Determination of Base Sequence in Nucleic Acids with the Electron Microscope. IV. Nucleoside Complexes with Certain Metal Ions*

A. Max Fiskin† and Michael Beer

ABSTRACT: The complex formation constants for Cu^{2+} and Pb^{2+} complexes involving the acidic and basic sites of the nucleosides are determined by *p*H-stat titration, and selectivity is demonstrated in complex formation at the basic sites. Spectrophotometric and conductometric studies indicate a maximum stoichiometry of two nucleosides per metal in these complexes.

Recently there has been considerable interest in the basic sites of the nucleosides, and in their possible involvement in complex formation with metal ions. The basic sites have been identified as N_1 for adenosine (Bryan and Tomita, 1962a), N_3 for cytidine (Miles *et al.*, 1963a; Jardetsky *et al.*, 1963; Bryan and Tomita, 1962b), and N_7 for guanosine (Sobell and Tomita, 1964; Miles *et al.*, 1963b; Jardetsky and Jardetsky, 1960).

Previous studies (Albert, 1953) suggested that weakly associated complexes of first transition series metal ions with the basic sites of the nucleosides do exist. No quantitative estimates have been given for their stabilities. Albert, and later Frieser and his collaborators (Harkins and Frieser, 1958; Cheney *et al.*, 1959) and Eichorn and Clark (1963), were unable to measure any proton release from basic sites in the presence of such ions. The charged complexes of the nucleosides with mercury salts have, however, been characterized (Ferreira *et al.*, 1961; Eichorn and Clark, 1963; Simpson, 1964), and complex formation constants have been measured. The conclusion that mercury complexes with guanosine and cytidine are more stable than those with adenosine supports the evidence of Frieden and Alles (1958) for preferential binding to guanosine.

This paper presents a method of determining formation constants of weakly associated complexes. This procedure is used to find the formation constants for nucleoside complexes with Cu^{2+} and Pb^{2+}. Finally, the implications of these results in an electron microscopic base-sequence determination are discussed.

Experimental

Materials. Adenine (A), cytosine (C), guanine (G), and uracil (U) ribonucleosides, and thymine (T) desoxyribonucleoside were obtained from the California Corp. for Biochemical Research. Reagent grade $Cu(NO_3)_2 \cdot 3\ H_2O$, $Pb(NO_3)_2$, $UO_2(NO_3)_2 \cdot 6\ H_2O$, and $Th(NO_3)_4 \cdot 4\ H_2O$ were obtained from Mallinckrodt Chemical Works. All solutions were prepared in 1 M sodium nitrate. The approximately 0.1 N sodium hydroxide was standardized with reagent grade potassium acid phthalate (J. T. Baker Chemical Co.), and this base was used to standardize an approximately 0.1 N nitric acid solution prepared by dilution of 70% nitric acid (J. T. Baker Chemical Co.).

Apparatus. The titration apparatus consisted of a Pyrex beaker loosely fitted with a transparent plastic cover, through which could be inserted a thermometer, a nitrogen inlet tube, and the buret delivery tube and electrode of an automatic titrating Radiometer *p*H meter, Type TTTl. A Radiometer Type GK 2021 B combination electrode was employed. A water bath maintained the temperature in the test solution at 20.0 ± 0.5°. The test solution was stirred with a magnetic stirrer and maintained in an atmosphere of carbonate-free nitrogen. The *p*H meter was standardized with Beckman standard buffer solution (*p*H 7.00 ± 0.02) and Fisher Scientific Co. standard buffer solutions (*p*H 2.00 ± 0.02 and *p*H 10.00 ± 0.02). Absorption spectra were measured on a Bausch and Lomb Spectronic 505 recording spectrophotometer using 10-cm, 1-cm, and 1-mm matched quartz cells. Pb^{2+} complexes were studied with a Radiometer conductivity meter, Type CDM 2d, equipped with a 1-ml microcell.

Job's Analyses (Job, 1928; Vosburgh and Cooper, 1941). Equimolar solutions (molarity dependent on solubility of nucleosides) of the metal ion and the nucleoside were prepared in 1 M sodium nitrate and adjusted to the chosen *p*H. These solutions were mixed in varying proportions to a chosen volume and compared to blanks with 1 M sodium nitrate (at the chosen *p*H) substituted for one reactant. Hyperchromic shifts

* From the Thomas C. Jenkins Department of Biophysics, Johns Hopkins University, Baltimore, Md. *Received December 23, 1964; revised March 23, 1965.* This investigation was supported by a research grant (GM-08968) from the National Institutes of Health, U.S. Public Health Service. A preliminary report was presented before the American Chemical Society, Chicago, August 29–September 4, 1964.

† Research Career Development Fellow (5-F1-GM-15, 403-03), U.S. Public Health Service.

in the cupric nitrate maximum at 695 mμ were used in the study of copper complexes, and differences in conductivity were used in studying Pb^{2+} complexes.

Titration Procedure. BASIC SITES. A given volume of standard nitric acid solution and 0.5×10^{-3} mole of nucleoside were diluted to 100 ml with carbonate-free 1 M sodium nitrate solution. This solution was titrated to an end point close to the expected pK_a for the nucleoside, and the volume of standard base required was recorded. A weighed quantity of the nitrate salt of the metal was then added, and the quantity of hydrogen ions displaced was measured by the volume of standard base required to maintain the *p*H at the end point. This procedure was repeated in the absence of nucleoside, and the difference between the volume of base required for the blank and the nucleoside solution was used to determine the acid dissociation constant and complex formation constant as described. Each titration was performed in triplicate at metal to ligand ratios of 1.5:1 and 3.0:1.

ACIDIC SITES. A given volume of standard nitric acid solution and a weighed quantity of metal nitrate were diluted to 100 ml with carbonate-free 1 M sodium nitrate solution and titrated to *p*H 5.0. Nucleoside (0.5×10^{-3} mole) was then added, and the quantity of hydrogen ions displaced was measured by the volume of standard base required to maintain this *p*H. The procedure was repeated in the absence of metal ion to verify the absence of hydrogen ion displacement upon addition of nucleoside to the sodium nitrate solution. Each titration was performed in triplicate at a 3.0:1 metal-to-ligand ratio. The K_a was determined in triplicate by adding 0.5×10^{-3} mole of nucleoside to 100 ml of 1 M $NaNO_3$ at *p*H 10.0, and recording the volume of standard base required to maintain this *p*H.

Calculation of Formation Constants. The notation given in Table I (Rossotti and Rossotti, 1961) is used.

$$K_a = \frac{a \cdot h}{ah} = \frac{1}{K_h} \tag{1}$$

$$K_n = \frac{ba_n}{ba_{n-1} \cdot a}, \quad \beta_n = K_1 \cdot K_2 \cdots K_n = \frac{ba_n}{b \cdot a^n} \tag{2}$$

$$H = C + jA - M = h - K_w \cdot h^{-1} + ah,$$

$$j = \text{dissociable hydrogen added per } A \tag{3}$$

$$\bar{n} = \frac{A - (a + ah)}{B} = \frac{\sum_1^n n \cdot \beta_n \cdot a^n}{\sum_0^n \beta_n \cdot a^n} \tag{4}$$

From (4) it is apparent that given a and K_a, and therefore $\bar{n}$, at n or more reactant concentrations, n equations with n unknowns (β's) are obtained, for which numerous methods of solution are available.

BASIC SITES. Considering the unprotonated nucleoside as ligand A, j is zero, and the difference between M for the nucleoside solution and for the blank in (3), considering changes in total volume, gives

$$\{ah\}_i = (v_0 - v_1)[(NV + \{C\})/(V + v_0)] \tag{5}$$

where subscripts 0 and 1 denote additions to blank and nucleoside solution, respectively; K_h is then given from (2) and h. When $\{B\}$ moles of cation are added to the nucleoside solution at the chosen *p*H, the dissociation of ah is driven by complex formation, releasing hydrogen ions. The increment in $\{ah\}$, $\Delta\{ah\}$, considering changes in total volume, is

$$\Delta\{ah\} = (v_3 - v_2)N + (v_3 - v_2)10^{-n} \tag{6}$$

The subscripts 2 and 3 denote additions to blank and nucleoside solutions, respectively, and the *p*H is chosen so that less than 0.005% of B is hydrolyzed ($\approx v_2$). The number of moles of protonated ligand $\{ah\}_f$ is given by (5) and (6), and with K_h determines $\{ah\}_f + \{a\}_f$ and $\bar{n}$.

ACIDIC SITES. Considering the anion as the ligand, and adding the neutral nucleoside to a solution at *p*H 10.0, gives $C = 0$, $j = 1$; (3) then gives, considering changes in total volume,

$$\{A\} - \{ah\}_i = \{a\}_i = v_4 N + 10^{-10} v_4 \tag{7}$$

from which K_h is readily obtained. When nucleoside is added to a solution of metal cation at a *p*H where the concentration of free anion is negligibly small (*p*H 5.0), the volume of standard base required to maintain the *p*H gives total bound ligand directly:

TABLE I: Notation for Calculation of Formation Constants.[a]

	Total Added Concentration	Equilibrium Concentration
Ligand	A	a
Central group	B	b
Dissociated acid	C	
Dissociable hydrogen	H	
Hydrogen ion		h
Standard base	M	
Products		ah, ab, etc.
Volume before standard base	V	
Volume of N-normal standard base added	v	
Moles of species x at equilibrium	$\{x\}$	
Subscripts denoting quantities prior to and following mixture of ligand and central group	i, f, respectively	

[a] After Rossotti and Rossotti (1961).

$$\{A\} - (\{a\}_f + \{ah\}_f) = \Delta\{ah\} = \nu_5 N + 10^{-5}\nu_5 \quad (8)$$

COMPUTATION. For the complexes studied it was convenient to express the data in the form:

$$K_1^* = \frac{r}{a(B - r)}, \text{ where } r = A - (a_f + ah_f) \quad (9)$$

as determined by (6) and (8). For $n = 2$, equation (2) can be expressed as

$$K_1^* = \beta_1 + \beta_2(2a + K_1^* \cdot a^2) \quad (10)$$

Then β_1 and β_2 are obtained by simultaneous solution of (10) at two concentrations. Note that K_1 must be less than K_1^*, and that for complexes with nucleoside anion where $B \approx 10^5 a$, K_1 is virtually equal to K_1^*.

Results

The dissociation constants for the protonated and acidic moieties of the nucleosides are given in Table II.

TABLE II: Dissociation Constants of Nucleosides.

Nucleoside	*p*H Used	Mean mM H[a] Bound[b]	pK_a
A, N1	3.50	0.3073 ± 0.0016[a]	3.703 ± 0.008
C, N3	4.00	0.3144 ± 0.0012	4.229 ± 0.004
G, N7	2.00	0.3077 ± 0.0134	2.20 ± 0.05
G, N1	10.00	0.4260 ± 0.0022	9.24 ± 0.01
T, N3	10.00	0.3463 ± 0.0034	9.65 ± 0.01
U, N3	10.00	0.4321 ± 0.0020	9.20 ± 0.01

[a] Standard error of the mean, after Snedecor (1956). [b] Equations (5) and (7).

These have been previously determined at variable ionic strength (Levene and Simms, 1925; Fox and Shugar, 1952; Albert, 1953; Harkins and Frieser, 1958; Cheney *et al.*, 1959; Ferreira *et al.*, 1961; Simpson, 1964). The values given here are in excellent agreement with those found in the literature, with the largest deviation (0.20 *p*H unit) observed for the basic pK_a of guanosine given by Simpson ($pK_a = 2.40$).

The estimated formation constants (K_1^*) for 1:1 complexes, determined with the mean values of the dissociation constants, are given in Table III with the measured $\Delta\{ah\}$ as obtained from equations (6) and (8). The dependence of the experimental error on the sensitivity of the automatic titrating potentiometer is reflected by the relatively high error at *p*H 2.0. Where data are not presented for any nucleoside-metal system, the hydrogen ion displacement was below the sensitivity of the apparatus. Applying the data to equation (10) reveals that the range of K_1^* permitted by the experimental error, for the weakly bound complexes studied, permits solutions varying from $K_1 \approx 0$, $K_2 \approx \infty$ to $K_1 \approx K_1^*$, $K_2 \approx 0$. The literature on the coordination characteristics of the bases (Harkins and Frieser, 1958; Cheney *et al.*, 1959) supports the prediction that $K_1 \geq K_2$ for the nucleoside complexes (Cotton and Wilkinson, 1962). Applying to this data the assumption that $K_1 \geq K_2$ shows K_1 must differ from K_1^* obtained at the 3.0:1 ratio by less than the experimental error. The only instance in which K_1^* appeared to vary with metal concentration in these experiments was with complexes of cytidine and Cu^{2+}; applying these data to equation (10) gives the upper limit for K_1 as

$$K_1 = 28 \; (K_2 = 3.2 \times 10^2)$$

Table IV presents the results of Job's method of continuous variations for the stoichiometry of A, C, and G complexes with Cu^{2+}, and Figure 1 presents the data for Cu^{2+}—C complexes. Figure 2 presents the results of conductivity studies of complexes of Pb^{2+} and C. The insolubility of G and the negligible association of Pb^{2+} with A prevented an analysis of the stoichiometry of these Pb^{2+} complexes. The intersection of the linear portions of the curves obtained for Cu^{2+} and Pb^{2+} complexes was from mole fraction of nucleoside 0.63–0.67, indicating 2:1 nucleoside-to-metal stoichiometry for all complexes studied.

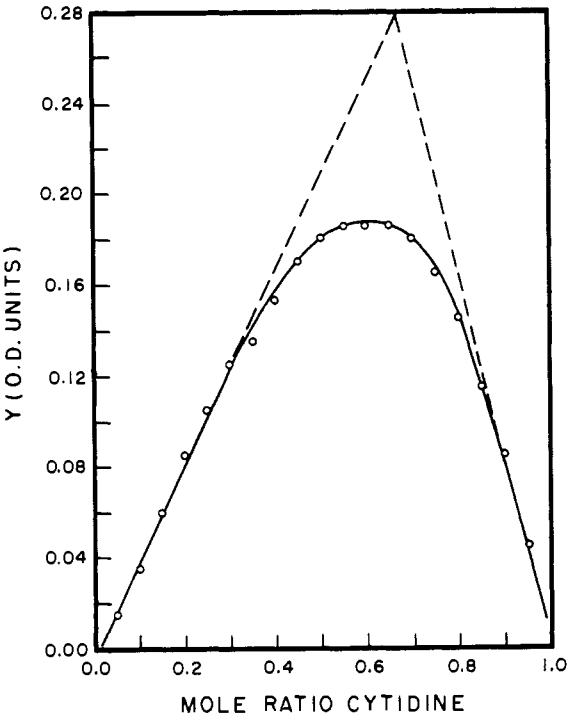

FIGURE 1: Job's method of continuous variations of Cu^{2+} and cytidine; 0.1 mole/liter 1 M $NaNO_3$; *p*H 4.0. Y = absorptivity of Cu^{2+} blank − absorptivity of reaction mixture at 695 mμ versus mole fraction of cytidine.

TABLE III: Formation Constants for Nucleoside Complexes.

Nucleoside	Cu^{2+} (mM)		Pb^{2+} (mM)	
	0.75	1.50	0.75	1.50
A, *p*H 3.50				
mM H^+ released[a]	0.0042 ± 0.0020	0.0085 ± 0.0008[b]		0.0000
K_1*	5.0 ± 2.3	5.1 ± 0.7		0.3[c]
C, *p*H 4.00				
mM H^+ released	0.0295 ± 0.0011	0.0499 ± 0.0023	0.0079 ± 0.0023	0.0148 ± 0.0017
K_1*	40.5 ± 1.5	36.6 ± 2.2	9.5 ± 3.0	9.2 ± 1.0
G, *p*H 2.00				
mM H^+ released	0.0667 ± 0.0034	0.1118 ± 0.0018		0.0045 ± 0.0014
K_1*	139.3 ± 10.0	140.3 ± 4.0		3.0 ± 1.0
G^-, *p*H 5.00				
mM H^+ released		0.0068 ± 0.0045		0.0000
K_1*		(2.2 ± 1.4)10^4		3×10^3[c]
T^-, *p*H 5.00				
mM H^+ released		0.0079 ± 0.0023		0.0008 ± 0.0005
K_1*		(4.8 ± 1.4)10^4		(5.0 ± 3.5)10^3
U^-, *p*H 5.00				
mM H^+ released		0.0068 ± 0.0023		0.0011 ± 0.0006
K_1*		(1.5 ± 0.5)10^4		(2.4 ± 1.3)10^3
G, *p*H 2.00[d]	UO_2^{2+} (1.5 mM)		Th^{4+} (1.5 mM)	
mM H^+ released	0.0079 ± 0.0012		0.0127 ± 0.0032	
K_1*	5.3 ± 1.8		8.8 ± 2.3	

[a] Equations (6) and (8). [b] Standard error of the mean, after Snedecor (1956). [c] Maximum value determined by machine sensitivity. [d] Hydrolysis of these ions limits quantitative studies to *p*H less than 3.00.

TABLE IV: Job's Analyses of Stoichiometry of Cu^{2+} Complexes.

(L) + (Cu^{2+})	*p*H	Mole Fraction for Maximum Spectral Shift at 695 mμ[a]
A 0.086 M	4.0	0.54–0.68
C 0.10 M	4.0	0.53–0.68
G 0.02 M	2.0	0.60–0.70

[a] Range in which spectral shift differed from the maximum by 0.5% of the maximum shift or less.

Discussion

It has been emphasized that the accuracy of these experiments is determined by the sensitivity and stability of the automatic titrating potentiometer, which depend strongly on the *p*H range employed. At *p*H 4.0 the titrator used responds to a change in h of less than 1×10^{-6} M and at *p*H 2.0 to a change of less than 5×10^{-5} M; this level of sensitivity is stable for up to 60 minutes. The standard-error calculations have been reported primarily as an index of the reproducibility of the experiments.

The relative stability of these complexes of Cu^{2+} and Pb^{2+} agrees with the empirical stability sequence given by Mellor and Maley (1947) (Cu ≥ Ni ≥ Pb ≥ Co ≥ Zn). The observation of formation constants for G and C complexes, at least an order of magnitude greater than those for A complexes, parallels Simpson's observations of mercury complexes (Simpson, 1964). The comparatively strong binding with methylmercury (log K = 3.0, 4.6, and 4.5 for A, C, and G, respectively) is expected because of its greater affinity for nitrogen-donor sites. It is interesting to note that the relative stability of Pb^{2+} complexes with C and G is the reverse of the stability of Cu^{2+} complexes.

The complexes of lead, uranyl, and thorium salts with biologically important molecules are particularly significant because these salts are frequently used to stain biological preparations for electron microscopic examination. In particular we are exploring the possibility of employing electron-dense reagents to determine the base sequence of nucleic acid molecules with the electron microscope (Beer and Zobel, 1961; Beer and Moudrianakis, 1962). This method requires conditions for selectively loading particular nucleotide components with electron-dense atoms. Accordingly, these experiments were designed for determination of the complex-formation constant K_1, which is required for prescribing staining conditions, and to compare the complexes of these heavy metal ions to those of the

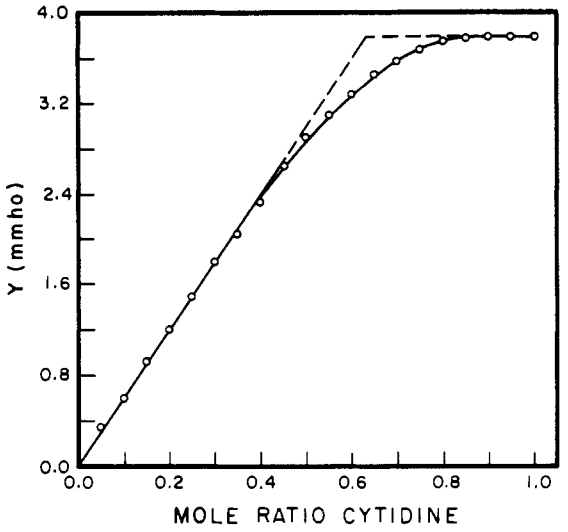

FIGURE 2: Job's method of continuous variations of Pb^{2+} and cytidine; 0.25 mole/liter 1 M $NaNO_3$; *p*H 4.0. Y = conductivity of Pb^{2+} blank − conductivity of reaction mixture versus mole fraction of cytidine.

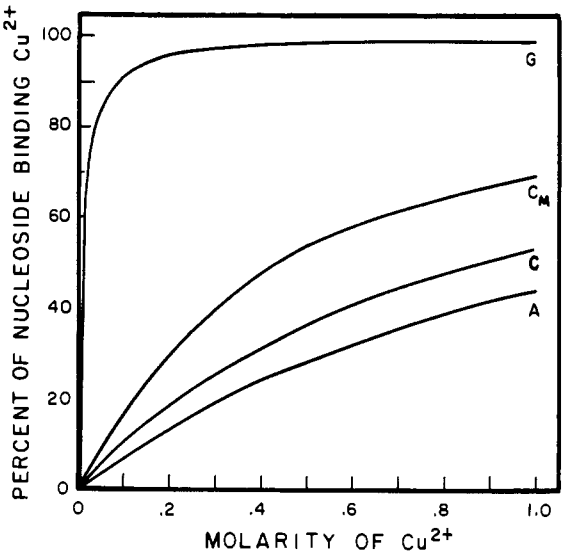

FIGURE 3: Binding of Cu^{2+} to the basic sites of the nucleosides at *p*H 3.00. A (adenosine), C (cytidine, using K_1), C_m (cytidine, using K_1*), and G (guanosine). All solutions 5×10^{-3} M nucleoside in 1 M $NaNO_3$.

more extensively studied cupric ion. (It should be pointed out that the limited solubility of the nucleosides precludes obtaining the ligand: metal ratios required for studying K_2 for these complexes).

It can readily be demonstrated that binding to the nucleosides in solution is dependent on the *p*H as well as relative concentrations. From K_1 and K_a for any nucleoside–metal ion system the fraction of ligand molecules binding metal ions can be calculated for any *p*H and metal ion concentration by the equation

$$\frac{ab}{A} = \frac{sQ}{sQ + Q + 1}, \qquad s = \frac{ab}{a} = K_1 b \qquad (11)$$

$$Q = \frac{a}{ah} = \frac{K_a}{h}$$

Using these relations the per cent of nucleosides which bind cupric ions has been plotted versus metal ion concentration at *p*H 3.0 (Figure 3) and *p*H 4.0 (Figure 4). It is seen that at 0.05 M Cu^{2+} about 90% of the guanosine basic sites are involved in complexes while less than 10% of the other nucleosides have bound metal. The *p*H sensitivity of this preferential binding is clear when these data are compared to those at *p*H 4.0. Here adenosine, cytidine, and guanosine are all extensively involved in complex formations at concentrations where uridine and thymidine are not. Thus the binding of metal ions to the basic sites of the nucleosides is selective and dependent on the *p*H. The formation constants for binding with G^-, T^-, and U^- indicate great selectivity for these ligands at high *p*H. However, with the metal ions used here this selectivity cannot be realized experimentally since the metal ions precipitate on hydrolysis.

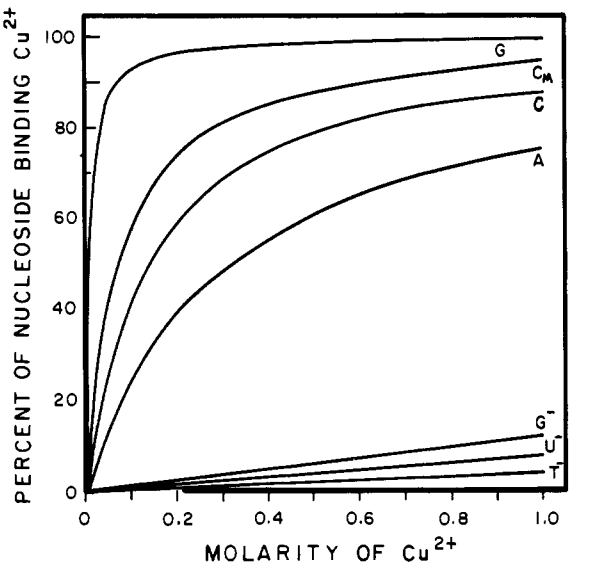

FIGURE 4: Binding of Cu^{2+} to the nucleosides at *p*H 4.00. A (adenosine), C (cytidine, using calculated K_1), C_m (cytidine, using K_1*), G (guanosine), G^- (guanosine anion), T^- (thymidine anion), and U^- (uridine anion). All solutions 5×10^{-3} M nucleoside in 1 M $NaNO_3$.

Our purpose in testing the selectivity of metal binding was to develop nucleoside-selective stains for an electron-microscopic determination of base sequence in nucleic acids. In a forthcoming paper we shall publish studies on a reagent giving selectivity as a consequence of differences in its formation constants which satisfies visibility criteria for electron microscopy.

References

Albert, A. (1953), *Biochem. J. 54*, 646.
Beer, M., and Moudrianakis, E. N. (1962), *Proc. Natl. Acad. Sci. U.S. 48*, 409.
Beer, M., and Zobel, R. C. (1961), *J. Mol. Biol. 3*, 717.
Bryan, R. F., and Tomita, K. (1962a), *Acta Cryst. 15*, 1179.
Bryan, R. F., and Tomita, K. (1962b), *Acta Cryst. 15*, 1174.
Cheney, G. E., Frieser, H., and Fernando, Q. (1959), *J. Am. Chem, Soc. 81*, 2611.
Cotton, F. A., and Wilkinson, G. (1962), Advanced Inorganic Chemistry, New York, Interscience, p. 544.
Eichorn, G. L., and Clark, P. (1963), *J. Am. Chem. Soc. 85*, 4020.
Ferreira, R., Ben-zui, E., Yamane, T., Vasilerskis, J., and Davidson, N. (1961), *in* Advances in the Chemistry of Coordination Compounds, Kirschner, S., ed., New York, Macmillan, p. 457.
Fox, J. J., and Shugar, D. (1952), *Biochim. Biophys. Acta 9*, 369.
Frieden, E., and Alles, J. (1958), *J. Biol. Chem. 230*, 797.
Harkins, T. R., and Frieser, H. (1958), *J. Am. Chem. Soc. 80*, 1132.
Jardetsky, C. D., and Jardetsky, O. (1960), *J. Am. Chem. Soc. 82*, 222.
Jardetsky, O., Pappas, P., and Wade, N. G. (1963), *J. Am. Chem. Soc. 85*, 1657.
Job, P. (1928), *Ann. Chem. 9*, 113.
Levene, P., and Simms, H. (1925), *J. Biol. Chem. 65*, 519.
Mellor, D. P., and Maley, L. (1947), *Nature 159*, 370.
Miles, H. T., Bradley, R. B., and Becker, E. D. (1963a), *Science 142*, 1569.
Miles, H. T., Howard, F. B., and Frazier, J. (1963b), *Science 142*, 1458.
Rossotti, F. J. C., and Rossotti, H. (1961), The Determination of Stability Constants, New York, McGraw-Hill.
Simpson, R. B. (1964), *J. Am. Chem. Soc. 86*, 2059.
Snedecor, G. W. (1956), Statistical Methods, Ames, Iowa, Iowa State Press.
Sobell, H. M., and Tomita, K. (1964), *Acta Cryst. 17*, 126.
Vosburgh, W. C., and Cooper, G. F. (1941), *J. Am. Chem. Soc. 63*, 437.