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The Effect of Metal Ions on the Hydrolysis of L-Asparagine by L-Asparaginase

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A variety of main group and transition metal ions have been studied as moderators of the enzymatic hydrolysis of L-asparagine by L-asparaginase. The greater deactivations were produced by mercury(II), copper(II) or tetrachloropalladate(II) ions when each moderator was incubated with the enzyme before the hydrolysis reaction was initiated. Different results were observed for copper(II) and mercury(II) when the moderator was incubated with the substrate before adding the enzyme; in this latter situation the copper(II) ion appears to act as a co-factor for the reaction. It has been shown that in the presence of each of the metal ions chosen the energy of activation of the moderated enzymatic hydrolysis reaction remains unchanged from that of the control reaction.

Introduction

The clinical use of L-asparaginase as an antineoplastic substance is based on the assumption that certain tumour cells have lost their ability to synthesize L-asparagine and thus have to rely on an exogenous supply of this amino acid [1]. L-Glutaminase has also been shown to possess antitumor activity [2]. We proposed [3] that L-asparagine or L-glutamine might be used in conjunction with cytotoxic metal ions to selectively "poison" tumor cells. Bis(L-asparaginato)copper(II) and bis(L-glutaminato)copper(II) were prepared in this laboratory [3] and an X-ray crystal structure of the former substance has been determined [4]. We described [3, 5] the use of induction of filamentous growth in *Escherichia coli* as a preliminary test-system for evaluating the potential antitumor activity of amino acid metal systems. Since L-asparaginase is a constituent of *E. coli*, we considered it worthwhile to investigate the effect of metal ions on the enzymatic activity of L-asparaginase.

Friedman and collaborators [6–9] have studied the effect of the tetrachloroplatinate(II) ion and some platinum(II) chelates on the enzymatic activity of malate dehydrogenase. There have been a number of recent reports on the effect of a variety of metal ions on the enzymatic activity of lipase [10], ribonuclease [11], carboxypeptidase [12] and cellulase [13]. Despite the considerable interest in the chemistry, biochemistry and clinical application of L-asparaginase [14, 15] the effect of metal ions on the activity of this important enzyme does not appear to have been investigated to any great extent, and in any event, some of the results are contradictory. Tower, Peters and Curtis [16] examined the effect of mercury(II), zinc(II), magnesium(II), calcium(II) and manganese(II) ions on the hydrolytic activity of guinea pig serum L-asparaginase. Zagats and co-workers [17] investigated the effect of some mercury compounds on the enzymatic activity of an *E. coli* L-asparaginase. Arens and co-workers [18] studied the effect of mercury(II) and copper(II) ions on the enzymatic activity of highly purified *E. coli* L-asparaginase. In this communication we report the effect of a variety of transition and main group metal ions on the hydrolytic activity of a commercial preparation of *E. coli* L-asparaginase used in Australian clinics for treating patients with leukemia.

Experimental

Materials

L-Asparaginase isolated from *E. coli* ATCC 9637 (lot numbers DC 3708 and G14) was obtained from May and Baker Chemical Company and used without further purification. L-Asparagine of reagent grade was obtained from the BDH Company. Potassium tetrachloroplatinate(II) and potassium tetrachloropallate(II) were prepared and purified in this laboratory according to Grube [19] from metallic platinum and palladium obtained from the Matthey Garrett Company. All other chemicals of reagent grade were obtained commercially.

Preparation of Solutions

Tris-HCl buffers of pH 8.6, 0.05 *M* and Tris-Malic Acid–NaOH buffers of pH 7.3, 0.05 *M* were prepared according to Gamori [20]. When zinc(II), cadmium(II) and manganese(II) were used as moderators it was necessary to use buffers of pH 7.3 to prevent precipitation. In all other cases, buffers of pH 8.6 were used.

Stock solutions (1 mg/ml) of the enzyme in 0.85% aqueous sodium chloride were prepared; these solutions could be kept at 0 °C for several weeks without loss of activity. In all experimental runs 2 ml portions of a stock solution were diluted with 23 ml of the appropriate buffer before use. Solutions of 0.01 *M* L-asparagine in the appropriate buffer were freshly prepared for each experimental run. Aqueous trichloroacetic acid (TCA, 1.5 *M*) was used to terminate enzymatic reactions.

Kinetic Studies

In control runs the substrate solution (20 ml) and the appropriate buffer solution (8 ml) were preheated to 37 °C and the reaction initiated by addition of 2 ml of the enzyme solution. At various time intervals 5 ml portions of the reaction mixture were withdrawn and 0.5 ml of TCA was added to each sample. Ammonia present in the different samples was estimated with an Orion ammonia electrode (model 95-10). Electrode potentials were recorded with an expanded scale of a pH-mV Radiometer (Type PHM 225).

Two different procedures were used for studying the hydrolysis of L-asparagine in the presence of metal ions:

(i) The moderator dissolved in water at the chosen concentration (5 ml) was incubated with the enzyme solution (5 ml) at 0 °C for 30 min. The substrate solution and buffer (6 ml) were preheated to 37 °C and the enzymatic reaction was initiated by addition of 4 ml of enzyme solution containing the moderator. The progress of the reaction was followed as described for the control runs.

(ii) A mixture of the substrate solution (20 ml) and a solution of the moderator in buffer at the chosen concentration (8 ml) was preheated to 37 °C. The enzymatic reaction was initiated by addition of 2 ml of the enzyme solution and the reaction was followed as described above.

Results

The data which we obtained for the effect of a variety of metal ions on the enzymatic activity of L-asparaginase are given in Table I. Figure 1 shows the time course curve for some metal salts. In a preliminary investigation we obtained similar plots using the Nessler reagent to follow the production of ammonia.

Since mercury(II), copper(II) and tetrachloropalladate(II) ions produced the greatest inhibition of L-asparaginase activity (Table I), the effect of change of concentration of these ions *versus* activity of the enzyme was investigated. The tetrachloroplatinate(II) ion was included in the study for comparison. The results are given in Table II and illustrated in Figure 2. There is a linear relationship between activity and concentration for the tetrachloropalladate(II) ion, which produces the greatest decrease in activity. The

TABLE I. Effect of Metal Ions on the Hydrolysis of L-Asparagine by L-Asparaginase at 37 °C.

[Substrate] = [Moderator] = $6.7 \times 10^{-3} M$, [Enzyme] = $4 \times 10^{-8} M$[a]

Moderator	Relative Activity[b] %	
$MgCl_2$	64[c]	66
$CaCl_2$	73[c]	74
$SrCl_2$	65[c]	
$BaCl_2$	74[c]	
$ZnSO_4$	65[d]	
$CdCl_2$	71[d]	
$HgCl_2$	0	
$MnCl_2$	86[d]	
$CoCl_2$	78	
$NiCl_2$	59	
$CuCl_2$	15	
K_2PdCl_4	0	

[a] Based on a molecular weight of 120,000 [21]. [b] Relative to controls without moderator taken as 100%. [c] Obtained by measuring disappearance of substrate according to Howard and Carpenter [22]. All other values obtained using the ammonia electrode. [d] Values obtained at pH 7.3. All other values obtained at pH 8.6.

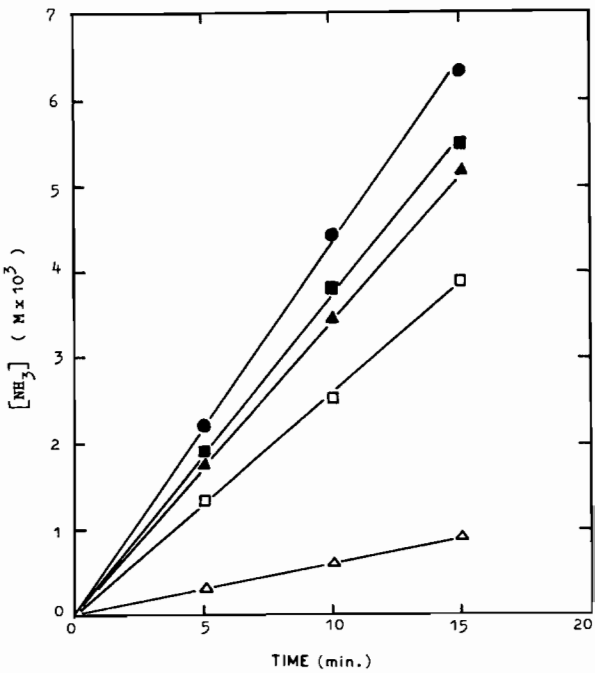

Figure 1. The time dependence rate of conversion of L-asparagine to L-aspartic acid by uninhibited (●) L-asparaginase, by $MnCl_2$ inhibited (■), by $CoCl_2$ inhibited (▲) by $NiCl_2$ inhibited (□), or by $CuCl_2$ inhibited (△) L-asparaginase.

TABLE II. Effect of Different Concentrations of Some Metal Ions on the Hydrolysis of L-Asparagine by L-Asparaginase at 37 °C.

pH 8.6, [Substrate] = $6.7 \times 10^{-3} M$, [Enzyme] = $4 \times 10^{-8} M$[b]

Moderator	Concentration $M \times 10^4$	Relative Activity[a] %
$CuCl_2$	5	84
	18	51
	20	48
	22	39
	27	29
	53	20
	67	15
$HgCl_2$	0.7	90
	1.3	85
	2.0	79
	3.3	74
	5.0	67
	6.7	62
	10	51
	20	33
K_2PdCl_4	0.45	97
	2.13	77
	4.27	52
	6.43	15
K_2PtCl_4	27	96
	41	90
	55	83
	82	57

[a]Relative to controls without moderator taken as 100%. [b]Based on a molecular weight of 120,000 [21].

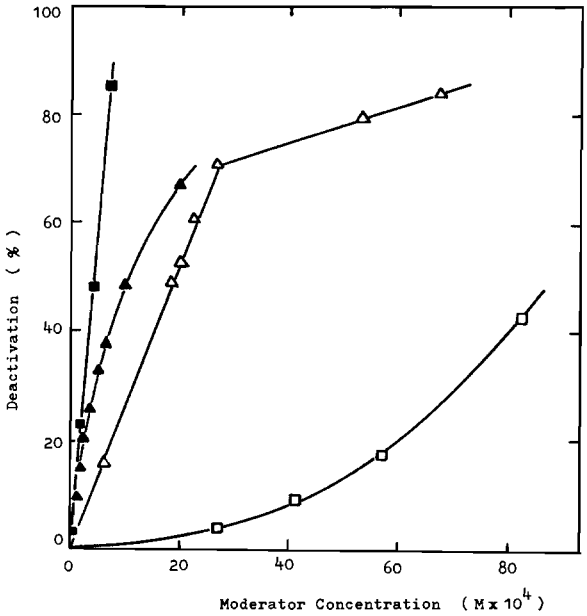

Figure 2. The concentration dependence of K_2PdCl_4 (■), $HgCl_2$ (▲), $CuCl_2$ (△), or K_2PtCl_4 (□) as inhibitors of the conversion of L-asparagine to L-aspartic acid by L-asparaginase.

mercury(II) ion has a greater inhibitory effect than the copper(II) ion, which in turn is greater than the tetrachloroplatinate(II) ion.

In order to gain some information concerning the mechanism of metal ion inhibition of L-asparaginase

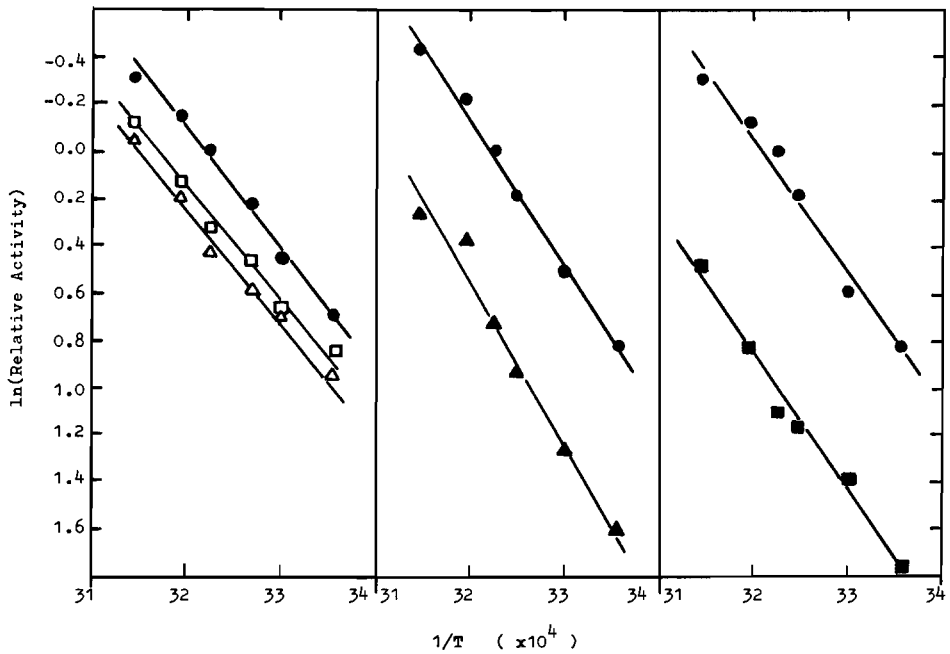

Figure 3. Arrhenius plots for the conversion of L-asparagine to L-aspartic acid by uninhibited (●) L-asparaginase, by $ZnCl_2$ inhibited (△), by $CdCl_2$ inhibited (□), by $CuCl_2$ inhibited (▲), or by $HgCl_2$ inhibited (■) L-asparaginase.

TABLE III. Effect of Temperature and the Presence of Metal Ions on the Hydrolysis of L-Asparagine by L-Asparaginase.

[Substrate] = $6.7 \times 10^{-3} M$, [Enzyme] = $4 \times 10^{-8} M$ [b]

Moderator	Concentration of Moderator $M \times 10^3$	pH	Temp. °C	Relative Activity [a] %	
				Control	Enzyme + Moderator
$MgCl_2$	6.7	8.6	25	50	42
			30	61	47
			37	100	66
			40	113	72
			45	134	104
$CaCl_2$	6.7	8.6	25	50	43
			30	61	49
			37	100	74
			40	113	79
			45	134	108
$CoCl_2$	6.7	8.6	30	55	49
			35	77	65
			37	100	78
			40	151	121
			45	182	148
$NiCl_2$	6.7	8.6	30	55	38
			35	77	50
			37	100	59
			40	151	108
			45	182	122
$CdCl_2$	6.7	7.3	25	50	43
			30	63	51
			33	80	62
			37	100	71
			40	116	87
			45	135	112
$ZnSO_4$	2.0	8.6	25	44	20
			30	60	28
			35	83	39
			37	100	48
			40	125	68
			45	154	76
$CuCl_2$	2.0	8.6	25	44	20
			30	60	28
			35	83	39
			37	100	48
			40	125	68
			45	154	76
$HgCl_2$	2.0	8.6	25	44	17
			30	55	25
			35	83	31
			37	100	33
			40	113	44
			45	135	62

[a] A relative activity of 100% refers to controls at 37 °C. [b] Based on a molecular weight of 120,000 [21].

activity, the effect of temperature changes was investigated. The results are shown in Table III. Energies of activation (E_a) were obtained from the slopes of Arrhenius plots (Figure 3) and are listed in Table IV. The E_a values for different samples of L-asparaginase vary between 9.9 and 16.5 Kcal mol^{-1}, although individual values did not vary for each particular batch. Citri and Zyk [23] recorded an E_a

TABLE IV. Energies of Activation for the Hydrolysis of L-Asparagine in the Presence of Metal Ions.

Moderator	E_a Kcal mol^{-1} to 90% Confidence Limits[a]	
	Control	Enzyme + Moderator
$CaCl_2$	9.9 ± 0.5	9.9 ± 0.4
$MgCl_2$	9.9 ± 0.5	9.9 ± 0.3
$CoCl_2$	16.5 ± 1.3	16.2 ± 1.2
$NiCl_2$	16.5 ± 1.3	16.6 ± 1.2
$ZnSO_4$	9.9 ± 0.4	9.3 ± 0.4
$CdCl_2$	9.9 ± 0.4	9.2 ± 0.3
$CuCl_2$	12.3 ± 0.4	13.5 ± 0.8
$HgCl_2$	11.2 ± 0.7	11.9 ± 0.4

[a]Calculated from the slope of Arrhenius plots obtained from the line of best fit using a Hewlett Packard 9810A calculator.

value of 12.9 Kcal mol^{-1} for *E. coli* L-asparaginase at pH 8.0, and Erikson [24] reported an E_a value of 1.2 Kcal mol^{-1} for *E. coli* L-asparaginase at pH 5.0. E_a values of 4.2 and 8.4 Kcal mol^{-1} have been reported for guinea pig serum L-asparaginase [16, 25]. It is noteworthy that the metal ions have little or no effect on the E_a values (Table IV).

In preliminary experiments with mercury(II) chloride we found that the moderator produced different inhibitions depending on whether it was incubated with the enzyme or whether it was incubated with the substrate. Table V shows the results for a number of different metal ions incubated with the substrate at several different concentrations. In the case of copper(II) and mercury(II) ions (molar ratio of metal to substrate 1:1) the results differ from those given in Table I. Increasing the metal to substrate ratio did not produce any marked effect with nickel(II), zinc(II) or cadmium(II) ions. With magnesium(II), calcium(II), cobalt(II) or mercury(II) ions, inhibition of the enzymatic activity increased when the ratio of metal ion to substrate was increased. An interesting result was obtained with the copper(II) ion; the enzymatic activity of L-asparaginase could be increased to 128% in the presence of excess of the metal ion (Table V).

It was found that the relative activity values were reproducible to a maximum error of 5%.

Discussion

Although the spectrophotometric method of Howard and Carpenter [22] gave results which were in good agreement with the ammonia electrode method when some alkaline earth metal ions were used as moderators (Table I), metal ions such as

TABLE V. The hydrolysis of L-Asparagine Metal Ion Mixture by L-Asparaginase.

[Substrate] = $6.7 \times 10^{-3}M$, [Enzyme] = $4 \times 10^{-8}M$[b]			
Metal Ion Added to L-Asparagine	Relative Activity[a] %		
	Molar Ratio of L-Asparagine: Metal Ion		
	1:1	2:1	3:1
Cu(II)	128	120	100
Mg(II)	81	93	104
Ca(II)	79	83	100
Co(II)	82	89	91
Ni(II)	80	80	85
Zn(II)	76	77	77
Cd(II)	79	79	83
Hg(II)	29	48	58

[a]Relative to activity of L-asparaginase without metal ion taken as 100%. [b]Based on a molecular weight of 120,000 [21].

mercury(II) were found to interfere with the spectrophotometric method. We have also found that magnesium(II), copper(II) or nickel(II) ions can interfere with Nessler's reagent. Most metal ions do not interfere with the estimation of ammonia by the ammonia electrode method. However, mercury(II) ions do complex ammonia sufficiently strongly under the conditions employed that it is necessary to add iodide ion before the ammonia determinations [26].

Since copper(II), mercury(II) and tetrachloropalladate(II) ions are known to have a strong affinity for sulfur [27, 28] their strong inhibitory effects may be due to interaction with sulfur-containing amino acid residues of the enzyme molecule. One possibility is that these ions might cleave the disulfide bonds of the enzyme [29]. The inhibitory effect of the tetrachoropalladate(II) ion is markedly different from that of the tetrachloroplatinate(II) ion (Figure 2). It is possible that the substrate is successfully competing with the enzyme for the platinum. A platinum(II) chelate of L-asparagine has been prepared by Volshtein and Anokhova [30].

An apparent agreement exists between the Irving–Williams order [31] and the deactivation values which we found for nickel(II), cobalt(II), manganese(II) and zinc(II) ions. This suggests that deactivation of L-asparaginase by these metals is controlled by metal–enzyme bonding equilibria. Shaw [32] has also noted that deactivation of some enzymes by metal ions can be related to the Irving–Williams rule.

Several enzyme-catalysed reactions follow the Arrhenius equation over a wide range of temperatures [33]. In the present study linear correlations between temperature and activity were observed for the temperature range 25–45 °C (Figure 3).

The E_a values obtained suggest that the enzymatic reaction mechanism is not changed in the presence of metal ions. Metal inhibition, therefore, would mean the depletion of free enzyme by either chelation or disulfide cleavage rather than a structural change in the active enzyme molecule leading to a mechanistic change with resultant diminished activity.

Whereas Tower and co-workers [16] observed an 83% deactivation of guinea pig serum L-asparaginase by $10^{-4}M$ mercury(II) chloride, Arens and collaborators [18] reported that the same metal salt, at a similar concentration, did not deactivate *E. coli* L-asparaginase. Neither paper gave sufficient details of the experimental procedure. Since we have shown that different experimental procedures, using the same metal ion, can result in different deactivations of *E. coli* L-asparaginase (Tables I and V), it is possible that the experimental procedure used by Tower and co-workers was different from that used by Arens and collaborators.

The observation that the degree of inhibition produced by metal ions depends on whether the metal ion is incubated with the enzyme or with the substrate strongly suggests that the inhibitions which we have observed are due to interaction of the metal ions with the enzyme rather than complexation of the metal ions by the substrate. The fact that in the case of copper(II) ions a 28% increase in enzyme activity is observed (Table V) suggests that in this situation the copper(II) ion may be acting as a co-factor for the enzymatic reaction.

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References

1 H. Oerkermann, W. D. Hirchmann and R. Gross, "Recent Results Cancer Res.", Vol. 33, pp. 125–130, Springer-Verlag, Berlin (1970).
2 J. Roberts, J. S. Holcenberg and W. C. Dolowy, *Nature, 227*, 1136 (1970).
3 A. J. Charlson, K. E. Trainor and E. C. Watton, *J. and Proc. Royal Soc. New South Wales, 108*, 6 (1975).
4 F. S. Stephens, R. S. Vagg and P. A. Williams, *Acta Cryst., B 31*, 841 (1975).
5 A. J. Charlson, R. J. Banner, R. P. Gale, N. T. McArdle, K. E. Trainor and E. C. Watton, *J. Clin. Hematol. and Oncol. (Wadley Inst.), 7*, 293 (1977).
6 M. E. Friedman, B. Musgrove, K. Lee and J. E. Teggins, *Biochim. Biophys. Acta, 250*, 286 (1971).
7 M. E. Friedman and J. E. Teggins, *Biochim. Biophys. Acta, 341*, 277 (1974).
8 M. E. Friedman and J. E. Teggins, *Biochim. Biophys. Acta, 350*, 263 (1974).
9 J. E. Teggins and M. E. Friedman, *Biochim. Biophys. Acta, 350*, 273 (1974).
10 T. Yamaguchi, N. Nuroya, M. Isobe and M. Sugiura, *Agr. Biol. Chem., 37*, 999 (1973).
11 H. Horitsu, Y. Higashi and M. Tomoyeda, *Agr. Biol. Chem., 38*, 933 (1974).
12 T. Nakadai, S. Nasuno and N. Iguchi, *Agr. Biol. Chem., 37*, 1237 (1973).
13 R. Ikeda, T. Yamamoto and M. Funatsu, *Agr. Biol. Chem., 37*, 1169 (1973).
14 D. A. Cooney and R. E. Handschumacher, *Ann. Rev. Pharmacol., 10*, 421 (1970).
15 J. C. Wriston and T. O. Yellin, *Advan. Enzymol., 39*, 185 (1973).
16 D. B. Tower, E. L. Peters and W. C. Curtis, *J. Biol. Chem., 238*, 983 (1963).
17 R. Zagats, I. Vina, R. Bluma and R. Platniece, *Khim. Prir. Soedin., 7*, 810 (1971).
18 A. Arens, E. Rauenbusch, E. Irion, O. Wagner, K. Bauer and W. Kaufman, *Z. Physiol. Chem., 351*, 197 (1970).
19 H. L. Grube, "Handbook of Preparative Inorganic Chemistry", 2nd Edn. (Brauer, C. Ed.) Vol. 2, pp. 1569–1584, Academic Press, New York (1965).
20 G. Gamori, "Methods in Enzymol.", Vol. 1, pp. 138–146, Academic Press, New York (1955).
21 E. Irion and A. Arens, "Recent Results Cancer Res.", Vol. 33, pp. 39–47, Springer-Verlag, Berlin (1970).
22 J. B. Howard and F. H. Carpenter, *J. Biol. Chem., 247*, 1020 (1972).
23 N. Citri and N. Zyk, *Biochemistry, 11*, 2103 (1972).
24 C. E. Eriksson, *Acta Chem. Scand., 21*, 2573 (1967).
25 A. P. Mistretta, G. C. Tassi and De Barbieri, *Biochim. Appl., 15*, 318 (1968).
26 Orion Research, "Instruction Manual, Ammonia Electrode Model 95-10", Orion Research Inc. (1974).
27 J. S. Thayer, *J. Organometal. Chem., 76*, 265 (1974).
28 D. R. Williams, *Chem. Rev., 72*, 203 (1972).
29 L. F. Lindoy, *Coord. Chem. Rev., 4*, 41 (1969).
30 L. M. Volshtein and L. S. Anokhova, *Zhur. Neorg. Khim, 4*, 325 (1959).
31 H. Irving and R. J. P. Williams, *Nature, 162*, 746 (1948).
32 W. H. R. Shaw, *Nature, 192*, 754 (1961).
33 I. W. Sizer, "Advan. Enzymol.", Vol. 3, pp. 35–62, Interscience, New York (1943).