange, aggregation of the polymer·dye complex is induced. It is also worthwhile to note that the limiting D_a/P_a values of 12–14 fall within the same number of 14–15 probable binding sites determined from the difference binding data presented in Fig. 4.

The identification of the primary sites of TBR attachment to the myelin basic protein can be arrived at from an analysis of the PMR and CD data. The extensive broadening of the arginine resonances while the lysine and histidine resonance bands are only slightly perturbed suggests that the arginine residues provide the majority of the primary sites of attachment. Bovine myelin basic protein contains 18 arginine residues [16]. If we tabulate these residues and their proximal aromatic residues as shown in Table II, it is possible to associate 8 of the 18 arginine residues with 12 of the 13 aromatic residues. An additional 9 arginine residues are located proximal to serine

TABLE II

A. Probable primary binding sites for TBR*	
0 to 1 residue separation	Arg (42)-Phe (43, 44)
	Arg (113)-Phe (114) Trp (116)
2 residue separation	Arg (10)-Tyr (13)
	Arg (24)-Phe (27)
	Arg (30)-Phe (27)
	Arg (130)-Tyr (127) Phe (125)
	Arg (130)-Tyr (134)
3 residue separation	Arg (48)-Phe (44)
	Arg (64)-Tyr (68)
B. Suggested secondary binding sites for TBR	
	Lys (5) Arg (6)-Ser (8)
	Lys (52) Arg (53)-Ser (55)
	His (31) Arg (32)-Thr (34)
	Arg (160)-Ser (162)
	Arg (163)-Ser (164)
	Arg (63)-Thr (64, 65)
	Arg (97)-Thr (98)
	Arg (169)-Arg (170)

* Assignments based on bovine basic protein amino acid sequence [16].

and threoine residues. The PMR spectra indicate that the resonances of protons from these residues are broadened and, therefore, these residues must also participate in some way to the stabilization of bound dye. The induced optical activity of TBR could then be explained in terms of a Kirkwood [17] coupled oscillator mechanism between the bound dye and the adjacent aromatic amino acid residues. Although the induction of an inherent dissymmetry or chirality of the TBR molecule upon binding cannot be ruled out, the CD data in conjunction with the PMR data makes this possibility highly unlikely. The PMR data clearly show the perturbation of the protein's aromatic residues upon dye binding and this perturbation is lost at elevated temperature. This result is in accord with the data of Schwarz and Seelig-Löffler [15] where elevated temperature was found to break down polymer·dye complexes of acridine orange and poly(α-L-glutamic acid).

Thus, the binding data, PMR and CD data in conjunction with the amino acid sequence of the basic protein can best be explained by assigning the arginine residues as the primary sites for TBR binding. The distribution of these residues throughout the basic protein is not incompatible with a metachromatic mechanism for the binding.

London and Vossenberg [18] showed that myelin basic protein is protected against the proteolytic action of trypsin and similar enzymes when the protein binds cerebroside sulfate or other acidic phospholipids. Based on the equal distribution of the arginine residues throughout the basic protein molecule these lipids may be attached primarily to the arginine residues thus providing further evidence for the parallelism between TBR and acidic phospholipid binding.

The effects of sodium dodecyl sulfate binding to the bovine myelin basic protein were followed by CD and PMR spectroscopy. Both methods gave evidence for only minor alteration from the random coil form to possible helical conformations and

revealed that 20.4 to 1 was the maximum ratio of sodium dodecyl sulfate to bovine myelin basic protein that gave tractable solutions for CD spectra at pH 4.8 where the net charge on the protein is 30. It is of interest, that at pH 7.4 where the net charge is reduced to 14, only a sodium dodecyl sulfate/basic protein molar ratio of 6.4 to 1 is achievable.

The PMR spectra of sodium dodecyl sulfate/basic protein solutions in ^2H_2O were also indicative of the introduction of a very small degree of helical character into the protein. In the presence of sodium dodecyl sulfate, the broadening of the aliphatic proton resonances in the 200 to 500 Hz region and the slight broadening of the aromatic resonances in the 1500–1600 Hz region confirms the small degree of conformational change observed in the CD spectra. The binding of sodium dodecyl sulfate to basic protein is not strong as evidenced by the temperature dependence of the PMR spectra. At 73 °C, the curves have the appearance of sodium dodecyl sulfate-free basic protein spectra. When the temperature is lowered to 31 °C, the perturbations elicited by sodium dodecyl sulfate binding reappear. In view of the highly charged nature of the myelin basic protein and the paucity of extended hydrophobic regions necessary to effect tight binding to sodium dodecyl sulfate, the weak binding of sodium dodecyl sulfate to bovine myelin basic protein which is analogous to the behavior of myelin lipids lends further support to the primary structure, physical behavior picture of this protein molecule.

The difference in the observed behavior of basic protein arising from TBR binding as opposed to the binding of sodium dodecyl sulfate is viewed as resulting from two different binding mechanisms. The binding behavior of TBR is primarily a consequence of charge-charge interaction while the binding effects of sodium dodecyl sulfate are a consequence of hydrophobic interactions. The sodium dodecyl sulfate binding acts as a shield which limits charge-charge interaction in the basic protein molecule thus preventing aggregate formation while TBR imposes no such restraints.

ACKNOWLEDGMENTS

The authors acknowledge the technical assistance of Francis Ferrari in helping to obtain the PMR spectra. We also wish to thank Dr. M. J. Hunter and Dr. J. L. Oncley for their helpful discussions and advice.

This work includes material from a thesis submitted by L. F. Liebes in partial fulfillment of the requirements for the degree of Doctor of Philosophy. This investigation was supported in part by PHS Training Grant No. GM-1355 from the National Institutes of Health.

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