It seems difficult to reconcile results obtained by the straightforward application of a method for which there is apparently more than adequate justification. One is led to inquire, then, if this be due to variation in F at constant ionic strength, or if it be possible to obtain a linear plot in the presence of a higher complex absorbing in the same region. The first explanation need not be pursued since it would require plots with different slopes but the same intercept. If a second complex, CuX_2, is present then (1) reads

$$a/D = 1/(\epsilon_1 + \epsilon_2 K_2 b)K_1 b + (1 + K_2 b)/(\epsilon_1 + \epsilon_2 K_2 b) \quad (2)$$

where $K_2 = (CuX_2)/(CuX^+)(X^-)$ and ϵ_2 is the molar extinction coefficient for CuX_2. In order that a plot of a/D *vs.* $1/b$ be linear in the presence of CuX_2 it is necessary that the second derivative of a/D with respect to $1/b$ be zero, which requires that

$$\epsilon_2 = \epsilon_1(1 \pm \sqrt{1 - 4R})/2R \quad (3)$$

where $R = K_2/K_1$. This condition need be considered only if $R \leqslant 1/4$. Equation 2 becomes

$$a/D = 1/\epsilon_1 K_1 b + (1 \mp \sqrt{1 - 4R})/2\epsilon_1 \quad (4)$$

and the interesting result is that the slope of such a plot is the same as for (1) where it was assumed no higher complex was present. Thus linearity must not be considered as proof of the absence of higher complexes, even in appreciable amounts.

That these conditions are not infrequently met is evident from the fact that the constant for a complex is often several times smaller than that for the next lower complex, and the value of ϵ_2 given by (3) is such that $\epsilon_1 \leqslant \epsilon_2 \leqslant 2\epsilon_1$ (if the minus sign is used). This raises a question as to the use of continuous variation experiments for ruling out the existence of all but the indicated species.

Equation 3 provides the basis for explaining equal slopes and different intercepts for the plots of series III and IV. Moreover if McConnell's results for $CuCl^+$ and $CuCl_2$ are accepted, it is seen that they are very nearly the values that make (3) applicable. In the solutions he uses for study of $CuCl^+$ it is far less likely that $CuCl_2$ is present than in series I. Using his K_1, K_2 and ϵ_1 at 2500 Å., one calculates that ϵ_2 should be 4300 to make (3) apply, and McConnell's experimental value of ϵ_2 is 4000.

Assuming that (3) will explain the difference between series III and IV with equal success, one obtains the following estimates of ϵ_2 and K_2 for $CuBr^+$ at 27°: $\epsilon_2 \sim 300$ or 1200, $K_2 \sim 0.25$. Since McConnell finds for $CuCl_2$ that $\epsilon_2 \sim 4\epsilon_1$, the value 1200 should probably be chosen for ϵ_2.

In conclusion, the assumption is found to be reasonable but incorrect that only the mono-complex is present in the case of low copper to halide ratios. The discrepancy between results at the two extremes of copper to halide concentration ratios is due to the unsuspected presence of CuX_2. When this is accounted for general agreement, even between experiments at different ionic strengths, is obtained. One is cautioned not to view as unequivocal continuous variation results or linearity in plots of extinction data.

The enthalpy change for the dissociation of $CuBr^+$ was calculated from series II and the van't Hoff equation. For $CuBr^+$, $\Delta H = -4.8 \pm 2.0$ kcal., which is a considerably larger enthalpy change than McConnell reports for $CuCl^+$.

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Binding of the Cobaltous Ion by Native and Modified Bovine Serum Albumins

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As a part of our general investigations on metal-protein interactions, we have studied the binding of the cobaltous ion by bovine serum albumin and by its methyl ester and N-acetylated derivative in an acetate buffer (*p*H 6.5 and ionic strength 0.2). Esterification and acetylation of the protein were carried out by the methods of Fraenkel-Conrat,[1,2] *et al.* Analyses showed 100% esterification of the free carboxyl groups in the former derivative and 83% acetylation of the free amino groups in the latter.

The addition of the methyl ester to cobalt chloride solution did not alter its absorption spectrum in the range studied (440–540 mμ). The native and acetylated albumins, however, increased its absorption, the latter having a more pronounced effect; the wave length of maximum absorption remained unaltered at 515 mμ (Fig. 1).

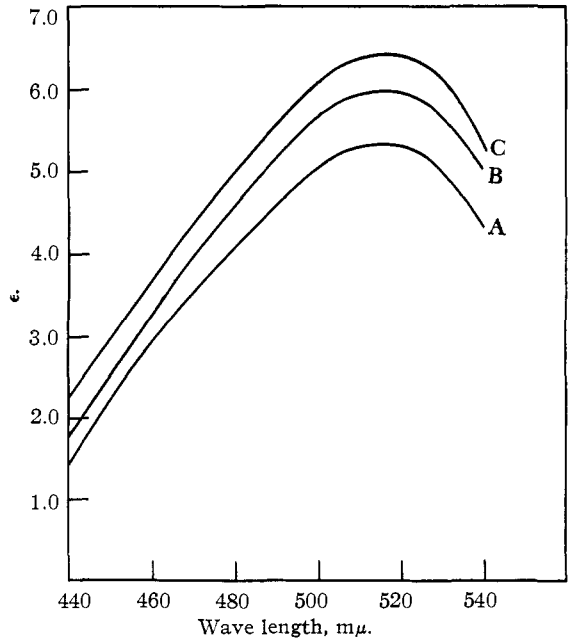

Fig. 1.—Absorption spectra in acetate buffer (*p*H 6.5, ionic strength 0.20): A, 0.057 *M* $CoCl_2$; B, 0.057 *M* $CoCl_2$ + 2% bovine serum albumin; C, 0.057 *M* $CoCl_2$ + 2% acetylated bovine serum albumin.

(1) H. Fraenkel-Conrat and H. S. Olcott, *J. Biol. Chem.*, **161**, 259 (1945).

(2) H. Fraenkel-Conrat, R. S. Bean and H. Lineweaver, *ibid.*, **177**, 385 (1949).

These results suggest that the acetylated derivative has a greater binding power for cobaltous ions than the native albumin, and that the methyl ester does not bind at all. The binding takes place probably through the carboxylate groups contributed by the aspartyl and glutamyl residues of the albumin molecule.

Binding studies were also made by the equilibrium dialysis technique of Klotz.[3] Cobalt was estimated colorimetrically using sodium β-nitroso-α-naphtholate as the coloring agent.[4] The methyl ester again did not show any interaction. The data for the native and acetylated albumins are given in Fig. 2, as a plot of the average number of metal ions (r) bound per mole of protein against the logarithm of the free metal ion concentration (A). A molecular weight of 70,000 has been assumed for both the proteins. It is seen that the acetylated albumin binds more than the native albumin. This may be due to breaking of the inter and/or intramolecular hydrogen bonding between NH_2 and COOH groups of the protein by acetylation, thus making more COOH groups available for binding.

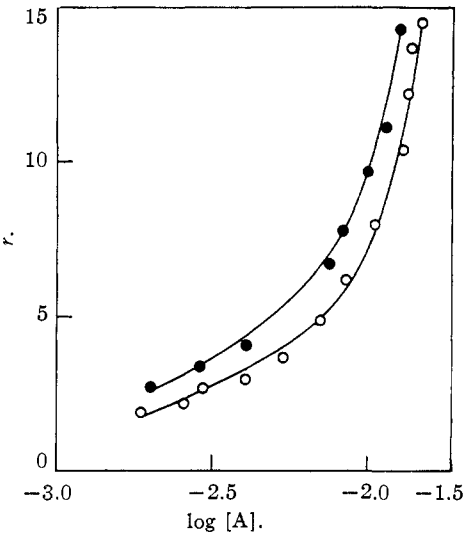

Fig. 2.—The binding of Co^{++} ions by serum albumins in acetate buffer at 25° (pH 6.5, ionic strength 0.20): -o-, bovine serum albumin; -●-, acetylated bovine serum albumin.

If statistical factors alone come into play in the binding of cobalt by the albumin molecule, one has[5]

$$1/r = K/n \times [1/A] + 1/n$$

where "n" is the maximum number of binding sites available on the protein molecule and "K" is an intrinsic dissociation constant. The plot of $1/r$ against $1/A$ shows a linear relationship up to an "r" value of 5. This contrasts with the behavior of cupric ions where no such linear relationship has been reported.[3] The association constant (k_1) and the free energy change (ΔF_1) for the first metal ion bound, both with the native and the acetylated albumin are given in the Table as also the values of "n."

TABLE I

Protein	k_1	ΔF_1, cal./mole	n
Native bovine serum albumin	1.0×10^3	−4090	15
Acetylated bovine serum albumin	1.5×10^3	−4330	15

At values of "r" greater than 5, the $1/r$ *vs.* $1/A$ plot deviates from linearity thus suggesting that the number of binding sites available has probably increased. This may be due to configurational changes in the protein molecule consequent on binding cobalt ions. The electrophoretic mobility data presented in Fig. 3 also support such a view. The negative mobility decreases linearly with increasing values of "r" up to $r \sim 5$ and then tends toward a steady value.

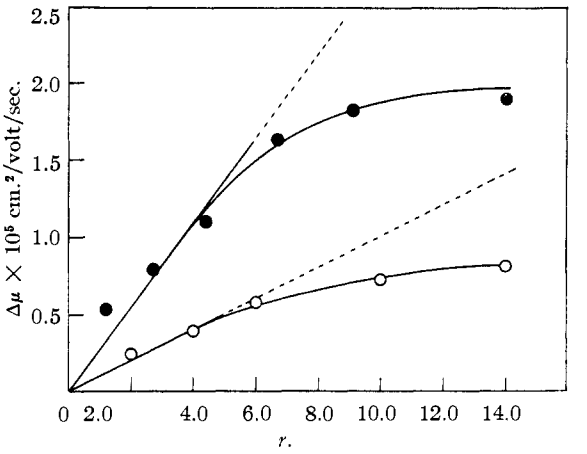

Fig. 3.—The effect of the binding of Co^{++} ions on the electrophoretic mobility of serum albumins (acetate buffer, pH 6.5, ionic strength 0.20); $\Delta\mu$ is the decrement in the negative mobility of the proteins consequent on binding Co^{++} ions. The mobility of bovine serum albumin was -3.03×10^{-5} cm.²/volt/sec., and of the acetylated serum albumin -7.70×10^{-5} cm.²/volt/sec.; -o-, bovine serum albumin; -●-, acetylated bovine serum albumin.

The mobility decrement per metal ion bound ($\Delta\mu/\Delta r$) is 0.10×10^{-5} cm.²/volt/sec. for the native albumin and 0.28×10^{-5} cm.²/volt/sec. for the acetylated derivative. This is in keeping with the larger binding capacity of acetylated albumin for cobalt ions. An exact interpretation of the results must, however, await a more detailed study of this as well as other metal–protein systems.

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