[CONTRIBUTION FROM THE LABORATORIES OF THE SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH]

Studies on the Structure of Nucleic Acids. VII. On the Identification of the Isomeric Cytidylic and Adenylic Acids[1]

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The densities of aqueous solutions of the isomeric cytidylic and adenylic acids and their apparent dissociation constants have been determined. In both pairs of nucleotides the *b* isomers manifest a greater distance between the nuclear NH_3^+ group and the singly-charged phosphate group. It is concluded that the *a* and *b* isomers are the 2′- and 3′-phosphates, respectively, in both cases. Further observations are presented which corroborate the conclusions reached.

In a preliminary communication[2] data were presented from which it was concluded that cytidylic acids *a* and *b*[3] are the 2′- and 3′-phosphates, respectively. In the present paper we record the details of those experiments and include data for similar measurements on the isomeric adenylic acids.[3]

It appears quite certain[4] that the isomerism which gives rise to the *a* and *b* nucleotides involves the 2- and 3-carbon atoms of the D-ribose moiety. On this basis, together with the fact that both cytidylic and adenylic acids exist largely as dipolar ions in aqueous solutions, it appeared that the isomers could be distinguished using techniques which would in effect measure distances between the charged groups of dipolar ions. For this we chose (a) high-precision density determinations, which afford a measure of the electro-striction process and hence the distance between the charged groups, and (b) potentiometric measurements from which the apparent dissociation constant of the 4-ammonium group can be calculated. The value of this pK_a will likewise depend on the distance between the 4-ammonium group and the singly-charged phosphate group.

Experimental

Materials.—Cytidylic acids *a* and *b* were fractionated from a mixture of the isomers[5] according to the procedure of Loring, Bortner, Levy and Hammell.[6] The respective isomers were recrystallized alternately as their ammonium salts and free acids to constancy with regard to ultraviolet spectral characteristics, optical rotation values, density measurements and *p*H titration curves. The optical densities at the wave lengths 250, 260, 280 and 290 mμ, in terms of the ratios 250/260, 280/260 and 290/260 were used to characterize the spectra. For the *a* isomer the values of these ratios were 0.47, 1.80 and 1.21; for the *b* isomer they were 0.43, 2.01 and 1.43, respectively[6]; all ultraviolet spectra were determined in 0.1 *N* hydrochloric acid with a Beckman spectrophotometer, model DU. Specific rotation, $[\alpha]^{25}D$ for *a* was −3°; for *b* 50°; *c* = 1, aqueous sodium hydroxide, *p*H 10.[6]

Adenylic acids *a* and *b* were purchased from the Schwarz Laboratories, Inc. The N/P ratio indicated >99% purity; no salts were present.[7] Paper chromatograms run in our laboratory showed no cross contamination.

Methods.—Density measurements on both pairs of nucleotides were carried out with a falling drop apparatus.[8] The size of the drop (delivered from an automatic pipet) was 5 mm.[3] and was timed for a 15-cm. distance with a stop watch reading to 0.01 second. The medium through which the drop fell was *m*-fluorotoluene. It would not be expected that these charged nucleotides would be extracted from the drop by the *m*-fluorotoluene; nevertheless the possibility was examined experimentally. The drop-time was observed at various distances in the tube and found to be constant.

Solutions were made up by weighing both solute (*ca.* 50 mg.) and solvent (doubly-distilled water, 10,000 mg.) with an accuracy of 0.03%. The samples were dried *in vacuo* at 100° and weighed in closed weighing vessels. Dilutions were made up by delivering known quantities of stock solution from a microburet into a weighed quantity of water. The bath temperature was 19.95° with a variation of <0.001°.

In any particular series, fifteen determinations were made and repeated alternately with different concentrations of nucleotides. The entire procedure was then repeated with similar but newly-made solutions. As a further check stock solutions of varying concentrations were prepared and diluted to the values of concentrations employed previously. This criss-cross technique thus provided a check both for the different weighings and dilutions. The final and penultimate samples in the recrystallization process for the cytidylic acids were used in the above procedure with no detectable differences.

Approximate apparent molal volumes were calculated as follows: The density of the *m*-fluorotoluene used was calculated with the Stokes' equation from the drop-time for pure water and the viscosity of *m*-fluorotoluene. In turn, the densities of the various solutions were calculated and from these the apparent molal volumes. The values are 235.82 and 234.94 ml. for cytidylic acids *a* and *b*, respectively (0.0067716 *M*); for adenylic acids *a* and *b*, the values are 253.77 and 250.84 ml., respectively (0.0064685 *M*). The experimental error is ±0.07 ml.

In the case of the cytidylic acids the apparent dissociation constants were calculated from data obtained with a Cambridge *p*H meter capable of a reproducibility of 0.006 *p*H units. Attempts to carry out the titration with a hydrogen electrode led to uncertain values due to an instability of the galvanometer. This was presumably due to reduction occurring at the hydrogen electrode. For the adenylic acids no such instability was observed and the pK_a values recorded were obtained using data obtained with this technique. The error was about 0.002 *p*H units. The temperature of titration was 24.5 ± 0.1° in both cases.

Results and Discussion

A dipolar ion occupies a smaller volume in a solution than does its uncharged isomer.[9] At

(1) This investigation was supported by grants from the National Cancer Institute, National Institutes of Health, United States Public Health Service, and from the Atomic Energy Commission, Contract AT(30-1)-910.

(2) L. F. Cavalieri, THIS JOURNAL, **74**, 5804 (1952).

(3) This nomenclature was introduced by C. E. Carter and W. E. Cohn, *Federation Proc.*, **8**, 190 (1949), and W. E. Cohn, THIS JOURNAL, **72**, 1471, 2811 (1950), and it refers to the location of the phosphate residue in the D-ribose moiety. Isomerism in the cytidylic acids was discovered simultaneously by H. S. Loring, N. G. Luthy, H. W. Bortner and L. W. Levy, *ibid.*, **72**, 2811 (1950).

(4) (a) H. S. Loring, M. L. Hammell, L. W. Levy and H. W. Bortner, *J. Biol. Chem.*, **196**, 821 (1952); (b) D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 44 (1952).

(5) Purchased from the Schwarz Laboratories, Inc., New York, N. Y.

(6) H. S. Loring, H. W. Bortner, L. W. Levy and M. L. Hammell, *J. Biol. Chem.*, **196**, 807 (1952).

(7) This information was kindly furnished by Dr. Louis Laufer of the Schwarz Laboratories, Inc., New York, N. Y.

(8) The author wishes to thank Dr. V. du Vigneaud for permission to use equipment in the Department of Biochemistry of Cornell Medical College.

(9) See J. T. Edsall in "Proteins, Amino Acids and Peptides," E. J. Cohn and J. T. Edsall, Reinhold Publ. Corp. New York, N. Y., 1943, p. 157.

equal molal concentrations, the former yields a solution of greater density. Further, it has been shown in the field of amino acids[9] that the greater the distance between the charges the greater the density of its aqueous solution. Since the difference between cytidylic acids *a* and *b* and adenylic acids *a* and *b* may essentially be resolved into a difference in distance between the charged groups, it was felt that density determinations would yield conclusive evidence for distinguishing the isomers. Experimentally, the drop-time was used as a measure of the differences in densities.

It will be seen from Table I that the *b* isomer in both cases exhibits a shorter drop-time than the *a*, *i.e.*, at the same molal concentration, the density of the solution is greater. This corresponds to a difference in density of about 2 units in the fifth decimal place. On the basis of these results we may conclude that the *b* isomers show a greater separation of charge.

TABLE I

DROP-TIME AS A FUNCTION OF CONCENTRATION

Time, sec.[a]		Concn., moles/l.	Time, sec.		Concn., moles/l.
Cytidylic acid			Adenylic acid		
a	b		a	b	
42.44	42.11	0.0067716	49.34	49.08	0.0048991
32.68	32.40	.010300	45.04	44.51	.0057636
27.68	27.47	.013544	42.10	41.23	.0064685
27.18	26.94	.013904			

[a] The probable error calculated from various series of runs was found to be 0.02 second.

It has been shown[10,11] that the field effect of a negatively charged group will tend to increase the pK_a of an ammonium group. As the distance between charged groups is increased this effect will decrease. The results are contained in Table II. It will be seen that both *b* isomers exhibit lower pK_a values. Thus it may also be concluded from these results that the *b* isomers show a greater separation of charge.

TABLE II

APPARENT DISSOCIATION CONSTANTS

Compound	pK_a(ammonium)	pK_a(secondary phosphoryl)
Cytidylic acid *a*[a]	4.44	6.19
Cytidylic acid *b*	4.31	6.04
Adenylic acid *a*	3.81[b,19]	6.17[b,19]
Adenylic acid *b*	3.74	5.92

[a] These values differ slightly from those previously reported.[2] The present constants were calculated by the method of Simms.[20] [b] Values of 3.80 and 3.65 have been reported for the ammonium groups of the *a* and *b* isomers, respectively; for the secondary phosphoryl dissociation values of 6.15 and 5.88 have been reported; *cf.* ref. 13. Both sets of values were calculated from titration data obtained at the glass electrode in 0.15 *M* sodium chloride. Considering these circumstances the agreement is good.

The pK_a of the ammonium group of muscle adenylic acid has been reported to be 3.80[12] and 3.74[13] while that of adenosine is 3.45[14] or 3.63.[13] Since the chain length between the ammonium and phosphate groups is great, *i.e.*, eight carbon atoms and one nitrogen atom, the inductive effect of the phosphate group operating through the chain will be negligible. The higher pK_a of the acid is clearly due to the proximity of the negatively charged phosphate group. This effect may readily be seen on the basis of the model for adenosine proposed by Furberg,[15] *i.e.*, sterically the 5′-phosphate can approach the ammonium group very closely. In the case of the isomeric cytidylic and adenylic acids this steric factor is also of significance. Here, too, the inductive effect will be negligible since there are 5 and 6 carbon atoms between the charged phosphate and ammonium groups of the *a* and *b* isomers, respectively.

The detailed structure of cytidine has been presented[15] on the basis of X-ray crystallographic analysis. One of the essential features of the structure is that the glycosidic bond (C_1–N_1) lies in the plane of the pyrimidine ring and is symmetrically disposed with respect to the 2 and 6 carbon atoms of the pyrimidine ring. Thus, regardless of the angle of rotation of the sugar ring about the pyrimidine ring the 3-position of the D-ribose will always be further from the 4-amino group than the 2-position. We conclude, therefore, that the cytidylic acid isomer showing the greater separation of charge (*i.e.*, the *b* isomer) is cytidine-3′-phosphate, while the *a* is cytidine-2′-phosphate.[16] It may also be concluded that adenylic acid *b* is adenosine-3′-phosphate.[17]

It should be emphasized that the two techniques employed in this investigation are actually dependent upon entirely different mechanisms. On the one hand, electrostriction is a measure of the ability of water molecules to orient themselves around the charged groups. This effect increases with increasing distance between charges and reaches a limiting value when the distance is infinite at which point the individual groups approach their maximum charge of one. On the other hand, the dissociation constant of the ammonium group is a measure of proton attraction and this will be affected by the negatively charged phosphate group in the vicinity. Since the results of the two methods corroborate each other, the probability that our conclusions are correct is accordingly increased. Recently a third independent method based on spectral shifts of cytidylic acids *a* and *b* in the high alkaline range has afforded results which lead to the same conclusion.[18]

(10) See reference 9, page 99.

(11) A. Neuberger, *Proc. Roy. Soc.* (*London*), **A158**, 68 (1937).

(12) Wassermeyer, *Z. physiol. Chem.*, **179**, 238 (1928).

(13) R. A. Alberty, R. M. Smith and R. M. Bock, *J. Biol. Chem.*, **193**, 425 (1951).

(14) P. A. Levene and H. S. Simms, *ibid.*, **65**, 519 (1925); *ibid.*, **70**, 327 (1926).

(15) S. Furberg, *Acta Chem. Scand.*, **4**, 751 (1950).

(16) Recently, H. S. Loring, M. L. Hammell, L. W. Levy and H. W. Bortner (*cf.* ref. 4a) suggested this relationship, taking into account the zwitterion formation. They state that the compound having the amino and phosphate acid groups in closest proximity to each other should show the greater tendency toward zwitterion formation with a concomitant decrease in solubility, acidity and ultraviolet absorption. The suggestion regarding solubility rests on firm grounds. However, the present author is not aware of any theoretical basis for these changes in ultraviolet absorption spectra. The suggestion of relative acidity, which refers to the pK_a values of the secondary phosphoryl dissociations, is without basis since the acidity of this group cannot be affected by the ammonium group which does not exist at *p*H values where the secondary phosphoryl hydroxyl group titrates (*ca.* *p*H 6).

(17) J. X. Khym, D. G. Doherty, E. Volkin and W. E. Cohn, THIS JOURNAL, **75**, 1262 (1953), have recently reported preliminary data from which the authors arrive at the same conclusion.

(18) J. J. Fox, L. F. Cavalieri and N. Chang, THIS JOURNAL **75**, 4315 (1953).

A further observation which is in conformity with our conclusions involves the pK_a values of the secondary phosphoryl dissociation of the isomeric nucleotides. It will be noted that the pK_a values in question are higher for the *a* isomers. This decreased acid strength is most probably due to an intramolecular interaction, probably involving hydrogen bonds between the secondary hydroxyl group of the phosphoric acid group and the purine or pyrimidine ring. The group closest to the ring will exhibit a higher degree of hydrogen bonding and thus have a higher pK_a value, or a decreased acid strength. This also leads to a formulation of 2′- and 3′-phosphates for the *a* and *b* isomers, respectively. That the melting point of adenylic acid *b* is higher than that of *a*[4,19,20] is a further confirmation of our conclusion.

Acknowledgment.—The author wishes to express his gratitude to Dr. George Bosworth Brown for helpful discussions and continued interest.

(19) W. E. Cohn, *J. Cellular Comp. Physiol.*, **38**, Suppl. 1, 21 (1951).

(20) H. S. Simms, THIS JOURNAL, **48**, 1239 (1926).

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[CONTRIBUTION FROM THE PHYSICAL RESEARCH LABORATORY OF THE DOW CHEMICAL COMPANY]

Organic Polyphosphorus Compounds. I. Synthesis of Aliphatic Amido Polyphosphates

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The possibilities of synthesizing aliphatic amido polyphosphates by phosphorylation of pyrophosphates were investigated. The following new polyphosphates were prepared: decamethyltriphosphoramide, linear and pyramidal dodecamethyltetraphosphoramide.

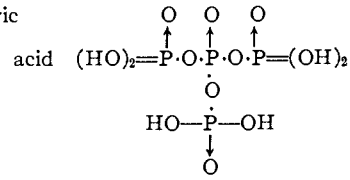
Introduction

While the chemistry of organic pyrophosphates has become well established during the past three decades, the chemistry of the higher organic polyphosphates is still at its beginning. In recent years the following compounds have been described: adenosine 5′-triphosphate,[1] thiamin triphosphate[2] hydroxythiamin triphosphate,[3] homothiamin and 2′-desmethylthiamin triphosphate,[4] O,N-di-(triphospho)-thiamin,[5] pyridoxamine-5′-triphosphate,[6] uridine triphosphate,[7] inosine triphosphate[8] and decamethyltriphosphoramide.[9] In view of the chemically interesting and biochemically important reactions of adenosine-5′-triphosphate we have attempted to synthesize simple organic derivatives of the polyphosphoric acids

triphosphoric acid $(HO)_2{=}P{\cdot}O{\cdot}\overset{\displaystyle OH}{P}{\cdot}O{\cdot}P{=}(OH)_2$

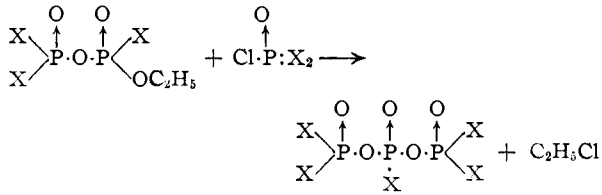
linear tetraphosphoric acid $(HO)_2{=}P{\cdot}O{\cdot}\overset{\displaystyle HO}{P}{\cdot}O{\cdot}\overset{\displaystyle OH}{P}{\cdot}O{\cdot}P{=}(OH)_2$

pyramidal tetraphosphoric acid

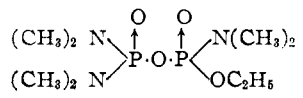


It is well-known that attempts to synthesize neutral esters of these parent structures have not been successful.[10] It was therefore decided to investigate the synthesis of their neutral dimethyl amides, *i.e.*, of decamethyltriphosphoramide and the two isomeric dodecamethyltetraphosphoramides.

Decamethyltriphosphoramide.—The synthesis of this compound was carried out on the basis of the reaction (X = dimethylamido group)



In this reaction only one reaction product was theoretically to be expected. The preparation of the starting products necessary has been described recently.[11] An equimolar mixture of O-ethyl hexamethyltriamidopyrophosphate and tetramethyldiamidophosphoryl chloride $[(CH_3)_2N]_2POCl$ was



(1) J. Baddiley, A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 582, 2487 (1949).

(2) L. Velluz, G. Amiard and J. Bartos, *Bull. soc. chim.*, **15**, 871 (1948).

(3) L. Velluz, J. Bartos and G. Amiard, *ibid.*, **17**, 297 (1950).

(4) P. Karrer, R. Schwyzer and K. Kostic, *Helv. Chim. Acta*, **33**, 1482 (1950).

(5) H. Roux and A. Callandre, *Experientia*, **6**, 386 (1950).

(6) M. Viscontini, C. Ebnother and P. Karrer, *Helv. Chim. Acta*, **34**, 2199 (1951).

(7) A. Kornberg, "Phosphorus Metabolism," Vol 1, The Johns Hopkins Press, Baltimore, Md., 1951.

(8) N. O. Kaplan, S. P. Colowick and F. E. Stolzenbach, *Federation Proc.*, **10**, 204 (1951).

(9) G. S. Hartley, D. F. Heath, J. M. Hulme, D. W. Pound and Mary Whittaker, *J. Sci. Food Agric.*, 303 (1951).

(10) S. A. Hall and M. Jacobson, *Ind. Eng. Chem.*, **40**, 694 (1948).

(11) G. Schrader, "Die Entwicklung neuer Insektizide auf Grundlage organischer Fluor- und Phosphor-verbindungen," Verlag Chemie, Weinheim, 1951.