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Binding of Ponceau 3R to Bovine Serum Albumin

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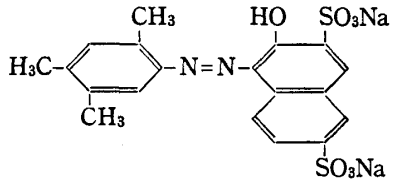
Interaction of Ponceau 3R with bovine serum albumin was studied by spectrophotometric, equilibrium dialysis and gel filtration method. In the presence of bovine serum albumin, absorption spectrum of Ponceau 3R shifted to higher wavelength from 503 mμ to 515 mμ with lowering optical density. The results of equilibrium dialysis and gel filtration showed that binding of Ponceau 3R to bovine serum albumin is essentially based on the Langmuir-type equation. Number of binding sites of bovine serum albumin to Ponceau 3R was approximately 5 over the pH range of 6 and 9, although it increased abruptly in further acidic region. Equilibrium constant of this reaction system was approximately $10^5 M^{-1}$ in neutral and alkaline region, whereas it increased in acidic region.

The interaction of organic ions with proteins has been studied extensively, since it is one of the fundamental subjects to know their nature and mechanism in the field of biology. In the field of drug research, for example, it has been recognized that interaction of drug with serum albumin plays an important role in the transport or membrane permeability in the blood streams. For this purpose dye has been used as a model of small molecules, since it is convenient for the determination of the amounts of binding and often reflects the nature of binding due to its spectral change in the interaction with proteins known as a metachromasy.

3-Hydroxy-4-[(2,4,5-trimethylphenyl)azo]-2,7-naphthalene disulfonic acid disodium salt (Ponceau 3R) is widely used as a reagent for the determination of various proteins in the electrophoresis and for the biological staining. Therefore, it is interesting to study the interaction of the dye with proteins. However, there have been few investigations[2] on this subject. In this paper, study has been made on the reversible interactions of the above azo dye with bovine serum albumin (BSA).



Ponceau 3R

Experimental

Reagents——Ponceau 3R was purchased from Wako Pure Chemical Industries, Ltd. and purified by recrystallization from EtOH.

Bovine serum albumin (BSA) used was "Fraction V" obtained from Sigma Chemical Company with further purification by the method of Cohn.[3] Water content was determined by drying to constant weight at 100°. A value of 70000 was used for the molecular weight of BSA.

Spectrophotometric Experiment——Optical density at the appropriate wavelength was measured with Hitachi Spectrophotometer, Model-124.

Equilibrium Dialysis——For the equilibrium dialysis experiment, commercial cellophane bag (Visking Company:18/32) was pretreated with hot saturated sodium bicarbonate solution and washed twice with

1) Location: *Shomachi-1, Tokushima.*
2) R.D. Strickland, T.R. Podleski, F.T. Gulule, M.L. Freeman and W.A. Childs, *Anal. Chem.*, **31**, 1408 (1959); H. Aizawa, *Fukushima-Daigaku Rika Hokoku*, **15**, 66 (1965).
3) E.J. Cohn, L.E. Strong, W.L. Hughes, Jr., D.J. Mulford, J.N. Ashworth, M. Melin and H.L. Taylor, *J. Am. Chem. Soc.*, **68**, 459 (1946).

hot water. The bags were filled with 4 ml of the reaction mixture containing the various concentration of Ponceau 3R and 10^{-4}M BSA in 0.1M phosphate buffer. Each bag containing the reaction mixture was immersed in 10 ml of 0.1M phosphate buffer and was kept at $4\pm0.5°$ for 72 hours. After equilibration the bags were removed and concentration of Ponceau 3R in the external solution of the bag was determined with spectrophotometer at 503 mμ. For each concentration of Ronceau 3R a controle experiment was done in the absence of BSA in the same procedure. Effect of phosphate ion in buffer on the binding affinity of Ponceau 3R was not detectable in the present experiment.

Gel Filtration Experiment——A column (10×150—600 mm) containing Sephadex G-100 was equilibrated with phosphate or acetate buffer. Samples (BSA-Ponceau 3R mixture, BSA or Ponceau 3R) were dissolved in the buffer and applied to the tops of the column and then eluted with buffer. Column was kept at $15\pm0.5°$ and effluents were collected in 5 ml fractions. Concentration of the effluents was determined by spectrophotometry.

Measurement of Optical Rotatory Dispersion——Measurement of optical rotatory dispersion was carried out with a JASCO spectropolarimeter, model ORD/UV-5. A value of 116 was used for mean residue molecular weight of BSA.

Result

Effect of BSA on the Spectral Property of Ponceau 3R

Absorption spectra of Ponceau 3R (concentration of Ponceau 3R: 4.10×10^{-5}M) at pH 7.0 were determined in the presence of various amounts of BSA, as shown in Fig. 1.

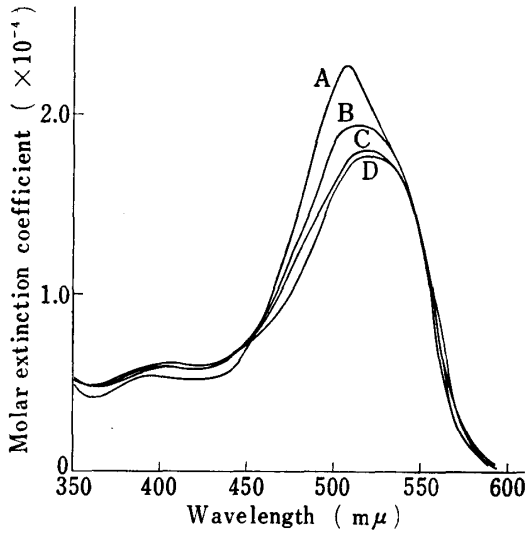


Fig. 1. Absorption Spectra of Ponceau 3R in the Presence of BSA at pH 7.0

concentration of Ponceau 3R: 4.10×10^{-5}M
A: ponceau 3R alone
B: in the presence of 0.44×10^{-5}M of BSA
C: in the presence of 1.20×10^{-5}M of BSA
D: in the presence of 2.80×10^{-5}M of BSA

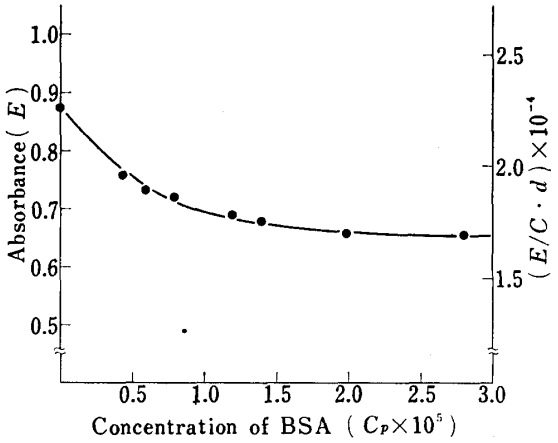


Fig. 2. Effect of BSA on the Optical Density of Ponceau 3R at 503 mμ (pH 7.0)

concentration of Ponceau 3R (C): 4.10×10^{-5}M

In the absence of BSA, λ_{max} was 503 mμ. In the presence of BSA (concentration of BSA: C_p), the value of optical density at 503 mμ decreased and λ_{max} of spectra shifted to higher wavelength with less absorbancy. When C_p was 3×10^{-5}M λ_{max} moved to 515 mμ. Fig. 2 shows the changes of the optical density at 503 mμ in the various concentration of BSA. Molar extinction coefficient of Ponceau 3R (ε_f) at 503 mμ was 2.26×10^4 M^{-1} cm^{-1}. A clear decrease in optical density was observed in the increasing value of C_p until about $C_p=10^{-5}$M. However, further increase in C_p caused smaller change and reached to a constant level. Absorbancy changes of Ponceau 3R caused by the presence of BSA was considered to be due to the effect of binding with protein and ratio of bound Ponceau 3R to total Ponceau 3R (α) is given in Eq. (1),[4] where C, C_f denote concentration of total and free Ponceau 3R respectively. E represents

$$\alpha = \frac{C-C_f}{C} = \frac{\varepsilon_f - E/C\cdot d}{\varepsilon_f - \varepsilon_b} \quad (1)$$

the optical density of the dye in the presence of various amounts of BSA (C_p). ε_b is a molar extinction coefficient of bound Ponceau 3R. If exact value of ε_b is obtained, the bound dye could be calculated from the spectrophotometric data. Decrease in E with the increasing in C_p reflects the increase in α in Eq. (1). From Fig. 2, it was suggested that an approximate value of ε_b is 1.70×10^4 M^{-1} cm^{-1}, although α was not evaluated in this experiment, since exact value of ε_b could not be obtained.

Binding of Ponceau 3R to BSA by Equilibrium Dialysis Method

Binding amount of Ponceau 3R to BSA was obtained directly by equilibrium dialysis method. Equilibrium dialysis experiments were carried out at 4° under various pH values. In Fig. 3, r/C_f versus r is plotted, and a relationship of straight line is observed in any pH. Results from the equilibrium dialysis also gave a linear relationship between $1/r$ and $1/C_f$ in any pH examined as discussed later. These facts indicate that Ponceau 3R binds to BSA according to Langmuir-type equation as shown in Eq. (2) and Eq. (3), where r is the moles of bound Ponceau 3R per one mole

$$r/C_f = n\cdot k - k\cdot r \quad (2)$$

$$1/r = (1/n\cdot k)\cdot(1/C_f) + 1/n \quad (3)$$

of BSA and k is an equilibrium constant of this binding. r is able to show in the following equation.

$$r = (C-C_f)/C_p \quad (4)$$

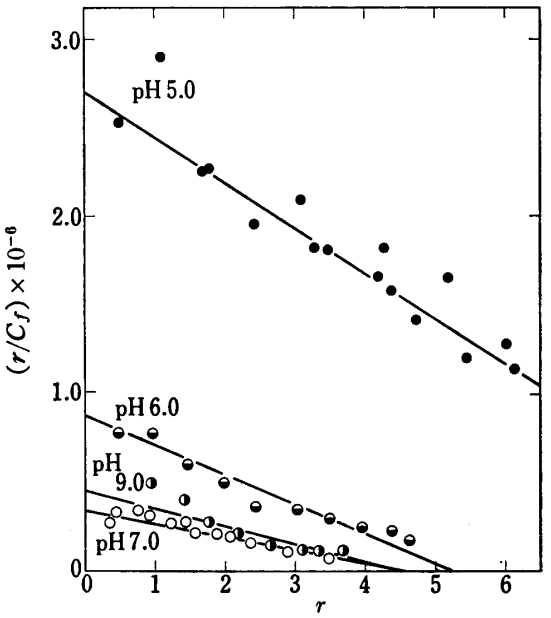


Fig. 3. Binding of Ponceau 3R to BSA at Various pH

Using Eq. (2), binding constant n and k were evaluated and are shown in Fig. 4 and Fig. 5. In the range of pH 6 and pH 9, the values of n took nearly constant and remained about 5, and at pH 5 the value of n increased abruptly reaching 11. From this result it is suggested that binding sites of BSA to Ponceau 3R are related to cationic residues of BSA, such as α-amino, ε-amino and guanidino groups.

The values of equilibrium constant k of this BSA-Ponceau 3R system were about 10^5 M^{-1} irrespective of pH values in the range of pH 7—9, whereas increased in acidic region. Such an increase in k in acidic pH was also observed in other protein-dye systems *i.e.*, BSA-methyl orange and lens proteins-methyl orange.[5] However, the increase of k in BSA-Ponceau 3R system is not striking as compared with those of other systems, since the value of k_{pH5}/k_{pH9} is 2.6 in the case of BSA-Ponceau 3R, while, 4.1 in β-crystallin-methyl orange and 13.1 in α-crystallin-methyl orange.[5]

In further acidic region less than pH 5, the binding experiments by equilibrium dialysis method could not be performed in the present experiment since the concentration of free Ponceau 3R outside of cellophane bags became hardly detectable due to the larger binding amounts of Ponceau 3R to BSA.

4) I.M. Klotz, *J. Am. Chem. Soc.*, **68**, 2299 (1946).
5) M. Nakagaki, N. Koga and H. Terada, *Yakugaku Zasshi*, **86**, 447 (1966).

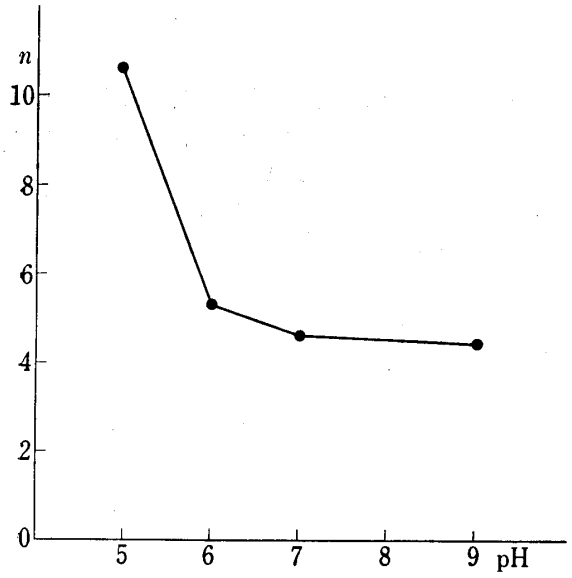


Fig. 4. Variation of n with pH

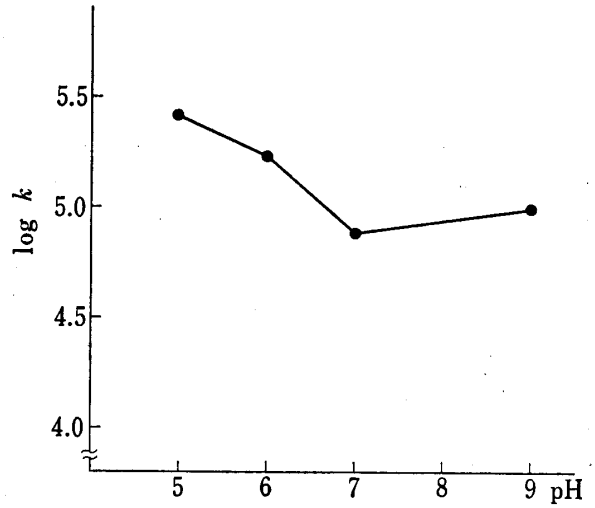


Fig. 5. Variation of k with pH

Gel Filtration of BSA-Ponceau 3R System under Acidic Conditions

As stated above, below pH 5 binding amounts of Ponceau 3R to BSA were not able to determine by equilibrium dialysis experiments, qualitative experiments by gel filtration were carried out. There have been some investigations on the interaction of various combinations of small molecules and proteins using gel filtration method recently, and many of them have been made by the method developed by Hummel and Dreyer.[6] However, in the use of this method, it seems rather difficult to elute dye solution through a gel filtration column in the case of BSA-Ponceau 3R system, probably due to the formation of insoluble protein-dye complex in acidic region which has often seen in the combination of small molecules and proteins.[7] Therefore, mixture of BSA and Ponceau 3R (C/C_p: 1—10) was applied to the column of Sephadex G-100, and was eluted with buffer as previously demonstrated by Lee and Debro[8] in the binding of phenol red with human serum albumin. The elution curve in the mixed system of protein and dye was compared with that of BSA or Ponceau 3R alone. Then the binding affinity was estimated qualitatively.

Gel filtration experiments were carried out at 15° using a column of 10×250 mm in pH 3.5—9.0. Fig. 6 shows the elution curves at pH 7.0 and pH 3.5, respectively.

At pH 7.0, elution curve in BSA-Ponceau 3R mixtures gave two peaks of Ponceau 3R, first peak (peak 1) was corresponded to that of BSA-Ponceau 3R mixed system and of BSA alone and second peak (peak 2) to that of Ponceau 3R alone. It is considered that Ponceau 3R eluted in peaks 1 and 2 indicates the movement of the BSA-Ponceau 3R system governed by Eq. (2). Therefore, it is suggested that in the elution process some part of bound Ponceau 3R becames free and is eluted mainly in peak 2. Amount of the released Ponceau 3R in the cource of gel filtration appeared to be proportional to column length and to reciprocal value of the binding affinity to BSA at the constant diameter of the column. Increasing in a height of column at pH 7.0, peak 1 was disappeared and all dye were found in peak 2. In the case of pH 9.0, similar result to pH 7.0 was obtained. However, in acidic region, for example at pH 3.5, Ponceau 3R was eluted only in peak 1, and not in peak 2. Subsequently, it was demonstrated that increasing column length caused an elution of small amount of Ponceau

6) J.P. Hummel and W.J. Dreyer, *Biochem. Biophys. Acta*, **63**, 530 (1962).
7) F.W. Putnum and H.J. Neurath, *J. Am. Chem. Soc.*, **66**, 692 (1944); K. Aoki and J. Hori, *Bull. Chem. Soc. Japan*, **29**, 104 (1956); R.G. Seals, T. Bynim, L. Watkins and J. Ross, *J. Dairy Sci.*, **48**, 737 (1965).
8) M. Lee and J.R. Debro, *J. Chromatog.*, **10**, 68 (1963).

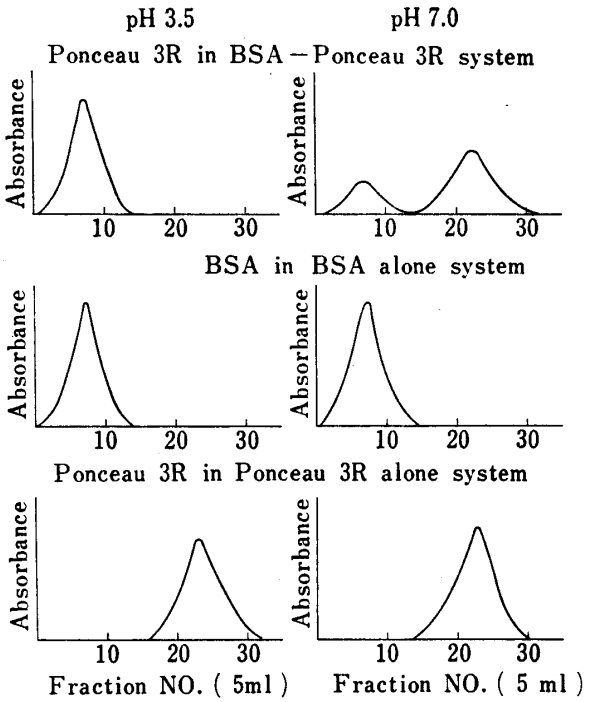


Fig. 6. Elution Pattern of a Mixture of BSA and Ponceau 3R, of BSA Alone and of Ponceau 3R Alone

Gel filtration experiments were carried out using 10×250 mm Sephadex G-100 column. Effluents were collected in 5 ml fractions. Amounts of Ponceau 3R and BSA were determined at 503 mμ and 280 mμ respectively with spectrophotometer.

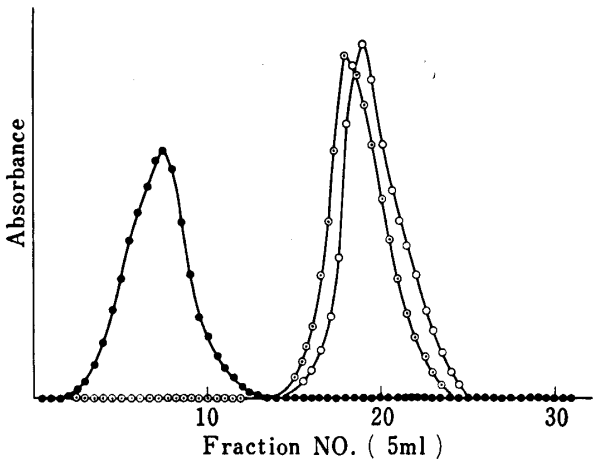


Fig. 7. Elution Pattern of a Mixture of BSA and Methyl Orange, of BSA Alone and of Methyl Orange Alone at pH 7.0

—●—: BSA in BSA-methyl orange and BSA alone systems
—⊙—: methyl orange in BSA-methyl orange system
—○—: methyl orange in methyl orange alone system
Gel filtration were carried out using 10×250 mm Sephadex G-100 column.

3R in peak 2. Therefore, it is suggested that binding mechanism in acidic region is essentially the same as in neutral region. Assuming that the amount of free Ponceau 3R in peak 1 is negligible, approximate amount of bound Ponceau 3R (r) can be estimated from the result of gel filtration experiment. In the case of 220 mm column length, r was 11 at pH 3.5 and was 1.5 at pH 7.0. These results indicate that binding affinity at pH 3.5 is estimated to be approximately 7 times greater than that of at pH 7.0.

Fig. 7 shows the elution curves of BSA-methyl orange system at pH 7.0 using 10×250 mm column; methyl orange has been commonly used in the binding study of proteins. In this case, methyl orange in BSA-methyl orange mixtures was not eluted in peak 1 but in peak 2, which differed from that of Ponceau 3R at pH 7.0. Binding of methyl orange with BSA is done according to Eq. (2) as has been shown previously[5] by equilibrium dialysis experiment. From the results of equilibrium dialysis, binding affinity of methyl orange for BSA is assumed to be 8 times smaller than that of Ponceau 3R at pH 7.0. The results in Fig. 6 and Fig. 7 show the difference of binding affinity between these two azo-dyes for BSA.

Discussion

In the present paper, the evidence of binding of organic dyes to protein was obtained from a decrease in optical density of dyes in the presence of protein. Subsequently, a quantitative consideration has been directed to get an amount of bound dye by spectrophotometric method and also by equilibrium dialysis.

The gel filtration technique described in this paper seems to be available for obtaining the information of the nature of binding. It was suggested that the elution pattern of dyes in the presence of protein directly depends on the binding affinity of dyes. Comparison of binding

of BSA-Ponceau 3R with BSA-methyl orange systems under various conditions were demonstrated using this method. It was expected to demonstrate the smaller elution volume (V_e) of BSA-Ponceau 3R complex than that of BSA alone especially under acidic conditions, since in the case of BSA-Ponceau 3R complex the sum of the molecular weight of the individual molecule is slightly greater than the molecular weight of BSA. However, it was failed to observe the difference between them in the present experiments. On the other hand, it was recognized that the peak of free Ponceau 3R in BSA-Ponceau 3R system (peak 2) located slightly ahead of the peak of Ponceau 3R alone in all experiments. This appears to be due to the gradual change of the equilibrium of these binding systems which is governed by the Eq. (2) in the filtration process. However, it was not successful to obtain any information of the binding from this in this paper.

Lee and Debro[8] have studied the binding of phenol red with human serum albumin using similar technique. In this case, phenol red binding was diminished as increasing in column length in a certain extent, but no decrease was demonstrated by further increasing in column length. Therefore, it is not likely that the same binding mechanism is present in BSA-Ponceau 3R and human serum albumin-phenol red systems. It may probably be due to the difference of the chemical structure of two dyes, *i.e.*, azo and sulfophthalein dyes, not due to the difference of proteins.

In view of the fact that the data of binding of Ponceau 3R to BSA can be fitted to both Eq. (2) and Eq. (3) at each pH examined, electrostatic repulsion among Ponceau 3R anions in the binding seems to be not participated in this system.[9]

TABLE I. λ_{max} of Absorption Spectra of Ponceau 3R in Various Solvent

Solvent	λ_{max} (mμ)
H_2O	503
40% methanol	507
40% ethanol	507
20% polyethylene glycol (400)[a]	508
BSA ($C_p=6.6\times10^{-5}$M) in H_2O	515

a) mean molecular weight: 380—420

The results of equilibrium dialysis and gel filtration experiments indicate that binding affinity of Ponceau 3R anions is nearly constant in neutral and alkaline region, but increases markedly in acidic condition. It is considered that this increase is mainly due to the increase in cationic charge of BSA in acidic region. Table I shows the values of λ_{max} of absorption spectra of Ponceau 3R in various organic solutions. The position of λ_{max} shifted to higher wavelength in any case by addition of organic solvent than that of aqueous solution of Ponceau 3R. It seems most likely that in these organic solvent, Ponceau 3R molecule is incapable of dissociation to an appreciable extent and remains such a form as RSO_3^-—Na^+. It is suggested, therefore, that a peak in the neighborhood of 510 mμ is a reflection of the short-range electrostatic interaction between Na^+ and sulfonate ion RSO_3^-, for in aqueous solution freely dissociated RSO_3^- shows a maximum at 503 mμ. These spectral shift in organic solvents are similar to that of BSA-Ponceau 3R system. On the basis of spectral properties, it should be expected that there is an interaction between sulfonate ion in Ponceau 3R molecule RSO_3^- and cationic loci of BSA P-NH_3^+ in this binding.[10] This suggets that electrostatic force plays an important role, though it does not always mean electrostatic force to be dominant in any pH. Regarding the value of $n\cdot k$ as a parameter of binding affinity,[11] relationship

9) I.M. Klotz, F.M. Walker and R.B. Pivan, *J. Am. Chem. Soc.*, **68**, 1486 (1946).
10) I.M. Klotz, R.K. Burkhard and J.M. Urquhart, *J. Phys. Chem.*, **56**, 77 (1952).
11) J. Koga, N. Morita and N. Kuroki, *Nippon Kagaku Zasshi*, **90**, 411 (1969).

between $n \cdot k$ and electrical charge of BSA (h) is shown in Fig. 8. In Fig. 8, decrease of $n \cdot k$ is linear as increasing in negative charge and electrostatic effect is observed in $-8 < h < 7$, but $n \cdot k$ is approximately constant in the range $-23 < h < -8$. If electrostatic factor is dominant in the range of $-23 < h < -8$, increase in negative charge may cause a decrease in $n.k$, since cationic residues of amino acid such as α-amino, ε-amino and guanidino groups are not completely dissociated.

To clarify the binding mechanism, thermodynamic treatment is considered to be useful. For this purpose binding data at different temperature must be necessary. In this paper equilibrium dialysis experiments at higher than 4° were not carried out to take into consideration of avoiding protein denaturation and rotting. It has often been observed an increasing in entropy in the binding of small molecules to protein or other high molecules.[13] In view of the fact that in the case of the dyes such as AG-1 and AG-2 which have similar molecular structure to Ponceau 3R induces positive entropy change in the BSA containing systems.[14] This entropy increase may be attributed to the presence of hydrophobic bonding. However, it is considered that conformational change of BSA in the binding process also gives increasing in entropy. Therefore, it seems to be not safe to emphasize the contribution of hydrophobic bonding from the thermodynamic data alone.

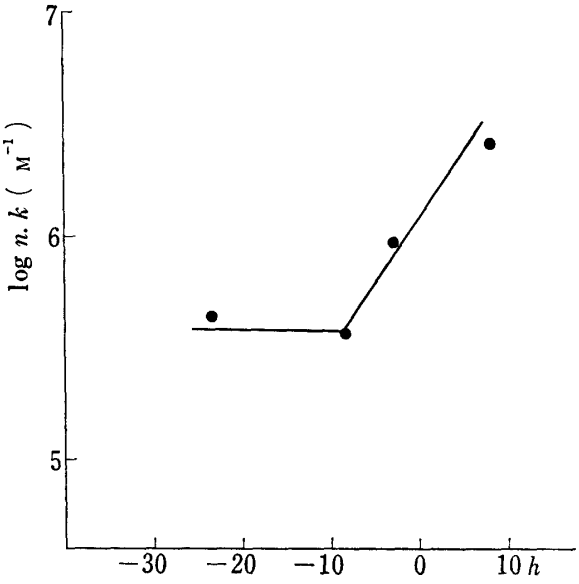


Fig. 8. Relationship between log $n \cdot k$ and Electrical Charge of BSA (h)

The values of h were calculated from the result of Tanford, *et al.*[12]

It has been observed by Markus, *et al.*[15] that presence of some kinds of dyes in BSA solution has induced a change in optical rotation of BSA molecule. But, Ponceau 3R from 2.11×10^{-5}M to 1.05×10^{-4}M has no effect on the optical rotation of BSA ($C_p = 2.62 \times 10^{-4}$M) in ultraviolet region near 233 mμ in any pH from pH 4.0 to pH 9.0. In the observation of difference spectra of BSA-Ponceau 3R mixture versus Ponceau 3R and BSA solution in the range of pH 4.0—10.0 using separate cell, there were significant differences between them at 268 mμ and 275 mμ. But, it is rather difficult to conclude the presence of conformational change of BSA from the results of difference spectra, since observed discrepancy may be overlapped by the effect of Ponceau 3R on the spectrum of BSA and vice versa. Nevertheless as has been pointed out by Karush,[16] there is a reversible structural alteration of BSA associated with binding process which is defined as a configurational adaptability. On the basis of the above considerations, it would be expected that some conformational change which are not detected by optical rotation measurement might be occured in the binding of Ponceau 3R, but it could not be successful to detect it distinctly at the present stage of the investigation.

12) C. Tanford, S.A. Swanson and W.S. Shore, *J. Am. Chem. Soc.*, **77**, 6414 (1955).
13) I.M. Klotz and J.M. Urquhart, *J. Am. Chem. Soc.*, **71**, 847 (1949); K. Nishida, T. Akimoto and H. Uedaira, *Kolloid Z. u. Z. Polymere*, **233**, 896 (1969).
14) T. Iijima and M. Sekido, *Kogyo Kagaku Zasshi*, **71**, 1199 (1968).
15) G. Markus and F. Karush, *J. Am. Chem. Soc.*, **80**, 89 (1958).
16) F. Karush, *J. Am. Chem. Soc.*, **72**, 2705 (1950); F. Karush, *J. Am. Chem. Soc.*, **76**, 5536 (1954).

Ponceau 3R has been commonly used for the quantitative determination of protein in electrophoretic separation with the use of such supporting medium as cellulose acetate membrane or acryl amide gels. Present results clearly show that presence of BSA gives error and its degree is the larger, the greater the amounts of protein present. In the case of the presence of BSA as shown in Fig. 2, its error became, for example, to be about 25% in the presence of 2.0×10^{-5}M of BSA. It is necessary to take care of the nature of binding affinity in the use of this method to determine the amounts of protein.