Magnetic Resonance Studies of the Interaction of the Manganous Ion with Bovine Serum Albumin*

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A new physical parameter of bound manganese, its effect on the longitudinal nuclear magnetic relaxation rate of water protons ($1/T_1$), has been measured by pulsed nuclear magnetic resonance. The values of this parameter are expressed in terms of enhancement ϵ_b, the ratio of the proton relaxation rate of bound Mn to that of free Mn at the same concentration. The observed enhancement values were used to determine the number of binding sites and the binding constants of Mn^{2+} to bovine serum albumin and to correlate changes in ϵ_b with structural changes induced in the protein. At *p*H 7.5, $\mu = 0.2$, $T = 24°$, bovine serum albumin binds one Mn^{2+} tightly, $K_a = 2.7 \pm 0.6 \times 10^4$ M^{-1}, with $\epsilon_b = 11.5 \pm 0.8$, and approximately five manganous ions weakly, $K_a = 3.3 \pm 0.6 \times 10^3$ M^{-1}, with $\epsilon_b = 6.5 \pm 0.3$. Binding constants and numbers of binding sites determined from measurements of *free* Mn^{2+} from electron spin resonance agreed well with those of *bound* Mn^{2+} determined from enhancement. For the first binding site, K_a increased monotonically with *p*H, suggesting ligands with pK values of 6.1, 6.8, and 8.5, but ϵ_b varied from 2 below *p*H 6 to 11 above *p*H 7.5, implicating a group with $pK = 7.0$. Titration of the one sulfhydryl group of bovine serum albumin with *p*-mercuribenzoate had no effect on K_a but slightly increased ϵ_b. Urea (≥ 2 M), guanidinium chloride (≥ 2 M), and decyl sulfate (0.1 M) decreased both K_a and ϵ_b. Guanidinium and urea each alter ϵ_b differently and in several stages as the concentration of denaturant is varied between 0.3 M and 6 M. Guanidinium (<3 M) appears to compete with Mn^{2+} for the tight binding site ($K_G = 18 \pm 6$ M^{-1}).

The longitudinal nuclear magnetic relaxation rate ($1/T_1$) of the protons of water is increased by the presence of paramagnetic ions. This effect of paramagnetic ions on protons is predominantly a short-range effect, inversely proportional to the sixth power of the distance between the paramagnetic ions and the protons (Bloembergen *et al.*, 1948). Rapid exchange of protons in the hydration sphere with those in the bulk of the solution results in an observed relaxation rate which is due to a weighted average of the different local environments of the protons, the dominant contribution occurring when the proton is within the hydration sphere of the ion. The enhancement of the effect of certain paramagnetic ions on the relaxation rate of water protons when the paramagnetic ions are bound to macromolecules was first observed with DNA by Eisinger *et al.* (1961) and with proteins by Cohn and Leigh (1962). This enhancement is ascribed to a restriction of the relative rotational motion of the hydration shell and the paramagnetic ion when the ion is bound to an external site of the macromolecule, with a consequent increase in the rotational correlation time of the magnetic interaction between the nuclear spin of the protons of the hydration shell and the electron spin of the paramagnetic ion (Eisinger *et al.*, 1961, 1962).

The present paper reports a quantitative study of enhancement and binding in a simple binary system consisting of a transition metal, the manganous ion, and a protein, bovine serum albumin. The results presented in this paper demonstrate that the measurement of enhancement, a rapid and sensitive physical method which does not disturb the equilibrium under investigation, can be used for determining association constants. These constants agree with association constants determined by an independent method. Enhancement data also give unique information about the structure of metal-protein complexes at the site of binding.

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Materials and Methods

All solutions were made in water which was redistilled in quartz and stored in polyethylene containers. No attempt was made to exclude oxygen from the solutions.

For experiments at *p*H 7.4, 0.05 M tris(hydroxymethyl)aminomethane, "Sigma 121," (Tris)–HCl buffer was used. At the manganese concentrations used (10^{-4} to 10^{-6} M), binding to Tris was negligible.[1] For the experiments in which *p*H was varied no buffer was added to the protein. The *p*H measurements were determined with a Leeds and Northrup 7664 *p*H meter with a glass electrode and an Ag/AgCl reference electrode. Variations in ionic strength were minimized by working at either 0.17 M or 0.50 M tetramethyl ammonium chloride, obtained from Eastman Organic Chemicals. The proton relaxation time (T_1) of solutions without Mn was 2.40 seconds, indicating negligible paramagnetic impurities in the water, salt, or buffer (Bloembergen *et al.*, 1948). Reagent-grade manganous chloride was used to make stock solutions. These were analyzed by the permanganate method with periodate as oxidizing agent (Sandell, 1959).

Crystallized bovine plasma albumin (Armour and Company, Lot No. W 69204) was used without purification. It was found to contain 0.87 ± 0.06 sulfhydryl groups per mole by titration with *p*-mercuribenzoate, in agreement with the value determined for a purified preparation by Boyer (1954). The proton relaxation time (T_1) of the solutions used in these experiments (2.40 seconds) was not altered by addition of bovine serum albumin at a final concentration of 3.5×10^{-4} M; this indicates minimal paramagnetic

[1] Hall and collaborators (1962) have shown that Tris base forms complexes with Cu^{2+} and Ni^{2+} with affinity constants of the order of 10^3 M^{-1}. Under the conditions used in the present study, the interaction between Tris base (10^{-2} M) and Mn^{2+} (10^{-5} to 10^{-3} M) was negligible, as shown by the fact that Tris had no detectable effect on (*a*) the amplitude or line width of the manganese EPR spectrum, (*b*) the proton relaxation rate of manganese solutions, (*c*) the measured binding constant of manganese to bovine serum albumin, or (*d*) the measured enhancement of the Mn-BSA complex.

impurities in the serum albumin (Bloembergen *et al.*, 1948). The concentration of serum albumin was measured by its extinction at 280 mμ (Cohn *et al.*, 1947).

Fresh solutions of reagent-grade urea were prepared daily. Recrystallized guanidinium chloride was given to us by Dr. B. C. Pressman. Sodium decyl sulfate, recrystallized three times, was a gift from Dr. F. Karush. *p*-Mercuribenzoate was purchased from the Sigma Chemical Company.

In the presence of bovine serum albumin (BSA)[2], the free manganese was measured by the intensity of its electron spin resonance (ESR) spectrum (Cohn and Townsned, 1954) using a Varian Model V4280A EPR spectrometer at 9.5×10^9 cycles/sec. The sample (approximately 0.1 ml) was placed in quartz capillary tubing of 1 mm internal diameter and 2 mm external diameter. The cavity temperature was controlled to ± 0.5° by air flow. The ESR measurements and relaxation time measurements were done at the same temperature.

The proton relaxation rate (PRR) of water was measured by the pulsed nuclear magnetic resonance method of Carr and Purcell (1954) at 25×10^6 cycles/second using 0.1-ml sample volumes. The temperature, determined by the magnet temperature, was found to vary less than ± 1° during the course of an experiment.

Analysis of Data

Enhancement (ϵ^*), defined by Eisinger *et al.* (1962) as the ratio of the PRR due to manganese in the presence and absence of complexing agent, was calculated from equation (1). T_1 is the observed relaxation

$$\epsilon^* = \frac{(1/T_1^*) - (1/T_1^*{}_{(0)})}{(1/T_1) - (1/T_{1(0)})} \qquad (1)$$

time in presence of manganese, $T_{1(0)}$ the observed relaxation time in absence of manganese (2.40 seconds). The terms with asterisks represent the same parameters in the presence of a complexing agent, in this case bovine serum albumin.

The observed enhancement (ϵ^*) of PRR in a mixture of free manganese, Mn_f, and bound manganese, Mn_b, is a weighted average of that due to both forms[3] (equation 2), where Mn_t is the total manganese concentration; ϵ_f, the enhancement of free manganese, is equal to 1 by definition (equation 1); and ϵ_b is the enhancement of manganese bound in the complex. One may solve equation (2) for the concentration of either free or bound manganese in terms of total manganese and the enhancement parameters (equations 3 and 4).

$$\epsilon^* = \frac{Mn_f}{Mn_t}\epsilon_f + \frac{Mn_b}{Mn_t}\epsilon_b \qquad (2)$$

$$Mn_f = \left(\frac{\epsilon_b - \epsilon^*}{\epsilon_b - 1}\right)Mn_t \qquad (3)$$

$$Mn_b = \left(\frac{\epsilon^* - 1}{\epsilon_b - 1}\right)Mn_t \qquad (4)$$

The association constant for manganese to protein (K_a) is defined in equation (5), where $[P_f]$ represents

$$K_a = \frac{[Mn_b]}{[Mn_f][P_f]} \qquad (5)$$

the concentration of free binding sites in solution and is equal to the concentration of total sites $nP_t - Mn_b$, n is the number of binding sites per mole of protein with the association constant K_a, and P_t is the total protein concentration. Substituting for Mn_f from equation (3) and for Mn_b from equation (4), one obtains from equation (6) equation (5). For the determination of binding constants from enhancement data using equation (6), one must know ϵ_b, the enhancement of the metal-protein complex, and n, the number of binding sites. Three procedures for determining ϵ_b were used: (1) titration of metal with protein: extrapolation of measured values of ϵ^* to infinite protein concentration yields ϵ_b.[4] (2) titration of protein with metal: extrapolation of measured values of ϵ^* to zero metal concentration yields a lower limit for ϵ_b.[5] (3)

$$K_a = \frac{(\epsilon^* - 1)(\epsilon_b - 1)}{(\epsilon_b - \epsilon^*)[nP_t(\epsilon_b - 1) + Mn_t(1 - \epsilon^*)]} \qquad (6)$$

[2] Abbreviations used in this paper: BSA, bovine serum albumin; PRR, proton relaxation rate; ESR, electron spin resonance.

[3] Equation (2) may be generalized for systems more complicated than a binary system to give (2A), where X_i

$$\epsilon^* = \Sigma X_i \epsilon_i \qquad (2A)$$

is the fraction of the i'th Mn-containing species and ϵ_i is the enhancement of the i'th species.

[4] At high protein concentration the reciprocal of observed enhancement ($1/\epsilon^*$) is a linear function of the reciprocal of total binding site concentration ($1/nP_t$), where n is the number of binding sites per mole of protein and P_t is the total protein concentration. For one-to-one binding this may be shown as follows: Since the dissociation constant (K_D) is the reciprocal of the association constant, from equation (5), one has (5A). Solving for Mn_b gives (5B). Substituting for Mn_b in terms of enhancement from equation (4) and solving for $1/\epsilon^*$ gives (5C). As the total protein concentration becomes large compared to K_D/ϵ_b, (P_f) approaches (P_t) and the quantity, $P_f + K_D/\epsilon_b$ may be approximated by P_t; therefore equation (5D) holds. Thus, at high protein concentration $1/\epsilon^*$ should be a linear function of $1/P_t$ with a slope equal to K_D/ϵ_b and intercept equal to $1/\epsilon_b$.

$$K_D = \frac{(Mn_f)(P_f)}{(Mn_b)} = \frac{(Mn_t - Mn_b)(P_f)}{(Mn_b)} \qquad (5A)$$

$$Mn_b = \frac{(P_f)(Mn_t)}{K_D + (P_f)} \qquad (5B)$$

$$\frac{1}{\epsilon^*} = \frac{1}{\epsilon_b}\left[\frac{K_D}{P_f + (K_D/\epsilon_b)} + \frac{P_f}{P_f + (K_D/\epsilon_b)}\right] \qquad (5C)$$

$$\frac{1}{\epsilon^*} = \frac{K_D}{\epsilon_b}\left(\frac{1}{P_t}\right) + \frac{1}{\epsilon_b} \qquad (5D)$$

[5] The amount of metal bound to protein on extrapolation to zero metal can vary from none to all, depending upon the total concentration of binding sites (nP_t) and the dissociation constant (K_D) of metal to protein. From the equilibrium equation we get equation (5E). Solving for Mn_b and dividing by Mn_t we have equation (5F). To avoid an indeterminate form as Mn_t approaches 0 as a limit, both numerator and denominator of equation (5F) are differentiated with respect to Mn_t, and, applying l'Hospital's rule, one obtains equation (5G). From equation (5G) one can see that if the total concentration of binding sites is large compared to the dissociation constant, all the manganese is bound on extrapolation to zero manganese. If nP_t is much smaller than K_D, however, none of the manganese is bound to protein in the limiting case.

$$K_D = \frac{1}{K_a} = \frac{(Mn_t - Mn_b)(nP_t - Mn_b)}{Mn_b} \qquad (5E)$$

$$\frac{Mn_b}{Mn_t} = \frac{(K_D + Mn_t + nP_t) \pm \sqrt{(K_D + Mn_t + nP_t)^2 - 4Mn_t nP_t}}{2\,Mn_t} \qquad (5F)$$

$$\lim_{Mn_t \to 0}\left[\frac{Mn_b}{Mn_t}\right] = \frac{nP_t}{K_D + nP_t} \qquad (5G)$$

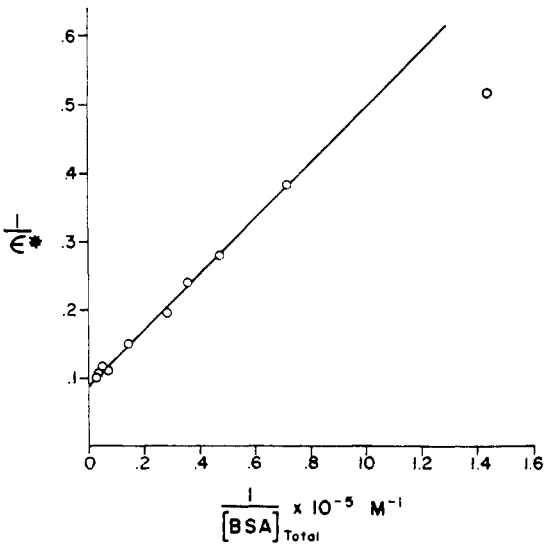

FIG. 1.—Titration of Mn^{2+} with BSA. Double reciprocal plot of observed enhancement versus total concentration of bovine serum albumin (see footnote 3). In addition to BSA, the solutions contained 5.0 $\times$ 10^{-5} M manganous chloride, 0.50 M tetramethyl ammonium chloride, and 0.05 M Tris base. The *p*H was adjusted to 7.5 with HCl in a final volume of 1.00 ml. 0.1-ml aliquots were used for the measurement of PRR at T = 24°.

measurement of ϵ^* at a single concentration of metal ion and of protein and independent measurement of the concentration of free metal ion concentration by ESR yields ϵ_b directly from the relationship in equation (3).

While all three methods give values for ϵ_b with reasonable agreement, method 2 is merely an approximation which may be iterated. Method 3 is the most convenient and involves the least number of measurements but requires two instruments.

The number of binding sites on the protein was determined graphically by the reciprocal plot used by Hughes and Klotz (1956), as illustrated in Figure 2B. In this figure the Mn_f values were determined directly by ESR but the Mn_f values can equally well be calculated solely from enhancement data (equation 3). When ϵ_b and n are known, one can then determine the binding constant either analytically (from equation 6) or graphically (from Figs. 1 and 2).

Of the two types of titration used to determine the enhancement, association constants, and number of binding sites, the first type, in which Mn was held constant and BSA was varied, provided data which were amenable to a simple analysis to yield the desired parameters for the tight binding site. The data from the second type of titration, in which BSA was held constant and Mn was varied, required a somewhat more complex analysis but yielded similar information for the weak binding sites as well as the tight binding site.

RESULTS

Titration of Manganese with Bovine Serum Albumin

Determination of the Enhancement of the Complex (ϵ_b), the Association Constant (K_a), and the Number of Binding Sites (n) from PRR Data.—Manganous chloride (5×10^{-5} M) was titrated with variable amounts of bovine serum albumin (0.7 to 35.0 $\times$ 10^{-5} M) at 24° and *p*H 7.5 in presence of 0.05 M Tris-HCl and 0.5 M tetramethyl ammonium chloride; values of the observed enhancement are given in Table I. The enhancement of the complex, ϵ_b, was obtained by extrapolation to infinite protein concentration, as shown in Figure 1. The reciprocal of the observed enhancement ($1/\epsilon^*$) was plotted against the reciprocal of the total serum albumin concentration and a value of ϵ_b = 11.5 was obtained from the intercept at infinite protein concentration.[3] Furthermore, the association constant for Mn-BSA, assuming a 1:1 complex, may be obtained from the same plot, since it is equal to the reciprocal of the concentration of free bovine serum albumin at an enhancement halfway between 1.00 and 11.5 (ϵ^* = 6.25). From Figure 1, one can obtain directly the total concentration of serum albumin at this value of the enhancement, namely, 5.6 $\times$ 10^{-5} M; since the concentration of Mn-BSA (half the total Mn concentration) is 2.5 $\times$ 10^{-5} M, the concentration of free BSA is 3.1 $\times$ 10^{-5} M. Thus, the association constant is 3.2 $\times$ 10^4 M^{-1} for a 1:1 Mn-BSA complex.

By an alternative graphical representation of the data in Table I, the validity of this assumption of one

TABLE I

VALUES OF FREE MANGANOUS ION CONCENTRATION AND ENHANCEMENT OF PROTON RELAXATION RATE WITH VARIOUS CONCENTRATIONS OF BSA AND CALCULATION OF K_a AND ϵ_b VALUES

The total concentration of $MnCl_2$ was 5 $\times$ 10^{-5} M in all solutions, which also contained 0.5 M tetramethyl ammonium chloride and 0.05 M Tris base. The *p*H of each solution was adjusted to 7.5 with HCl.

[BSA] M $\times$ 10^5	(*a*) $[Mn]_{free}$ M $\times$ 10^5	(*b*) $K_a \times 10^{-4}$ M^{-1}	(*c*) ϵ^*	(*d*) ϵ_b	(*e*) $K_a \times 10^{-4}$ M^{-1}
0	5.00	—	1.00	—	—
0.7	4.53	4.50	1.94	11.0	3.91
1.4	4.30	2.32	2.62	12.6	2.90
2.1	3.86	3.08	3.58	12.3	3.75
2.8	3.48	3.42	4.18	11.4	3.38
3.5	3.45	2.30	5.14	14.4	4.25
7.0	2.25	2.98	6.63	11.2	2.67
14.0	1.55	2.12	9.10	12.7	3.33
21.0	1.30	1.64	8.58	11.2	1.50
28.0	0.90	1.91	9.35	11.2	1.63
35.0	0.55	2.65	9.92	11.0	1.84
Average ± 1 S.D.	—	2.7 ± 0.8	—	11.7 ± 1.1	2.9 ± 1.0

[a] Measured by electron spin resonance. [b] Calculated from $[Mn]_{free}$, column *a*. [c] Measured by PRR. [d] Calculated from equation (3). [e] Calculated from equation (6) assuming n = 1 and ϵ_b = 11.5 from Figure 1.

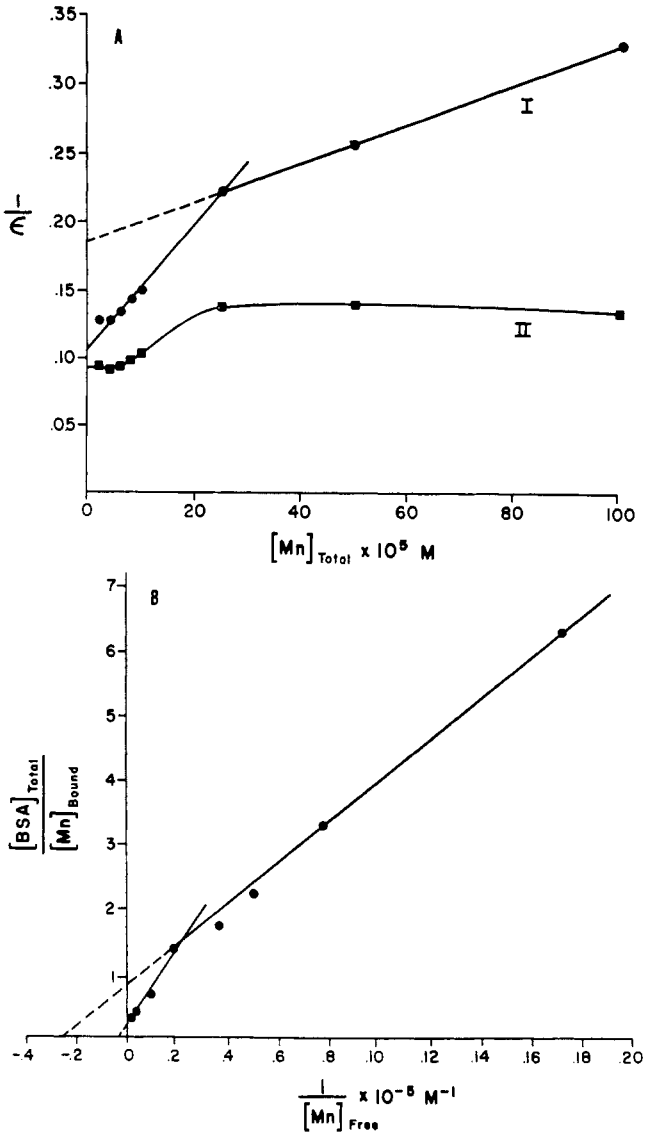

FIG. 2.—(A) Titration of BSA with variable concentrations of Mn^{2+} in a series of solutions containing 8.96×10^{-5} M bovine serum albumin, 0.17 M tetramethyl ammonium chloride, and 0.05 M Tris-HCl buffer, *p*H 7.5, at $T = 22°$ and $MnCl_2$ concentration as indicated. I, Reciprocal of observed enhancement (ϵ^*) versus total Mn^{2+} concentration. II, Reciprocal of enhancement of the bound manganese (ϵ_b) versus total Mn^{2+} concentration. (B) Hughes and Klotz type plot of the concentrations of free and bound manganese as obtained by electron spin resonance on 0.1-ml aliquots from the series of solutions described in Figure 2A.

binding site per molecule may be established. From the double reciprocal plot of Hughes and Klotz (1956), one can determine n and K_a (as exemplified in Figure 2B). For this plot one determines the concentrations of free and bound manganese at each protein concentration from equations (3) and (4) by using the value of $\epsilon_b = 11.5$ which has been determined from Figure 1. The resulting extrapolation gives $n = 1.1$ and $K_a = 2.7 \times 10^4$ M^{-1} for this binding site.

The association constant for each point may also be determined analytically from equation (6), assuming one tight binding site and letting $\epsilon_b = 11.5$ as determined graphically (Fig. 1). This method gives equal weight to each point and indicates the deviation of the average $K_a = 2.9 \pm 1.0 \times 10^4$ M^{-1} as shown in Table I.

Determination of the Binding Constant for Tight Binding by ESR.—In the same series of solutions, the concentration of free manganous ion was measured by the intensity of its electron spin resonance spectrum. This method has been used previously for determining association constants of Mn complexes of small molecules (Cohn and Townsend, 1954) and of Mn-enolase (Malmström *et al.*, 1958). The concentrations of free manganese in these solutions as determined by ESR are listed in Table I. Assuming one-to-one binding, the association constant directly calculated from these data using equation (5) is $K_a = 2.7 \pm 0.8 \times 10^4$ M^{-1}. The binding constant determined by this independent method is, therefore, in good agreement with that determined from enhancement (Table II).

Determination of ϵ_b of the Tight Binding Site Using Both ESR and PRR.—Using the value of free manganese, Mn_f, measured by ESR and the observed enhancement ϵ^* of each solution in equation (3), one obtains a set of determinations of ϵ_b for the tight binding site (Table I) which appear to be constant. The average of these values yields $\epsilon_b = 11.7 \pm 1.1$.

A summary of the various procedures and the constants calculated from data obtained by ESR and enhancement is presented in Table II. From these data it is apparent that the association constants obtained graphically or analytically from enhancement data alone are within the statistical variation for these con-

TABLE II
SUMMARY OF NUMBER OF BINDING SITES, ASSOCIATION CONSTANTS, AND ENHANCEMENT VALUES DERIVED BY VARIOUS METHODS

Method	Titration Variable	Treatment of Data	Tight Binding			Weak Binding		
			n	$K_a \times 10^{-4}$ M^{-1}	ϵ_b	n	$K_a \times 10^{-4}$ M^{-1}	ϵ_b
ESR	BSA	Analytic (eq. 5) $n = 1.0$	—	2.7 ± 0.8	—	—	—	—
	Mn	Graphic (Fig. 2B)	1.2	2.7 ± 0.6	—	5	0.33 ± 0.06	—
PRR	BSA	Graphic (Fig. 1)	—	3.1	11.5	—	—	—
		Graphic (Fig. 2B)[a]	1.1	2.7	—	—	—	—
		Analytic (eq. 6) $n = 1.0, \epsilon_b = 11.5$[a]	—	2.9 ± 1.0	—	—	—	—
	Mn	Graphic (Fig. 2A)	—	$\geqslant 1.1$	$\geqslant 9.25$	—	$\geqslant 0.18$	$\geqslant 5.4$
		Graphic (Fig. 2A)[b]	—	3.2	—	—	0.38	—
		Graphic (Fig. 2B)[c]	$\leqslant 1.2$	$\leqslant 5.5$	—	$\leqslant 6.2$	$\leqslant 0.19$	—
		Graphic (Fig. 2B)[d]	1.06	2.5	—	—	—	—
		Analytic (eq. 5G)	—	—	12.0	—	—	6.2
Both ESR and PRR	BSA	Analytic (eq. 3)	—	—	11.7 ± 1.1	—	—	—
	Mn	Analytic (eq. 3 and eq. 2A)	—	—	10.7 ± 0.5	—	—	6.5 ± 0.3
Average[e]			1.1 ± 0.1	2.8 ± 0.2	11.5 ± 0.6	5	0.36 ± 0.03	6.4 ± 0.2

[a] From eq. (3) and (4), assuming $\epsilon_b = 11.5$ as obtained from extrapolation to infinite [BSA] (Fig. 1). [b] Assuming $n = 1.0$ and $\epsilon_b = 11.7$ for tight binding and $n = 5$ and $\epsilon_b = 6.5$ for weak binding as calculated from ESR and PRR data (Table I, Fig. 2A, II and eq. 2A). [c] From eq. (3) and (4), assuming $\epsilon_b = 9.25$ for tight binding and $\epsilon_b = 5.4$ for weak binding as obtained from extrapolation to $Mn_t = 0$ (Fig. 2A, II). [d] From eq. (3) and (4), assuming $\epsilon_b = 11.7$ for tight binding and $\epsilon_b = 6.5$ for weak binding as calculated from ESR and PRR data (Table I, Fig. 2A, II and eq. 2A). [e] Average excludes values which are upper or lower limits.

stants obtained by electron spin resonance and that ϵ_b extrapolated from enhancement data alone agrees with that determined analytically using both enhancement and electron spin resonance data.

Titration of Bovine Serum Albumin with Manganese

Determination of ϵ_b from PRR Data. —The enhancement (ϵ^*) was measured in a series of solutions containing 9×10^{-5} M BSA with increasing amounts of manganous ion from 2×10^{-5} to 100×10^{-5} M at 22° and *p*H 7.5 in the presence of 0.05 M Tris-HCl and 0.17 M tetramethyl ammonium chloride. The variation of $1/\epsilon^*$ as a function of total Mn concentration is shown in Figure 2A,I. At this concentration of BSA, Mn is never completely bound[4] and therefore the extrapolation of such a curve to zero manganese concentration yields only a lower limit for the enhancement of the complex ϵ_b. The enhancement curve (Fig. 2A,I) has a discontinuity at a total manganese concentration of 2.5×10^{-4} M, indicating two types of sites. Extrapolation to zero manganese concentration from the low range of manganese concentrations yields $\epsilon_b \geqslant 9.25$ for the tight binding site. Similar extrapolation from the high range of manganese concentrations yields $\epsilon_b \geqslant 5.4$ per site for the weak binding sites. Correcting these values for the amount of manganese which remains unbound[6] as the total manganese approaches zero, one obtains enhancement values, ~12.0 for the tight complex and 6.2 for the weak complex, in good agreement with the most reliable values for these constants (Table II). Since the corrections for free Mn could not have been made without prior knowledge of the binding constants, the following calculations will be made with the uncorrected values of ϵ_b.

Determination of the Approximate Number of Binding Sites (n) and the Approximate Binding Constants (K_a) from PRR.—The number of binding sites and the binding constants can be determined analytically from the observed enhancement (ϵ^*) by using equation (6) to set up simultaneous equations in the two unknowns, n and K_a. A less laborious graphical method which gives the same values is the double reciprocal plot of Hughes and Klotz (1956) as exemplified in Figure 2B. For such a graphical analysis, the enhancement data are used in equations (3) and (4) to calculate the free manganese (Mn_f) and the bound manganese (Mn_b). Because the values for the enhancement of bound manganese (ϵ_b) obtained from Figure 2A,I represent lower limits, the number of binding sites and the binding constants determined in this way tend to be high and are, therefore, considered to be upper limits.

By this method the number of tight binding sites, derived from the enhancements observed at the low range of manganese concentrations, is $\leqslant 1.2$ sites per mole of serum albumin, and the binding constant $K_a \leqslant 5.5 \times 10^4$ M^{-1}.

To study the weak binding, the enhancements observed at the high range of manganese concentration were first corrected for the enhancement due to the tightly bound manganese using equation (2A) (see footnote 2) assuming $K_a = 5.5 \times 10^4$ M^{-1} and $\epsilon_b = 9.25$ for the one tight binding site. The concentration of free and bound manganese obtained with the corrected enhancement values from equations (3) and (4) were plotted as in Figure 2B to yield the number of weak binding sites, $n \leqslant 6.2$/mole, with an average binding constant $K_a \leqslant 1.9 \times 10^3$ M^{-1}. Constants for both strong and weak binding are in reasonable agree-

[6] For the tight binding site, using equation (5G) (footnote 4), the dissociation constant ($K_D \sim 3 \times 10^{-5}$ M), and the concentration of tight binding sites in the present experiment ($nP_t \sim 9 \times 10^{-5}$ M), one calculates that three-fourths of the manganese is tightly bound in the limiting case. The corrected value of ϵ_b is, therefore, (9.25-0.25)/0.75 = 12.0. For the five weak binding sites one first corrects the extrapolated value of 5.4, which is a weighted average of the enhancement of the one tight binding site and of the five weak binding sites using equation (2A) (footnote 2). This gives a value of $\epsilon_b > 4.14$ at zero metal concentration. From equation (5G) (footnote 4), using the values of K_D (3×10^{-4} M) and nP_t (4.5×10^{-4} M) for the weak binding sites, one calculates that 60% of the manganese is bound weakly in the limiting case. Therefore the corrected value of ϵ_b = (4.14-0.40)/0.60 = 6.2.

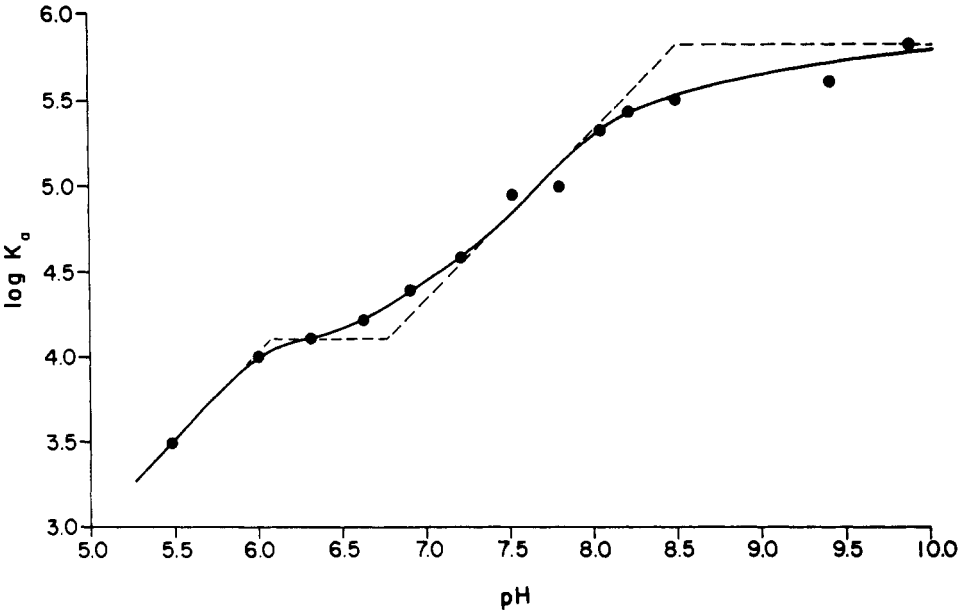

FIG. 3.—Variation of the K_a of the tight binding site of Mn-BSA with pH determined by electron spin resonance measurements. Broken line segments represent tangents of zero and unit slope which intersect at pH 6.06, 6.75, and 8.46. The solutions contained $MnCl_2$, 5 or 10 $\times$ 10^{-5} M; BSA, 4.6 or 10 $\times$ 10^{-5} M; and tetramethyl ammonium chloride, 0.17 M. The pH was adjusted with HCl (final concentration < 0.01 M) or tetramethyl ammonium hydroxide (final concentration $\leq$0.03 M). T = 24°.

ment with the constants obtained from measurements of free manganese by ESR (cf. Table II).

In addition, if the number of binding sites of each type is known, the association constants can be derived by obtaining Mn_f, the concentration of free manganese at half-maximal enhancement, from Figure 2A,I. From the high manganese range of the biphasic curve, Mn_f at ϵ = 3.2 (half maximal for weak binding sites) is equal to $Mn_t - Mn_b$. The bound manganese concentration is equal to the total concentration of the strong binding sites plus half the concentration of the weak binding sites. The numerical value of Mn_f is 56.6 $\times$ 10^{-5} M, which represents the upper limit of the dissociation constant for the weak binding sites. For these sites, therefore, the average $K_a \geq 1.8 \times 10^3$ M^{-1}. Analogous treatment of the data in Figure 2A,I at the low range of manganese concentrations gives $K_a \geq 1.1 \times 10^4$ M^{-1} for the tight binding site. Both constants are in reasonable agreement with those obtained from independent measurements of free manganese by ESR (see Table II).

Determination of Precise Number of Binding Sites (n) and Binding Constants K_a from Enhancement.—All of the above calculations of the number of binding sites (n) and the association constants (K_a) are approximations because the values for the enhancement of the complex (ϵ_b) were lower limits.[4] Using the most reliable value of ϵ_b = 11.7 for the tight binding site, in equations (3) and (4) and by the graphical method of Figure 2B, one obtains n = 1.06 and K_a = 2.5 $\times$ 10^4 M^{-1} for the tight binding site, in good agreement with the most reliable values for these constants (Table II).

The enhancement data available for the weak binding sites limit the accuracy of the double reciprocal plot (Fig. 2B) to the approximations in the preceding section. However, more precise values may be obtained by using the most reliable value of ϵ_b, 6.5, and n, 5 (Table II) in the graphical procedure of Figure 2A,I. By this procedure, one obtains an average weak binding constant, K_a = 3.8 $\times$ 10^3 M^{-1}. An analogous treatment of the data for the tight binding site yields K_a equal to 3.2 $\times$ 10^4 M^{-1}.

Determination of n and K_a by ESR.—In the same titration of serum albumin (9 $\times$ 10^{-5} M) with variable concentrations of manganese, the free manganese was measured by the intensity of its electron spin resonance spectrum. The data derived from the free Mn^{2+} concentration are plotted in Figure 2B in a modified form of Hughes and Klotz's procedure (1956). The y intercept of this curve is the reciprocal of the number of binding sites and the x intercept is the association constant. Like the enhancement data (Fig. 2A,I) the ESR data are represented by two straight lines intersecting at a concentration of total manganese of 2.5 $\times$ 10^{-4} M. The extrapolations of the ESR data indicate that BSA binds approximately one (1.2) manganous ion tightly with an association constant of 2.7 $\pm$ 0.6 $\times$ 10^4 M^{-1}, and approximately five manganous ions weakly with an average association constant of 3.3 $\pm$ 0.6 $\times$ 10^3 M^{-1}. These constants are in agreement with those determined from enhancement (Table II).

Determination of ϵ_b for Both Types of Sites by a Combination of ESR and PRR Data.—A direct determination of ϵ_b for each site can be made by inserting both the directly observed free manganese concentration and the observed enhancement data in equation (3). The results of these calculations are plotted in curve II of Figure 2A, where ϵ_b varies from a value of 10.7 at low manganese concentration to 7.3 at high manganese concentration. The former represents the enhancement of the tight binding site. The latter represents the weighted average of the enhancements of the manganese bound to the one tight binding site and to the five weak binding sites. Using equation (2A)[2] and the binding constants of manganese to each type of site one obtains an average enhancement for the weak site of 6.5 $\pm$ 0.3.

Effect of pH on the Association Constant and Enhancement of the Tight Binding Site

The logarithm of the association constant of manganese to the tight binding site of bovine serum albumin (determined by electron spin resonance) as a function of pH is plotted in Figure 3. Tangents of zero and

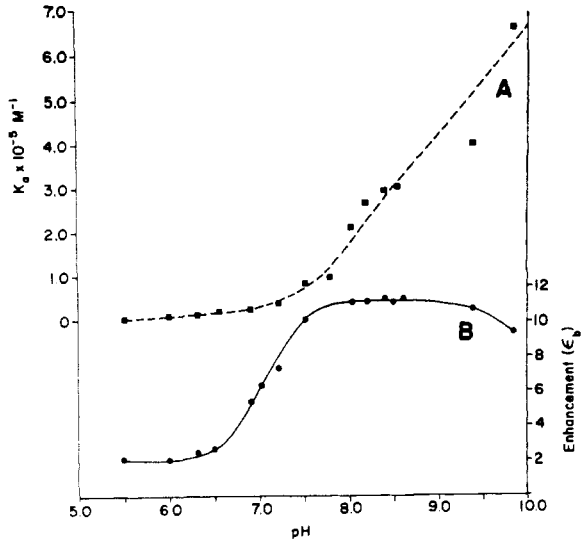

FIG. 4.—A, Variation of tight binding constant (K_a) with *p*H. B, Variation of enhancement of the bound manganese (ϵ_b) with *p*H. Experimental conditions are as described in Figure 3. Calculation of ϵ_b was from ESR and PRR data using equation (3).

unit slope reveal, at their intersections, three dissociating groups, presumably ligands for manganese, whose *pK* values are approximately 6.1, 6.8, and ~8.5. The data for the *pK* values of the first and third groups are less satisfactory than those for the second. Neglect of the hydrolysis of the manganese aquocation, *pK* = 10.6 (Chaberek *et al.*, 1952), introduces a maximum error of 1.2% in log K_a at *p*H 9.85. At lower *p*H levels the correction for the hydrolysis of the manganese aquocation is completely negligible. Using equilibrium dialysis, Borg and Cotzias (1958) observed a ligand for manganese on bovine serum albumin with a *pK* = 6.8.

The variation of ϵ_b with *p*H differs from that of K_a. Both are plotted for comparison in Figure 4, where it may be seen that ϵ_b varies from a value of 2 below *p*H 6 to a value of 11 above *p*H 7.5, implicating a group with a *pK* = 7.0. Above *p*H 9, ϵ_b decreases slightly. Unlike ϵ_b, the association constant is a monotonically increasing function of *p*H over the range studied.

Effect of pCMB and Denaturants on the Binding Constant and Enhancement of the Tight Binding Site

When BSA has been titrated with *p*-mercuribenzoate (Table III), there is no change in the binding constant

TABLE III

EFFECT OF *p*-MERCURIBENZOATE ON K_a AND ϵ_b AT TWO *p*H VALUES

All solutions contained 0.05 M Tris-HCl buffer, 0.17 M tetramethyl ammonium chloride, 4.8×10^{-5} M BSA, and 5×10^{-6}M $MnCl_2$. T = 22°.

*p*H	*p*-Mercuribenzoate M × 10^5	SH titrated[a] mole BSA	$K_a \times 10^{-5}$ [b] M^{-1}	ϵ_b [c]
8.1	0	0	2.0	11.0
	10	0.88	2.2	10.0
	20	1.00	2.0	10.4
7.4	0	0	0.47	11.2
	10	0.71	0.43	12.5
	20	0.93	0.48	13.1

[a] Diluted aliquots were examined spectroscopically at 250 mμ to determine the sulfhydryl titer (Boyer, 1954). [b] Calculated from ESR data. [c] Calculated from ESR and PRR data using equation (3).

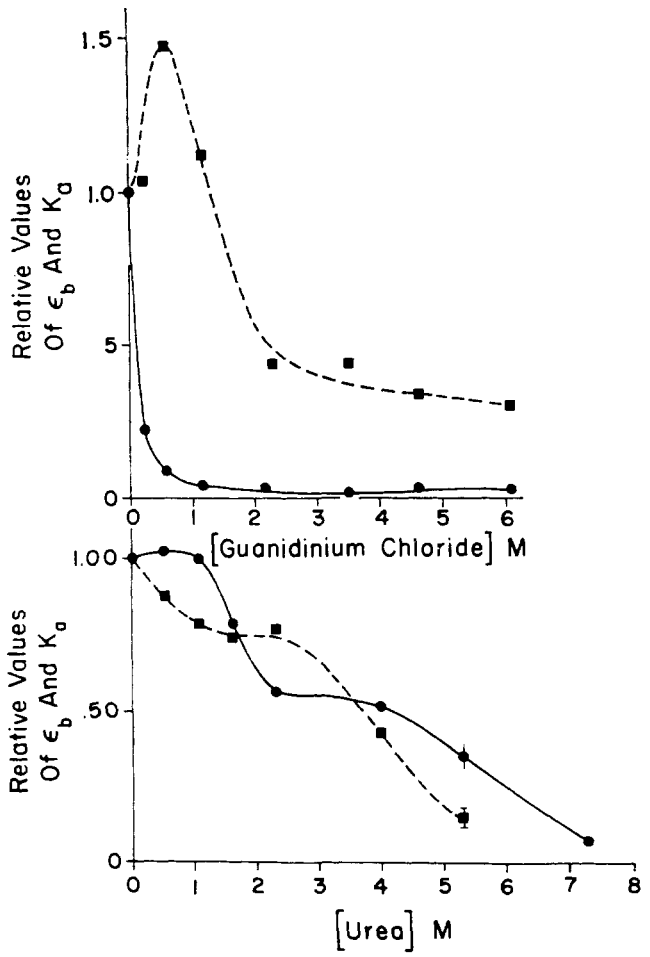

FIG. 5.—Effect of denaturants on ϵ_b and K_a of the tight binding site. *Upper curves*, Relative values of ϵ_b (broken line) and of K_a (solid line) as a function of guanidinium chloride concentration; the value of ϵ_b and K_a in absence of denaturant is taken as 1. The solutions contained 1×10^{-4}M $MnCl_2$, 1×10^{-4} M BSA, 0.17 M tetramethyl ammonium chloride, and 0.05 M Tris base; *p*H was adjusted to 7.5 with HCl. Temperature range of these experiments, 21–27°. *Lower curves*, Urea concentration as variable; parameters and experimental conditions were as described for the upper curve.

TABLE IV

EFFECT OF DENATURANTS ON K_a AND ϵ_b

All solutions contained 0.17 M tetramethyl ammonium chloride, 0.05 M Tris-HCl buffer, 1.0×10^{-4} M BSA, and 1.0×10^{-4} M $MnCl_2$. The *p*H was adjusted, when necessary, with dilute HCl and the final volume adjusted to 3.00 ml. The temperature was 29° in the urea and decyl sulfate experiments and 21° in the guanidinium experiment.

Denaturant	Concentration (M)	$K_a \times 10^{-4}$ [a] M^{-1}	ϵ_b [b]
None	—	4.5	10.9
Urea	5.3	1.4	1.5
Guanidinium chloride	0.2	1.4	10.7
Decyl sulfate	0.1	1.4	6.6

[a] Calculated from ESR data. [b] Calculated from ESR and PRR data using equation (3).

TABLE V

COMPARISON OF THE EFFECTS OF HIGH IONIC STRENGTH, UREA, AND GUANIDINIUM CHLORIDE ON K_a AND ϵ_b

Each sample contained 1×10^{-4} M BSA, 1×10^{-4} M $MnCl_2$, and 0.05 M Tris-HCl buffer. The final *p*H was adjusted to 7.5, when necessary, with HCl (final concentration <0.01 M). $T = 28°$.

Expt. No.	[TMA Cl] [a] M	[Urea] M	[Guanidinium Cl] M	$K_a \times 10^{-4}$ [b] M^{-1}	ϵ_b [c]
1	0.16	0	0	4.8	11.1
2	2.30	0	0	3.1	10.5
3	0.16	2.30	0	2.5	8.3
4	2.30	2.30	0	2.1	7.3
5	0.16	0	2.30	0.15	4.5

[a] Tetramethyl ammonium chloride. [b] Calculated from ESR data. [c] Calculated from ESR and PRR data using equation (3).

of manganese, indicating that the Mn is not bound to the sulfhydryl group. There is, however, a slight increase in the enhancement of the manganese–bovine serum albumin complex at *p*H 7.4 but not at *p*H 8.1.

At concentrations of the three denaturants sufficiently high to produce changes in such physical parameters of serum albumin as rotatory dispersion (Kauzmann and Simpson, 1953; Markus and Karush, 1957) and viscosity (Neurath *et al.*, 1944; Frensdorff *et al.*, 1953), namely, urea >2 M, guanidinium chloride >2 M, and decyl sulfate 0.1 M, marked lowering of the binding of Mn and the enhancement of the complex was observed (Fig. 5 and Table IV). However, differences in the effects of individual denaturants on the two parameters are shown in Table IV, where the concentration of each denaturant required to reduce K_a to one-third of its value had quantitatively dissimilar effects on ϵ_b.

Urea and guanidinium chloride, whose effects were studied in greater detail, were found to show marked differences in effects on K_a and ϵ_b, especially at low concentrations. As shown in Figure 5, guanidinium chloride, at the lowest concentration used, 0.23 M, caused a large decrease in the binding of manganese; this trend continued until a concentration of 2.3 M was reached; beyond this concentration only small changes were noted. The product of K_a and guanidinium concentration was constant below 3.5 M guanidinium (0.28 ± 0.09), which suggests competition between manganese and guanidinium for the tight binding site in this concentration range. Assuming competition, one may calculate a binding constant for guanidinium $K_G = 18 \pm 6$. Guanidinium chloride had a complex effect on the enhancement of manganese–bovine serum albumin depending on the concentration range as shown in Figure 5. In the lowest concentration range, ϵ_b was increased by 50% at 0.6 M. This was followed by a precipitous decrease in ϵ_b between 0.6 and 2.3 M. As the concentration of guanidinium chloride was increased above 2.3 M, further small decreases in ϵ_b occurred. That these effects of guanidinium chloride were not due to specific chloride ion nor to nonspecific ionic strength effects is indicated by the data of Table V. Although 2.3 M guanidinium chloride decreased K_a and ϵ_b greatly (Expt. 5), 2.3 M tetramethyl ammonium chloride alone (Expt. 2) or together with 2.3 M urea (Expt. 4) did not produce comparable decreases.

The lower curves in Figure 5 reveal a complex pattern of urea effects on binding and enhancement, differing considerably from the effects of guanidinium chloride. Below 1 M urea the binding of manganese was not affected; in the region of urea concentration from 1.6 to 7 M, K_a progressively decreased. Urea had a biphasic effect on the enhancement of the manganese–bovine serum albumin complex, producing a progressive reduction in ϵ_b to 75% of the control until the concentration reached 1.6 M. In the range from 1.6 M to 2.3 M, urea did not affect ϵ_b. At higher concentrations urea again produced a progressive reduction in ϵ_b.

DISCUSSION

The PRR technique should be applicable to studies of binding of those transition metals, including Mn^{2+}, Cu^{2+}, and Cr^{3+}, whose electron spin relaxation time is sufficiently long that their effect on the longitudinal nuclear magnetic relaxation rate of water is determined primarily by the rotational correlation time. This class of metal ions would be expected to exhibit the phenomenon of enhancement upon binding to external sites of macromolecules such as proteins and nucleic acids (Eisinger *et al.*, 1962).

The agreement in the binding constants of manganese to bovine serum albumin when measured by determining either the free manganese (by electron spin resonance) or the bound manganese (by enhancement of the proton relaxation rate) indicates that the latter is a sensitive independent technique for such quantitative determinations. By titrations with the proper choice of variable as shown above, the number of binding sites as well as the binding constants for both weak and strong binding sites may be obtained.

In addition to its use in determining the number of binding sites and binding constants, enhancement gives some information concerning the structure of the metal-protein complex. The enhancement of the bound metal (ϵ_b) is a characteristic property of the site of binding not necessarily related to the binding constant for the site, as may be seen from the effects of *p*H, urea, and guanidinium chloride. Above *p*H 7.5 ϵ_b remains constant while the binding constant K_a continues to rise. Concentrations of urea below 1 M cause a decrease in ϵ_b while having no effect on K_a. Concentrations of guanidinium chloride below 1 M cause an increase in ϵ_b while decreasing K_a. Furthermore, comparison of the metal complex of albumin with that of other proteins, in particular metal-binding enzymes (Cohn, 1963), reinforces the concept that the magnitude of the enhancement and the stability constants of metal-protein complexes are parameters which are determined by different structural properties.

Some insight into the structural features of Mn complexes underlying the enhancement may be gained from

a consideration of the parameters involved in enhancement. As formulated by Eisinger *et al.* (1962),

$$\epsilon_b = \frac{(p\tau_c)_b}{(p\tau_c)_0} \qquad (7)$$

where p is the ratio of the number of water protons and other rapidly exchanging protons in the coordination sphere about the cation to the total number of water protons in the medium, and τ_c is the correlation time for the interaction. The terms in the numerator, with the subscript b, refer to the complex; those in the denominator refer to the hydrated manganous ion in solution. The correlation time for magnetic-dipole interaction (τ_c) depends on the rotational correlation time τ_r and the electron spin relaxation time τ_s as shown in equation (8). For $Mn(H_2O)_6^{2+}$, $\tau_s \sim 100\ \tau_r$ and the

$$\frac{1}{\tau_c} = \frac{1}{\tau_r} + \frac{1}{\tau_s} \qquad (8)$$

correlation time τ_c is dominated by the rotational correlation time τ_r. Although it is known from ESR measurements that τ_s is shorter in the Mn complex than in the Mn aquocation, even in the complex the value of τ_c is dominated by τ_r. The displacement of water from the hydration shell by ligands from the protein decreases p but increases τ_c. The increase of τ_c in the complex must be greater than the decrease of p when an $\epsilon_b > 1$ is observed. The observation that ϵ_b for the tightly bound manganese is 11.5 and the average ϵ_b for the weakly bound manganese is 6.4 implies that both sites of Mn binding remain accessible to water. The difference in ϵ_b for the two sites is interpreted to be primarily due to the difference in rotational correlation time, i.e., the hydration shell around the tightly bound manganese is more hindered in its rotation than the hydration shell around the loosely bound manganese.

Hindrance of the relative rotation of manganese and its hydration shell and the resulting enhancement could result from several chemical properties of the metal-macromolecule complex. Among these are chelation of the metal by several ligands from the protein, steric effects of the protein surface upon the hydration shell of the metal, hydrogen bonding of the hydration shell to the protein, or hydrogen bonding of the hydration shell to water "immobilized" by the protein.

Chelation per se is unlikely to be the predominating cause of the large enhancement observed because ATP and ADP provide at least two ligands to manganese (Cohn and Hughes, 1962) and produce an enhancement of only 1.5 (Cohn and Leigh, 1962). Chelation, however, may permit the other mechanisms listed above to operate more effectively in hindering the rotation of the water molecules in the hydration shell of the Mn. Binding of manganese by bovine serum albumin in chelate form would be expected to affect both the association constant and the enhancement, while the other interactions listed involve the water ligands and would be expected to exert an effect primarily upon the enhancement rather than on binding. Chelation would increase the binding constant but could either increase or decrease the enhancement, as may be seen from equation (7).

Chelation in the Mn–bovine serum albumin complex is suggested by the tightness of binding and by the pH effect on K_a. The pH effect may be interpreted to indicate three ligands whose apparent pK values are approximately 6.1, 6.8, and 8.5, assuming no shift has occurred in these pK values due to metal binding (Klotz, 1954). The pK values are consistent with the assignment of imidazole groups for the first two ligands and an α-amino group to the third.

The sulfhydryl group, which also has a pK near 8.5 (Cecil and McPhee, 1959), is eliminated because the binding of manganese is unchanged when the one sulfhydryl group of bovine serum albumin has been titrated with p-mercuribenzoate. Holeysovska (1961) has studied the binding of manganese to human serum albumin by equilibrium dialysis and has found one tight binding site with $K_a = 2 \times 10^4\ M^{-1}$ and fifteen weak binding sites with an average $K_a = 66\ M^{-1}$. On the basis of one-to-one binding, Holeysovska suggested the binding of manganese to sulfur for the tight binding site.

One may consider the tightly bound manganese as being chelated by two of the sixteen imidazole groups of bovine serum albumin. The pK near 7.0 in the ϵ_b curve may be explained by a change in rotational correlation time due to the introduction of the second ligand onto the manganese which results in an increase in ϵ_b from 2 to 11.6. An alternative explanation for the striking effect of a group with a pK of 7.0 on ϵ_b is that one is titrating a group other than the ligand which controls the configuration of the protein around the manganese binding site.

The ability of bovine serum albumin to chelate manganese depends upon the spatial arrangement of the ligands as determined by the folding of the protein. The fact that various denaturants, in concentrations which produce changes in the physical properties of serum albumin (Neurath *et al.*, 1944; Frensdorff *et al.*, 1953; Kauzmann and Simpson, 1953; Markus and Karush, 1957), decrease both the binding of manganese and the enhancement of the complex supports the view that an intact secondary and tertiary structure of the protein is necessary for both the characteristic binding and enhancement exhibited by the tight binding site.

The protein surface to which the manganous ion is bonded clearly decreases the freedom of rotation of the hydration shell by steric hindrance. Furthermore, certain theoretical and experimental work suggests that a layer of water may be bonded to polar groups on the surface of proteins (Tanford, 1961) or be oriented near nonpolar groups into a lattice structure (Frank and Evans, 1945; Scheraga, 1960). It is possible that the water of hydration about a bound ion might participate in such immobilized arrangements of water molecules. Such weak bonds would further hinder the rotation of the hydration shell and thus increase τ_r. Although one or more of these mechanisms must be operating to explain the large enhancement observed, we are, at present, unable to determine the relative contribution of each. The biphasic effect of urea and the triphasic effect of guanidinium on ϵ_b suggest that enhancement may be the result of several processes and that these denaturants act in several stages or by several mechanisms.

The profound effect of low concentrations of guanidinium on the binding of manganese by serum albumin is best explained by a direct competition between the manganese and guanidinium cations for the tight binding site. The affinity constant for guanidinium indicates a free energy of binding of 1.7 kcal/mole at 25°, compatible with hydrogen bond formation (Pimental and McClellan, 1960). Thus guanidinium may compete with manganese by hydrogen bonding to one of its imidazole ligands.

Finally, it should be pointed out that effects on enhancement of the PRR of the manganese albumin complex occur at concentrations of urea and guanidinium below those required to produce changes in rotatory dispersion, indicating that the former is a more sensitive indicator of local changes of protein structure.

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The Formation of Hydridocobalamin and Its Stability in Aqueous Solutions

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Hydridocobalamin, the two-equivalent reduction product of cyanocobalamin, was prepared employing chromous chloride reagent and by electrolysis at a mercury pool cathode. Amperometric titration and electrolysis with measurement of the amount of electricity passed have shown that it is also the product of the reduction of cyanocobalamin at the dropping mercury electrode. Hydridocobalamin is oxidized to B_{12r} at the dropping mercury electrode with a half-wave potential at -0.87 v vs. saturated calomel electrode. It slowly decomposes water to yield hydrogen and B_{12r}. In aqueous solution hydridocobalamin has a half-life which varies from 355 minutes at *p*H 10.0 to 87 minutes at *p*H 8.0.

The stepwise reduction of vitamin B_{12}, (cyanocobalamin), first yields vitamin B_{12r}, an orange-brown product which contains +2 cobalt, and finally a green product, which possesses remarkable reducing properties. Vitamin B_{12r} was obtained by Diehl and Murie (1952) by catalytic hydrogenation of vitamin B_{12}, and its polarographic behavior has been fully investigated by Jaselskis and Diehl (1954). Because of its hydridelike properties, the green product has been called *hydridocobalamin* by Smith and Mervyn (1963), a name which we shall employ herein. Boos *et al.* (1953) doubtless produced B_{12r} as the brown product of one-equivalent reduction of vitamin B_{12} by chromous ion in EDTA solution. In some experiments, however, these workers must have formed hydridocobalamin since their spectrum of the "reduced vitamin B_{12}" is closer to that of hydridocobalamin than B_{12r}. Beaven and Johnson (1955), showed that hydridocobalamin was obtained on further reduction of B_{12r} with chromous acetate and thus clarified the relation between the reduction products obtained by Diehl's group and by Boos *et al.* Schindler (1951), found that B_{12r} was produced by the action of bisulfite or dithionite ions on the original vitamin, while treatment with zinc in NH_4Cl solution gave a *hellblau* product, which we assume was hydridocobalamin. Recent synthetic work in the area of cobalamin coenzymes by Smith *et al.* (1962) and Mueller and Mueller (1962) has shown that hydridocobalamin is a valuable intermediate in the synthesis of compounds which contain a carbon-cobalt bond. Chemical reduction of cyanocobalamin or hydroxocobalamin by means of zinc or sodium borohydride is ordinarily used to produce hydridocobalamin.

In the present work we shall show that hydridocobalamin is the final product in the titration of cyanocobalamin with chromous chloride in alkaline medium. Furthermore, the evidence is strong that hydridocobalamin is the product of electrolysis of cyanocobalamin at the dropping mercury electrode. This fact has led to the successful application of controlled potential electrolysis at a mercury pool cathode as a means of producing hydridocobalamin. Finally we shall present data on the rate of oxidation of hydridocobalamin by water in solutions of varying *p*H.

RESULTS AND DISCUSSION

Formation of Hydridocobalamin.—When one equivalent of chromous ion is added to cyanocobalamin in

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