[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF PITTSBURGH]

Adenine–Metal Complexes[1,2]

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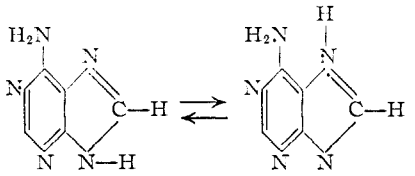
The reaction between adenine and certain divalent metal ions has been described. The acidic group of the imidazole ring apparently is involved in the reaction. A chelate structure is proposed for the complexes formed and formation constants of the metal complexes have been evaluated.

The Calvin[3]–Bjerrum[4] potentiometric titration technique has been utilized in a study of the behavior of adenine (6-aminopurine) toward metal ions.



Such an investigation was thought desirable because of the occurrence of this purine in nucleic acids. A very limited amount of work has been reported on the possible complexes formed between adenine and metal ions. Liquier-Milward[5] has isolated a 1:1 cobalt–adenine complex from aqueous solution. Albert[6] has titrated adenine in the presence of copper(II) and cobalt(II), concluding that a weak complex is formed with copper (log K_1 = 2.5) which is more likely to be of the type given by many other amines with this metal, rather than one involving the acidic group of the imidazole ring. It is interesting in this respect that Laufer and Charney[7] have found that purine compounds possessing an unsubstituted imino nitrogen (*i.e.*, those purines having a potential acidic group) formed insoluble compounds with copper(I), whereas those purines which had a substituent other than hydrogen in the 9-position did not form such insoluble compounds.

Experimental

The titration apparatus is the same as that previously described.[8] Potentiometric measurements of *p*H were made using a Beckman model G *p*H meter equipped with a glass-saturated calomel electrode pair.

The purification of dioxane, standardization of sodium hydroxide and perchloric acid also have been described.[8]

Stock solutions of approximately 0.01 *M* metal ions were prepared by dissolving their reagent grade perchlorates (G. Frederick Smith Co.) in water. The copper(II) and cobalt(II) solutions were standardized by electrodeposition. The nickel(II) solution was standardized by precipitation with dimethylglyoxime.

Adenine (Nutritional Biochemicals Corp.) was dried at 110°. *Anal.* Calcd. for $C_5H_5N_5$: C, 44.5; H, 3.7. Found: C, 44.4, 43.9; H, 3.7, 3.4. Adenosine (Nutritional Biochemicals Corp.) was dried *in vacuo* over calcium chloride. *Anal.* Calcd. for $C_{10}H_{13}N_5O_4$: C, 44.9; H, 4.9. Found: C, 44.9, 44.2; H, 4.9, 4.8. D-Ribose (Nutritional Biochemicals Corp.) was dried *in vacuo* over calcium chloride. *Anal.* Calcd. for $C_5H_{10}O_5$: C, 40.0; H, 6.7. Found: C, 39.8; H, 6.5.

The titration procedure is essentially the same as that described previously[8]: 55 ml. of water (or dioxane), 50 ml. of 0.01 *N* perchloric acid and 5 ml. of 0.01 *M* metal perchlorate were added to a weighed quantity of the reagent. Standard sodium hydroxide (0.1 *N*) was added in small increments to the stirred solution. For the titration of reagent alone 5 ml. of water was substituted for the metal perchlorate solutions.

The method of calculation of the formation constants of the metal complexes has been summarized previously.[8]

Discussion

Acid Dissociation Constants.—The pK_a values determined are summarized in Table I; pK_{a_1} refers to the dissociation of the imidazole N–H group.

TABLE I

SUMMARY OF ACID DISSOCIATION CONSTANTS

Compound	Solvent	Temp., °C.	pK_{a_1}	pK_{a_2}
Adenine	50% dioxane	25	3.43	10.7
	Water	10	4.33	
		25	4.18	9.7
		40	4.02	
Adenosine	Water	10	3.61	
		25	3.51	
		40	3.37	
D-Ribose	Water	25	<2	

There are a number of nitrogen atoms in the adenine molecule which might possibly serve as a basic center. Albert and Brown[9] have suggested that the principal basic center of adenine may be located on the pyrimidine ring, with a possible contribution from the amino group in position 6. This suggestion is based on a comparison of the pK_a's of imidazole, benzimidazole, pyrimidine, purine and adenine. X-Ray crystallographic data on adenine hydrochloride indicate that the proton is attached to the number 1 nitrogen of the pyrimidine ring.[10]

Adenosine is somewhat less basic than adenine, indicating that the ribosyl group is located in such a manner as to tend to diminish the electron density of the basic center. By way of comparison it might be noted that substitution of a β-D-ribosyl group for the imino hydrogen of 5,6-dimethylbenzimidazole reduces the basicity of the pyridine nitrogen by 1.3 pK_a units (*i.e.*, from pK_{a_1} = 5.98 to 4.70[11]). Indeed, on this basis one might expect a greater decrease than 0.7 pK_a units for the β-D-ribosyl derivative of adenine, if the basic center is the

(1) Abstracted from the thesis submitted by T. R. Harkins in partial fulfillment of the requirements for the Ph.D. degree at the University of Pittsburgh, June, 1956.

(2) Presented in part at the 128th National Meeting of the American Chemical Society, Minneapolis, Minn., 1955.

(3) M. Calvin and K. W. Wilson, THIS JOURNAL, **67**, 2003 (1945).

(4) J. Bjerrum, "Metal Amine Formation in Aqueous Solution," P. Haase and Son, Copenhagen, 1941.

(5) J. Liquier-Milward, *Nature*, **167**, 1068 (1951).

(6) A. Albert, *Biochem. J.*, **54**, 646 (1953).

(7) L. Laufer and J. Charney, U. S. Patents 2,379,913-4, 1945.

(8) H. Freiser, R. G. Charles and W. D. Johnston, THIS JOURNAL, **74**, 1383 (1952).

(9) A. Albert and D. J. Brown, *J. Chem. Soc.*, 2060 (1954).

(10) W. Cochran, *Acta Cryst.*, **4**, 81 (1951).

(11) M. T. Davies, P. Mamalis, V. Petrow and B. Sturgeon, *J. Pharm. Pharmacol.*, **3**, 420 (1951).

tertiary nitrogen of the imidazole ring. The pK_a values are not inconsistent with the latter possibility, however, since the base-weakening effect of the pyrimidine nucleus in reducing the electron density of the imidazole ring would be expected to make any further electron withdrawal from that center more difficult.

Thermodynamic data indicate the difference in basicity of adenine and adenosine lies solely in the enthalpy term since changes in entropy accompanying the two reactions are the same.

THERMODYNAMICS OF FORMATION OF CONJUGATE ACID IN WATER

Temperature = 25° and μ = 0.005

Compound	$-\Delta F$, kcal.	$-\Delta H$, kcal.	ΔS, cal./deg.
Adenine	5.71	4.2	5
Adenosine	4.77	3.4	5
2,2′-Bipyridine[12]	5.91	2.8	10

It is interesting that the entropy changes for these reactions are one-half the values obtained for 2-(2-pyridyl)-imidazoline and 2,2′-bipyridine. Furthermore, even though 2,2′-bipyridine is slightly more basic than adenine (pK_{a_1} = 4.33 and 4.18, respectively), a comparison of the ΔH terms for the two reactions would seem to indicate a reverse order. These results may reflect the tendency of the two nitrogen atoms of the bipyridine-type compounds to combine with only one proton at these acidities.

Formation of Metal Complexes.—Bonding of a metal ion to the adenine molecule might also occur at various sites. From observations made on certain imidazole derivatives (*e.g.*, 2-(2-pyridyl)-benzimidazole)[13,14] it was thought that a comparable situation might exist in the case of adenine, with complex formation occurring between the metal and the tertiary nitrogen of the imidazole ring. This type of bonding was found to affect the acidity of the N–H group of the imidazole ring. Because of the inherently greater acidity of the N–H group of adenine as compared with that of the imidazole derivatives, there is the possibility of an ionization of this hydrogen ion during complex formation rather than following complexation. There is also a distinct possibility of forming a five-membered ring between the 6-amino group, the metal and a nitrogen atom of the imidazole ring. Such a chelate structure would be expected to have a greater stability than any simple coördination type complex formed between the number one nitrogen atom of the pyrimidine ring and the metal.

Titration curves of adenine in the presence of copper(II), nickel(II), and cobalt(II), are given in Fig. 1. Although adenine and 2-(2-pyridyl)-benzimidazole have identical basicities (pK_{a_1} = 3.43 and 3.44, respectively, in fifty volume per cent. dioxane–water), their behavior toward metal ions is not the same in the low *p*H region. Titration curves for the latter compound and copper(II), for example, indicate that a complex of the type CuR_2^{++} is initially formed in acidic solution.[13]

(12) T. R. Harkins and H. Freiser, THIS JOURNAL, **77**, 1374 (1955).
(13) T. R. Harkins and H. Freiser, *ibid.*, **78**, 1143 (1956).
(14) T. R. Harkins, J. L. Walter, O. E. Harris and H. Freiser, *ibid.*, **78**, 260 (1956).

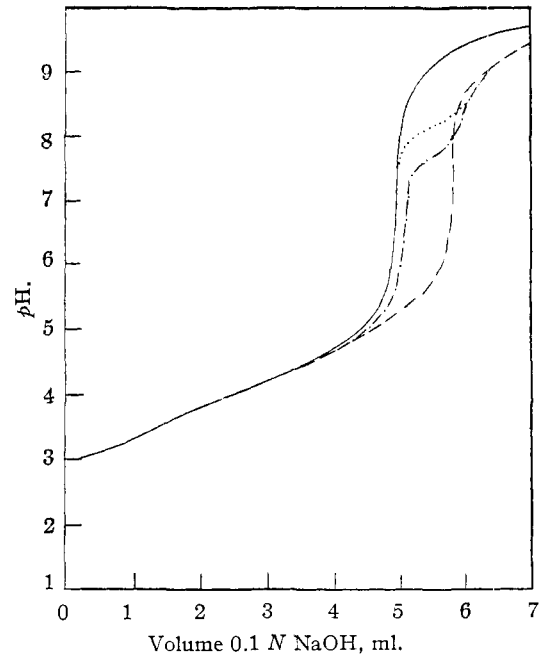


Fig. 1.—Titration curves of adenine (0.40 mmole) + $HClO_4$ (0.50 mmole) in water: ———, no metal; ——— copper(II); ——— nickel(II); - - - - cobalt(II); metal = 0.05 mmole.

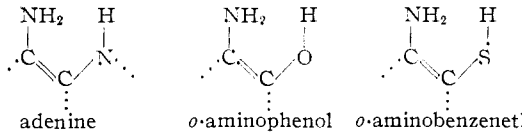
Figure 1 indicates that adenine has no appreciable tendency to form charged complexes of this type. Albert's[6] observation that the acidic group of the imidazole ring is not involved in complex formation is not justified on the basis of the data obtained in this study. It is evident from Fig. 1 that the acidic N–H group is indeed involved in the reaction between adenine and copper(II), as evidenced by the displacement of the equivalence point at 5 ml. of base.

The displacement of hydrogen ion from adenine was also verified in a more direct manner. The *p*H value of a 0.01 *M* $Cu(ClO_4)_2$ solution was found to drop from an initial value of 5.46 to a value of 4.88 upon the addition of sufficient solid adenine to give a 1:2 adenine to copper mole ratio. The *p*H dropped to 4.80 upon further addition of adenine at a 1:1 adenine to copper ratio, but remained at this value upon increasing the adenine to copper ratio as high as 8:1.

The titration curves are markedly similar to those obtained by Charles and Freiser[15,16] for dimethylglyoxime, *o*-aminophenol and *o*-aminobenzenethiol, all of which form complexes with metal ions of the type MR_2. The latter two compounds have structures comparable with adenine. This similarity can be shown as follows, along with the respective acid dissociation constants in 50 volume per cent. dioxane–water.

Charles and Freiser[16] have noted that except for copper, chelate formation with *o*-aminophenol occurred in the *p*H region in which metal hydrolysis took place, so that formation constants calculated from titration data were maximum rather than true values. By increasing the ratio of reagent to

(15) R. G. Charles, Ph.D. Thesis, University of Pittsburgh, 1952.
(16) R. G. Charles and H. Freiser, THIS JOURNAL, **74**, 1385 (1952).



	adenine	*o*-aminophenol	*o*-aminobenzenethiol
pK_{a_1}	3.43[a]	3.75	<2
pK_{a_2}	10.66	11.57	7.90

[a] This value does not necessarily reflect the basicity of the amino group.

metal ion they were able to lower the *p*H range of formation and thereby minimize this error. This is also the case for metal–adenine interactions, so that the formation constants summarized in Table II are maximum values.

TABLE II

SUMMARY OF CHELATE FORMATION CONSTANTS OF ADENINE AT 25°

Metal	Solvent	log K_1	log K_2	log K_{av}
Cu(II)	50% dioxane	9.0	8.0	8.6
Cu(II)	Water	7.1	6.4	6.8
Cu(II)[a]	Water	7.3	..	7.1
Ni(II)	Water	4.8	..	4.6
Co(II)	Water	4.2	..	4.2

[a] Data obtained at a ratio of 4 moles of reagent per metal ion. All other data are for a molar ratio of 8:1.

The value of log K_{av} for the copper–adenine complex (8.6) is very similar to that obtained for the copper–*o*-aminophenol chelate (8.9).[16] The lower acidity of *o*-aminophenol would account for its slightly greater metal binding capacity.

During the titration of the copper–adenine mixture in dioxane solution a reddish color appeared after the addition of 0.44 mole of base and increased in intensity as the titration progressed. No color effect was noted in aqueous solution. A spectral transmittance curve of the red solution in the visible region shows a maximum absorbance at 560 mμ. The absorbance of the solution at this wave length was examined over a *p*H range of 6 to 9 and found to be constant. Assuming that all of the copper present is incorporated in the complex, a molar absorptivity of 230 at 560 mμ was calculated. The intensity of the 560 mμ band was found to depend upon the dioxane concentration. A maximum absorbance was observed at 20 volume per cent. dioxane. This would correspond to an extremely large molar ratio of dioxane to adenine and copper. It is quite probable that the dioxane does not compete for a coördination position of the copper ion but in some way effects an equilibrium, possibly by changing the dielectric constant of the medium.

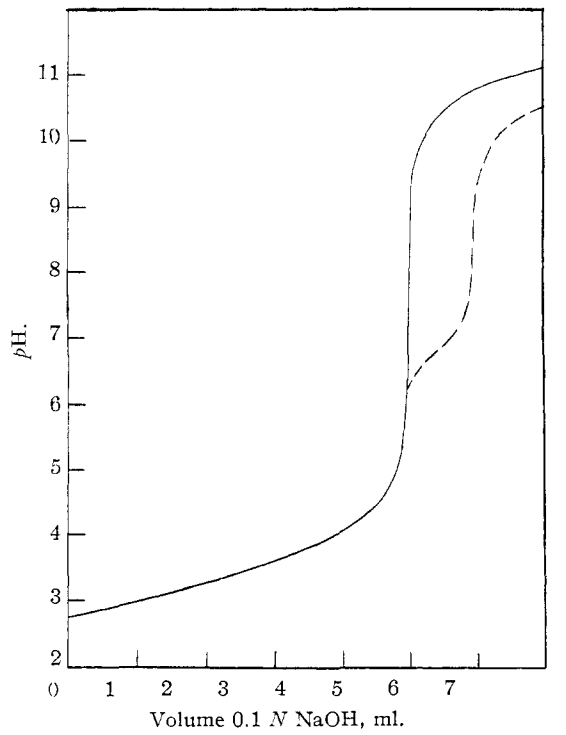
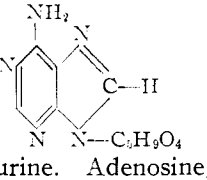


Fig. 2.—Titration curves of adenosine (0.40 mmole) + $HClO_4$ (0.50 mole) in water: ———, no metal; – – – –, copper(II) (0.05 mmole).

In order to elucidate the behavior of the N–H group in adenine, adenosine also was studied. The structural formula of adenosine, a ribonucleoside, shows this molecule to be a derivative of adenine, having a β-D-ribofuranose residue located at the 9-



position of the purine. Adenosine, which has no imino hydrogen, would not be expected to parallel the behavior of adenine if the acidic group of the latter compound is involved in its reactions with metal ions.

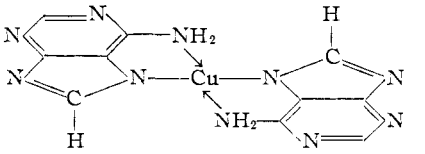
Whereas an immediate drop in *p*H was observed when adenine was added to a copper(II) solution, this effect was not noted in the case of adenosine. The titration curve for adenosine and copper(II) verifies the fact that this reagent does not behave in quite the same manner as does adenine (Fig. 2). For adenosine and copper(II) the liberation of protons occurring from 0.5 to 0.6 mole of added base is at a much higher *p*H than that which was observed for the titration of copper and adenine. Because adenosine has no measurable acidic group, it is of interest to account for its behavior as an acid in the presence of copper(II). The titration curve of adenosine and copper is practically identical with the hydrolysis curve of copper(II). Rather than the characteristic blue precipitate of copper hydroxide appearing during the titration of adenosine and copper, however, the solution being titrated assumed a definite green color. This fact could be observed more clearly in a test-tube reaction. An aqueous solution of adenosine, to which some copper(II) salt was added, had little, if any, color. Upon the addition of base, the solution turns green and becomes viscous.

To test the possibility that the copper ion combines with the ribose group of adenosine, a titration curve of D-ribose and copper(II) was determined. This was found to be practically identical with that of copper and adenosine. Further studies along this line may help explain reactions of cuprammonium ion with carbohydrates.[17] In these in-

(17) R. E. Reeves, "Advances in Carbohydrate Chemistry," Vol. 6, Academic Press, Inc., New York, N. Y., 1951.

teresting reactions, solubilization of cellulose and other carbohydrates may result from the formation of copper(II) complexes similar to that of ribose. The reaction apparently is reversible in nature since a back-titration with 0.1 *N* perchloric acid exhibits no hysteresis.

On the basis of the foregoing discussion the following structure is proposed as probably representing the copper–adenine complex in aqueous solution



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[CONTRIBUTION FROM THE INSTITUTE OF MICROBIOLOGY, RUTGERS, THE STATE UNIVERSITY, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY, AND THE UNIVERSITY OF ALABAMA]

Precipitation of the Specific Polysaccharide of *Cryptococcus neoformans* A by Types II and XIV Antipneumococcal Sera[1]

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The type specific polysaccharide of *Cryptococcus neoformans* A, which is known to contain xylose, galactose, mannose and glucuronic acid, has been shown to be a mixture of at least two polysaccharides, since the proportion of galactose in the mixture was increased by fractional precipitation with Type XIV antipneumococcal serum. Since neither cetyltrimethylammonium bromide nor Type II antipneumococcal serum effected any fractionation, it is concluded that both polysaccharides contain glucuronic acid.

The specific polysaccharides of *Cryptococcus neoformans*, Types A, B and C, although serologically different, are quite similar in composition and contain mainly mannose and xylose, with smaller amounts of glucuronic acid and galactose.[5] A contaminating galactan had also been separated from the immunologically reactive material, so that it became of interest to determine, if possible, whether or not the galactose and perhaps also the glucuronic acid were derived from impurities. Since precipitation with an appropriate antiserum has been shown to be a powerful means for the fractionation of otherwise difficultly separable mixtures of polysaccharides, for instance, gum arabic[6] and lung galactan,[7] advantage was taken of the cross precipitation of the specific polysaccharide of Cryptococcus A in Types II[5] and Type XIV[8] antipneumococcal[9] sera. An attempt also was made to fractionate the polysaccharide with cetyltrimethylammonium bromide, cetavlon, since this reagent has been shown useful in the separation of acidic from non-acidic polysaccharides.[10] However, the original polysaccharide, the cetavlon-precipitated fraction and the cetavlon-soluble fraction all exhibited the same cross reactivity with Type XIV anti-Pn serum and qualitative paper chromatographic analysis of the hydrolysates showed no differences in composition. Recent preliminary experiments suggest that it may be possible to separate the mixture into two components by electrophoresis. The results are of interest in showing once more both the possibilities and the limitations of the methods used.

Since only about 300 μg. of polysaccharide was recovered from either of the specific precipitates, even though relatively large amounts of antiserum were used, and glucuronic acid was present, differential colorimetric reactions appeared to be the method of choice. Moreover, preliminary hydrolysis to the free sugars is not required, an especial advantage in the presence of glucuronic acid.

Inasmuch as the original polysaccharide is known to contain only xylose, mannose, galactose and glucuronic acid, xylose and total hexose were estimated by the basic cysteine reaction,[11] in which mannose gives 0.8–0.9 as much color as galactose. The ratio of mannose to galactose was then determined with the help of the secondary cysteine reaction,[12] in which mannose gives 0.10–0.11 as much color as galactose. Solution of two simultaneous equations then gives the content of each sugar.

Glucuronic acid was determined separately by the carbazole reaction.[13]

Experimental

Materials and Methods.—The Type II anti-Pn horse serum was kindly supplied by the Bureau of Laboratories,

(1) These studies were carried out under a grant to Rutgers University from the National Science Foundation.

(2) Rockefeller Foundation Fellow, 1955–1956, from the University of Birmingham, England.

(3) Department of Ophthalmology, College of Physicians and Surgeons, Columbia University, New York.

(4) University of Alabama Medical Center, Birmingham.

(5) E. E. Evans, *J. Immunol.*, **64**, 423 (1950); E. E. Evans and J. W. Mehl, *Science*, **114**, 10 (1951); E. E. Evans and J. F. Kessel, *J. Immunol.*, **67**, 109 (1951); E. E. Evans and R. J. Theriault, *J. Bacteriol.*, **65**, 571 (1953); E. E. Evans, L. J. Sorensen and K. W. Walls, *ibid.*, **66**, 287 (1953).

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(9) Herein designated anti-Pn.

(10) B. C. Bera, A. B. Foster and M. Stacey, *J. Chem. Soc.*, 3788 (1955); J. E. Scott, *Chem. and Ind.*, 168 (1955); A. S. Jones, *Biochim. Biophys. Acta*, **10**, 607 (1953); M. Stacey and S. A. Barker, "Biochemistry of Nitrogen," *Acad. Sci. Fennica*, 262 (1955).

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(13) Z. Dische, *J. Biol. Chem.*, **167**, 189 (1947).