The difference in the amount of clottable protein in PBF and ABF can be due in part to the fact that the determinations on PBF were made after the sample had been stored in the lyophilized form in the refrigerator at about 5° for several years. The determinations on ABF were made on the fresh preparation since the preparation was never lyophilized. Clottability of fibrinogen does not indicate homogeneity either with regard to sedimentation or with regard to electrophoresis. It would seem that one can obtain a homogeneous preparation of fibrinogen with low clottability. In the case of PBF, the original intent was to purify a quantity of fibrinogen to be stored in the lyophilized form so that a number of studies could be made on the same material. Some clottability is lost during the drying process. Although the clottability of PBF was low, its physical properties are similar to those of ABF. By all standards ABF was a good preparation of fibrinogen. Furthermore, the sedimentation and electrophoretic homogeneities of both PBF and ABF were similarly improved on purification. The results for ABF agree rather well with those of Casassa[31] and Sturtevant *et al.*[32] Both groups of workers found variations in the clottability of fibrinogen samples prepared by purification of commercial bovine fibrinogen.

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Summary

Definite changes in the sedimentation, viscosity and light scattering properties of two preparations of bovine fibrinogen are observed in solutions of NaCl at various ionic strengths.

The changes are discussed in relation to shape, volume, hydration, and interaction of the protein molecules. The influence of the presence of impurities is also discussed.

The molecular parameters were calculated from sedimentation, viscosity and partial specific volume data. The weight-average molecular weights were calculated from light scattering data. The average molecular weight from both sets of data at an ionic strength of 0.1 is 249,000 for both preparations of fibrinogen.

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CHICAGO 11, ILLINOIS

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, WASHINGTON UNIVERSITY]

Adenosine-3′:5′-phosphoric Acid: A Proof of Structure[1]

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One of the products obtained in the degradation of adenosine-5′-triphosphoric acid (ATP) in aqueous barium hydroxide at 100° was shown conclusively to be adenosine-3′:5′-phosphoric acid, a six-membered cyclic phosphate. This compound is identical with the cofactor for the interconversion of liver phosphorylase–dephosphophosphorylase isolated from natural sources. It also has been found to mediate the adrenocorticotropic hormone activation of adrenal cortical phosphorylase. The proof of structure of adenosine-3′:5′-phosphoric acid was obtained by the use of a variety of enzymatic and chemical degradations, as well as by the direct determination of the molecular weight of the compound by ultracentrifugation. Adenosine-3′:5′-phosphoric acid was prepared also by the action of dicyclohexylcarbodiimide on adenosine-5′-phosphoric acid. A new technique for degrading adenine nucleotides by means of liquid, anhydrous hydrogen fluoride is described.

A new adenine ribonucleotide with unexpected properties has been isolated from the products obtained on degradation of adenosine-5′-triphosphoric acid (ATP) with aqueous barium hydroxide.[4,5] This new substance was assigned a cyclic dinucleotide structure as the result of a preliminary study. Further experiments reported in this paper show conclusively that the original structural assignment is incorrect and that the new nucleotide actually is the monomeric cyclic phosphate, adenosine-3′:5′-phosphoric acid (A-3′:5′-P).

Sutherland, *et al.*, have reported the formation of a heat-stable factor by tissue particles which stimulates the interconversion of liver phosphorylase and the dephosphophosphorylase.[6] Using a variety of criteria, they have demonstrated that this factor and the A-3′:5′-P obtained by the degradation of ATP[4,5] are identical.[7] Haynes has found that A-3′:5′-P serves as an intermediate agent in the induced stimulation of adrenal phosphorylase by adrenocorticotropic hormone (ACTH).[8]

It was readily demonstrated that A-3′:5′-P is an adenine nucleotide. The following observations led to this conclusion: (1) Its ultraviolet absorption spectrum is essentially identical with the spectra of the adenylic acids: (2) deamination by nitrous acid yields a product with the characteristics of an inosine derivative: (3) adenine is obtained on acid hydrolysis: (4) elementary analyses for carbon, hydrogen, nitrogen and phosphorus are those expected for an adenine nucleotide: and, (5) adenosine is slowly formed by the action of *Crotalus adamanteus* venom. All other evidence which was obtained is in agreement with this conclusion.

Configuration of the Anomeric Carbon Atom.—The specific rotation of A-3′:5′-P[5] is the same in sign (negative) and order of magnitude as the spe-

(1) Presented at the 133rd Meeting of the American Chemical Society, San Francisco, Calif., April, 1958.

(2) Universal Match Co. Fellow, 1958–1959.

(3) On leave from the Agricultural Research Council Virus Research Unit, Cambridge, England, and who would like to thank the Wellcome Foundation for a travel grant.

(4) W. H. Cook, D. Lipkin and R. Markham, THIS JOURNAL, **79**, 3607 (1957).

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(6) T. W. Rall, E. W. Sutherland and J. Berthet, *J. Biol. Chem.*, **224**, 463 (1957); T. W. Rall and E. W. Sutherland, *ibid.*, **232**, 1065 (1958).

(7) (a) E. W. Sutherland and T. W. Rall, THIS JOURNAL, **79**, 3608 (1957); (b) *J. Biol. Chem.*, **232**, 1077 (1958).

(8) R. C. Haynes, Jr., *ibid.*, **233**, 1220 (1958).

cific rotation of other adenosinephosphoric acids,[9] in which the configuration of the glycosidic linkage is beta. In the case of diphosphopyridine nucleotide (DPN), configurational inversion of one of the anomeric carbon atoms changes the specific rotation from $[\alpha]^{23}D$ −34.8° (H_2O, *c* 1) for the β- to $[\alpha]^{25}D$ +14.3° (H_2O, *c* 1) for the α-isomer. The specific rotation, $[\alpha]^{23}D$, of the nicotinamide mononucleotide from β-DPN is −38.3° (H_2O, *c* 1), while that of the corresponding compound from α-DPN is +58.2° (H_2O, *c* 1).[10] Furthermore, it is worth noting that the specific rotation of 9-(β-D-ribofuranosyl)-adenine is negative ($[\alpha]^{28}D$ −65.5° (H_2O, *c* 0.6),[11] while that of the α-isomer is positive ($[\alpha]D$ +24° (H_2O, *c* 0.65)).[12]

Additional evidence for the β-configuration in A-3′:5′-P is that the compound also can be prepared by the action of dicyclohexylcarbodiimide (DCC) on a trialkylammonium salt of adenosine-5′-phosphoric acid (A-5′-P) in anhydrous pyridine solution. The A-3′:5′-P prepared in this way is not only identical by the usual criteria with the material prepared from ATP, but it also exhibits the biological activity characteristic of A-3′:5′-P.[13] It should be noted that the thymidine analog of A-3′:5′-P (T-3′:5′-P) has been prepared by the action of DCC on an anhydrous pyridine solution of thymidine-5′-phosphoric acid[14] (T-5′-P).

Degree and Positions of Esterification of the Phosphoric Acid.—It was readily demonstrated that A-3′:5′-P is a diester of orthophosphoric acid. The compound was not attacked by prostate phosphomonoesterase, but it was slowly degraded either by whole snake venom or by a purified diesterase fraction from venom.[15] The electrophoretic mobility on paper of A-3′:5′-P relative to A-5′-P at various *p*H's indicated that the compound has only one acid dissociation constant in the *p*H region 3.5–9.2. In addition, direct potentiometric titration of A-3′:5′-P with barium hydroxide showed only one sharp break in the titration curve corresponding to a pK'_a of 3.82. The fact that A-3′:5′-P has a high R_f in solvent A[5] was a further indication that it was not a monoester of orthophosphoric acid.

Evidence concerning the positions to which the phosphoric acid is attached was obtained in a variety of ways. Deamination of A-3′:5′-P with nitrous acid yields inosine-3′:5′-phosphoric acid (I-3′:5′-P). This same compound is obtained by digestion of inosine-5′-triphosphoric acid (ITP) with barium hydroxide. These observations clearly indicate that the phosphorus is not attached to the heterocyclic amino nitrogen. The resistance of A-3′:5′-P to oxidation by periodate shows that in the conversion of ATP to A-3′:5′-P the 2′- or 3′-hydroxyl of ATP has been substituted. As mentioned previously, A-3′:5′-P is degraded slowly by either whole snake venom or a purified diesterase fraction from the venom. The products obtained by the action of whole venom are adenosine-3′-phosphoric acid (A-3′-P) and adenosine while, as expected, the purified diesterase fraction leads to A-3′-P and A-5′-P as products. No trace of adenosine-2′-phosphoric acid (A-2′-P) is found. The fact that the diesterase breaks both 3′- and 5′-phosphate ester bonds is surprising, but parallel observations have been made for the snake venom-catalyzed hydrolysis of T-3′:5′-P and uridine-3′:5′-phosphoric acid.[14]

A more classical approach also demonstrated that the phosphoric acid is esterified to the 3′- and 5′- positions of adenosine. A-3′:5′-P was methylated exhaustively by the Purdie method.[16] The methylation product then was degraded by the action of liquid, anhydrous hydrogen fluoride.[17] The methylated ribose obtained was shown by paper chromatography to be a monomethylribose which behaved like 2-deoxy-D-ribose and somewhat different from authentic 5-O-methyl-D-ribose.[18] Paper electrophoresis of the methylribose using borate buffer (*p*H 9.2) demonstrated conclusively that it was 2-O-methylribose.[19] It is worthy of note that Purdie methylation of uridylic acid leads to migration of the phosphate between the 2′- and 3′-positions, presumably because of the formation of a 2′:3′ cyclic intermediate.[19] In the case under discussion here there is no evidence for the migration of the phosphate to the 2′-position. A study of a Stuart–Briegleb model[20] of A-3′:5′-P indicates that the oxygen atom of the 2′-hydroxyl group cannot come within bonding distance of the phosphorus atom because the latter is held rigidly in a six-membered ring.

Configuration of the Pentose.—In order to eliminate the possibility that the configuration of the 3′-carbon atom was inverted in the conversion of ATP to A-3′:5′-P, the stereochemical configuration of the pentose was determined. This was done by degrading a sample of A-3′:5′-P with liquid, anhydrous hydrogen fluoride. The pentose which was recovered was shown to be ribose by a combination of paper electrophoresis using borate buffer (*p*H 9.2) and by paper chromatography. As control experiments, A-5′-P and A-2′(3′)-P also were degraded with liquid, anhydrous hydrogen fluoride. It was demonstrated that in both of these cases the pentose recovered was ribose and, therefore, the hydrogen fluoride does not cause inversion of configuration during the dephosphorylation of the sugar.

Additional evidence that the pentose in A-3′:5′-P is ribose, rather than xylose, was obtained on the A-3′-P produced by the snake venom degradation

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(13) We wish to thank Dr. Earl W. Sutherland for testing this sample for biological activity.

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(15) We wish to thank Dr. Morris E. Friedkin for a sample of the diesterase.

(16) F. J. Bates, *et al.*, "Polarimetry, Saccharimetry and the Sugars," U. S. Government Printing Office, Washington, D. C., 1942, p. 506; A. S. Anderson, G. R. Barker, J. M. Gulland and M. V. Lock, *J. Chem. Soc.*, 369 (1952).

(17) Further studies are under way on the action of aqueous and liquid, anhydrous hydrogen fluoride on nucleic acids and their derivatives.

(18) D. Lipkin, E. B. Rauch and W. H. Hunter, unpublished results.

(19) D. M. Brown, D. I. Magrath and A. R. Todd, *J. Chem. Soc.*, 1442 (1954).

(20) Obtained from Arthur S. La Pine and Co., Chicago, Ill.

of A-3′:5′-P. Treatment of the A-3′-P with DCC (dicyclohexylcarbodiimide) converted it to a cyclic phosphate ester. At *p*H 12.2 and 37° the cyclic phosphate was readily hydrolyzed to a mixture of A-2′-P and A-3′-P. If the product of the DCC reaction were a xyloside-3:5-phosphoric acid, it would be stable under the hydrolysis conditions which were used.[21]

Molecular Weight.—Several experiments indicate that A-3′:5′-P has the monomeric structure proposed in this paper, rather than the cyclic dimeric structure originally proposed. First, an attempt was made to prepare a mixed cyclic dimer containing one mole of adenine and one mole of hypoxanthine by heating a mixture of ATP and ITP with aqueous barium hydroxide. No evidence was found for the formation of the mixed dimer, although A-3′:5′-P and I-3′:5′P were detected in the reaction mixture. Second, a sample of A-3′:5′-P was deaminated by means of nitrous acid. The reaction was stopped when it was one fourth to one third complete and the reaction products were examined. Once again, only A-3′:5′-P and I-3′:5′-P were found, but no adenine-hypoxanthine dimer. Third, A-3′:5′-P is identical with adenosine-2′:3′-phosphoric acid (A-2′:3′-P) both in its behavior on paper chromatography using a variety of solvents and on paper electrophoresis at various *p*H's. Similarly, I-3′:5′-P and inosine-2′:3′-phosphoric acid are identical in their behavior on paper electrophoresis at *p*H 3.5 and 7.4. Finally, it was found that hydrolysis of A-3′:5′-P by means of aqueous barium hydroxide at 100° yields A-3′-P and A-5′-P; no A-2′-P is found in the hydrolyzate. If A-3′:5′-P had the dimeric structure originally proposed, the hydrolyzate should have contained A-2′-P in addition to adenosine, A-3′-P and 2′,5′- and 3′,5′-di-*O*-phosphoryl-adenosines. Furthermore, A-5′-P would not have been observed as one of the hydrolysis products.

Conclusive evidence that A-3′:5′-P is monomeric, rather than a cyclic dimer, was obtained by an ultracentrifugal determination of the molecular weight.[22] The molecular weight obtained by sedimentation to equilibrium in the ultracentrifuge was 320. This value is in reasonable agreement with that of 370 ± 28 obtained by the Archibald method[23] and also with the theoretical value for the monomeric compound of 329. A single determination of the molecular weight from measurements of the diffusion coefficient and sedimentation rate, which is the least reliable technique, gave the value 448.

Experimental

Most of the experimental techniques which were used have been described previously.[5] In addition to the solvent systems for paper chromatography described in the preceding paper,[5] the following solvent systems (v./v.) also were used: 1-butanol–water, 86:14 (solvent E)[19]; pyridine–water–ethyl acetate, 1:2:2 (solvent F)[19]; 1-butanol-saturated aqueous boric acid, 85:15 (solvent G)[24]; 1% aqueous ammonium sulfate–propanol-2, 1:2 (solvent H)[25]; and, pyridine–water–ethyl acetate, 2:1:8 (solvent J). Periodate–Schiff reagent sprays were used to detect vicinal glycol groups.[26] Aniline hydrogen phthalate spray was used to detect pentoses and methylated pentoses after paper chromatography or electrophoresis.[27]

Paper Chromatography.—The following values represent the ratios of the R_f of A-3′:5′-P to the R_f of adenosine in a given solvent system: solvent A, 0.91; solvent B, 0.43; solvent C, 0.06; solvent D, 0.72; and solvent H, 0.67.

Samples of A-3′:5′-P which were presumed to be pure frequently yielded double spots on chromatography in solvent B. It was found, however, that these same samples gave only single spots on chromatography in this solvent if the papers were pre-soaked in a 1% solution of the disodium salt of Versene ((ethylenedinitrilo)-tetraacetic acid) in solvent B and then were allowed to dry before spotting the samples. That metal ions may affect the R_f of A-3′:5′-P was demonstrated directly with the ammonium and barium salts of a given sample of A-3′:5′-P using filter paper which had not been pretreated with Versene. The R_f of the barium salt relative to that of adenosine was 0.19, instead of the 0.43 reported above for the ammonium salt or the free acid. In solvent A the effect of barium ion is very much less, but it still decreases somewhat the R_f of A-3′:5′-P.

Paper Electrophoresis.—The mobility of A-3′:5′-P relative to A-5′-P is 1.20 at *p*H 3.5, 1.0 at *p*H 5.1, 0.57 at *p*H 7.2, and 0.49 at *p*H 9.2 (borate).

Preparation from A-5′-P.—One hundred and eighty-three mg. (0.50 mmole) of A-5′-P was warmed on the steam-bath with 2 ml. of anhydrous tri-*n*-butylamine. After most of the solid dissolved, 7 ml. of a 1 *M* solution of DCC (7 mmoles) in anhydrous pyridine was added to the hot A-5′-P solution. The reaction mixture was kept at 100° and aliquots were taken at 4, 21, 48 and 78 hr. These aliquots were streaked on separate filter papers and developed in solvent A. The A-3′:5′-P on each of these chromatograms was determined by standard techniques. The yield of A-3′:5′-P was found to be 4% in 4 hr., 17% in 21 hr., 21% in 48 hr., and 29% in 78 hr.

Potentiometric Titration.—An aqueous solution of A-3′:5′-P was titrated with standard barium hydroxide solution in an automatic recording potentiometric titration apparatus[28] with glass and calomel electrodes. The experimental titration curve was in excellent agreement with a curve calculated for an acid of pK_a' 3.82. Using the same apparatus and the same barium hydroxide solution, the pK_a' of a sample of A-5′-P was found to be 4.05.

Deamination by Means of Nitrous Acid.—An 8-mg. sample of the barium salt of A-3′:5′-P (0.0094 mmole) and 8.5 mg. of potassium nitrite (0.10 mmole) were dissolved in 2.0 ml. of citric acid–disodium phosphate buffer, *p*H 3.0. After 13.5 hr. the ultraviolet absorption maximum of the reaction mixture indicated that the reaction was one-quarter complete. Paper electrophoresis at *p*H 9.2 (borate) of a portion of the reaction mixture taken at this time showed that only two nucleotides were present, A-3′:5′-P and I-3′:5′-P. After 18 hr., when the reaction was one-third complete, electrophoresis gave the same result except that the I-3′:5′-P band was more intense relative to the A-3′:5′-P band than at 13.5 hr. Chromatograms in solvent A of the 18-hr. reaction mixture were in agreement with the electrophoresis results.

Preparation of I-3′:5′-P from ITP.—One ml. of saturated barium hydroxide solution was added to 50 mg. of ITP (Sigma Chemical Co., St. Louis, Mo.). The mixture, which was heterogeneous, was agitated occasionally while it was heated for 30 min. at 100°. The barium ion was precipitated and the supernatant was chromatographed on paper using solvent A. The two fastest moving bands, running very close together, were removed separately and subjected to electrophoresis at *p*H 9.2 (borate). The faster-moving band was identical with known inosine (elec-

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(28) Di-Functional Recording Titrator, International Instrument Co., Canyon, Calif.

trophoretic mobility relative to A-5′-P, 0.72). The other band (electrophoretic mobility relative to A-5′-P, 0.79) was identical with I-3′:5′-P obtained by nitrous acid deamination of A-3′:5′-P. The electrophoretic mobility at *p*H 5.1 of I-3′:5′-P relative to A-5′-P is 1.10.

Enzymatic Degradations.—Using prostate phosphomonoesterase under the conditions described by Markham,[29] A-3′:5′-P was found to be resistant to the action of this enzyme.

Three ml. of solution containing 25 mg. of A-3′:5′-P and 100 mg. of *Crotalus adamanteus* venom (Ross Allen's Reptile Institute, Silver Springs, Fla.) was brought to *p*H 9.5 with 5 *N* NH_4OH. After 42 hr. at 37° the degradation of the A-3′:5′-P is essentially complete. Paper chromatography in solvents A and B and electrophoresis at *p*H 9.2 (borate) showed that the digest contained a small amount of unchanged A-3′:5′-P, adenosine and A-2′(3′)-P. Chromatography in solvent D showed, furthermore, that no A-2′-P was present, only the 3′-isomer. In a similar experiment using a purified diesterase fraction from snake venom,[15] A-3′:5′-P was converted to a mixture of A-3′-P and A-5′-P.

There are several features of the snake venom degradation of A-3′:5′-P which are of interest. First, the molar ratio of A-3′-P to adenosine was not constant from run to run, but varied between the extreme values 0.7–1.2. The reasons for this variation are not known. Second, the snake venom degradation of A-3′:5′-P is abnormally slow for an enzymatic reaction. Third, magnesium ion does not affect the rate of degradation, but barium ion inhibits the reaction. Evidence was obtained that the snake venom degradation of A-3′:5′-P is actually an enzyme-catalyzed reaction by demonstrating that the reaction does not take place if the venom is pretreated with 0.1 *N* alkali for 1 hr. at 37° and then neutralized. Neither A-5′-P nor A-3′:5′-P was degraded by the alkali-treated venom, but both were degraded in the usual manner by the untreated venom.

Degradation of Methylated A-3′:5′-P to 2-O-Methylribose.—A 27-mg. sample of A-3′:5′-P was methylated exhaustively by the Purdie method using absolute methanol as the reaction medium.[16,19] The methylated A-3′:5′-P, which was recovered as a sirup, was transferred to a polyethylene test-tube and it was dried *in vacuo* over phosphorus pentoxide. When liquid, anhydrous hydrogen fluoride was added to the dried sirup, it dissolved instantly. After most of the hydrogen fluoride was evaporated by a stream of dry air, the tube and its contents were transferred to a vacuum desiccator containing soda lime. After remaining in the desiccator overnight, excess calcium carbonate and then water were added to the residue in the polyethylene tube. The mixture was centrifuged and the supernatant was removed for examination by paper chromatography and electrophoresis. The electrophoretic and chromatographic data on the carbohydrate in the supernatant are summarized in Table I. The data clearly indicate that 2-O-methyl-ribose is obtained by the degradation of the exhaustively methylated A-3′:5′-P.

TABLE I

PROPERTIES OF O-METHYLRIBOSE FROM THE DEGRADATION OF EXHAUSTIVELY METHYLATED A-3′:5′-P

Compound	Chromatography R_f, solvent F	Chromatography R_f, solvent E	Chromatography M_R,[a] solvent G	Electrophoresis M_R,[a] *p*H 9.2 (borate)
Methylribose from A-3′:5′-P	0.50	0.33	1.79	0.44
2-Deoxy-D-ribose	.47	.31	1.64	.42
				.46[19]
2-O-Methylribose	.49[19]	.34[19]	..	.49[19]
3-O-Methylribose	.55[19]	.38[19]	..	.90[19]
5-O-Methylribose	.57	.40	1.21	1.01
	.58[19]	.40[19]		0.99[19]
D-Ribose	.41	.17	1.00	1.00
	.37[19]	.18[19]		

[a] Mobilities relative to D-ribose.

Determination of Configuration of the Pentose.—Approximately 1 ml. of liquid, anhydrous hydrogen fluoride was added to 5 mg. of A-3′:5′-P in a polyethylene test-tube. The solution was then treated as described in the previous paragraph. Paper chromatography (Whatman No. 1 paper) of the supernatant and a known sample of D-ribose using solvent J (development time, 30 hr.) showed that the only pentose present in the supernatant was ribose.[30] The R_f values of the other three pentoses relative to ribose are lyxose, 0.74; arabinose, 0.50; and xylose, 0.66. Electrophoresis in 0.05 *M* borate buffer (*p*H 9.2) confirmed the identity of the pentose in the supernatant as ribose. The electrophoretic mobilities (17 volts/cm., 3 hr.) of the other three pentoses relative to ribose are lyxose, 0.92; arabinose, 1.17; and xylose, 1.26. As controls, A-5′-P and A-2′(3′)-P also were degraded by liquid, anhydrous hydrogen fluoride. The pentoses recovered in both of these experiments also were shown to be ribose.

Evidence for the ribose configuration of the pentose in A-3′:5′-P was obtained in two other ways. First, the nucleoside isolated by paper chromatography from a snake venom degradation of A-3′:5′-P was oxidized rapidly in aqueous solution by periodate. Pentose nucleosides which do not have a *cis*-glycol configuration are not rapidly oxidized under the same conditions.[31] Second, the nucleoside-3′-phosphate from the snake venom digestion of A-3′:5′-P described in the previous section was separated by paper chromatography (solvent B). It was dissolved in 300λ of dry formamide; 250λ of 1 *M* DCC in anhydrous pyridine was added to the resulting solution.[32] After 22 hr. at room temperature, another 250λ of the DCC solution was added. After three more days the reaction mixture was chromatographed on paper (solvent A). The fastest-moving and principal band, which was shown by electrophoresis at *p*H 9.2 (borate) to be identical with authentic A-2′:3′-P, was isolated. It was dissolved in disodium phosphate–sodium hydroxide buffer, *p*H 12.2. After several hours at 37°, it was found by electrophoresis at *p*H 9.2 (borate) that the hydrolysis of the cyclic phosphate from the DCC reaction was essentially complete. The hydrolyzate was subjected to electrophoresis in borate buffer. The band which had the same mobility as authentic A-2′(3′)-P was removed from the electrophoresis paper and was chromatographed on fresh paper using solvent D. Two ultraviolet-absorbing spots were found which were identical with authentic A-2′-P and A-3′-P samples.

Degradation of Mixtures of ATP and ITP by Means of Barium Hydroxide.—Mixtures of ATP and ITP in the ratio of 10:1 were treated with 0.4 *N* barium hydroxide at 100°. The 10:1 ratio was used in order to ensure that, if a reaction occurred between two molecules of nucleoside triphosphate, there was a good chance of a mixed adenosine–inosine compound being formed. On chromatography the reaction mixtures were not found to contain such a compound, but they did contain the substances identified as I-3′:5′-P and A-3′:5′-P.

Specific Volume of A-5′-P and A-3′:5′-P.—A 0.294-g. sample of A-5′-P[33] (phosphorus, 8.23%) was dissolved in water and the solution was made up to a volume of 25.00 ml. A "flask" pycnometer,[34] approximately 15 ml. in volume, was filled with this solution at 24°. The weight of the pycnometer plus solution was 34.1258 g. The weight of the pycnometer filled with distilled water at 24° was 34.0444 g. The empty pycnometer weighed 18.4956 g. Using these data and a value of 1.0027 ml./g. for the specific volume of water at 24°, the specific volume of A-5′-P was found to be 0.53 ml./g.

A 0.2088-g. sample of A-3′:5′-P.1.75 H_2O (phosphorus, 8.59%) in 25 ml. of aqueous solution was used for determining the specific volume using the same pycnometer and procedure as for the A-5′-P; pycnometer plus solution at 24°, 34.0926 g. The specific volume of A-3′:5′-P is calculated to be 0.60 ml./g.[35]

(29) R. Markham in K. Paech and M. V. Tracey, "Modern Methods of Plant Analysis," Springer–Verlag, Berlin, 1955, Vol. IV, p. 288.

(30) Ribose itself on being subjected to the action of liquid, anhydrous hydrogen fluoride is converted in part to what appears to be a series of polyriboses. These give a positive test with the aniline phthalate spray.

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(32) M. Smith, J. G. Moffatt and H. G. Khorana, *ibid.*, **80**, 6204 (1958).

(33) Sigma Chemical Co., St. Louis, Mo.

(34) N. Bauer in A. Weissberger, "Physical Methods of Organic Chemistry," Interscience Publishers, Inc., New York, N. Y., 1949, Part I, p. 265.

(35) The specific volumes required for the calculation of molecular weights are those of the anhydrous compounds. Because of this, the

The above values for the specific volumes are in agreement with values found by Tennant and Vilbrandt[36] for a number of different nucleic acid samples. They do not agree, however, with the values given by Cavalieri for the apparent molar volumes of A-2′-P, A-3′-P and the isomeric cytidylic acids.[37] While Cavalieri's values are very reliable relative to one another, their reliability on an absolute scale is questionable.

Ultracentrifugal Determination of Molecular Weight.[22]—A Spinco analytical ultracentrifuge with schlieren optics was used for the molecular weight determinations.

The A-3′:5′-P solution used for the specific volume determination (*p*H 4) was used for the molecular weight determinations by the Archibald method and by equilibrium sedimentation. For these determinations the sample was subjected to 280,000 *g* for 72 hr. During the early part of the sedimentation, four pairs of measurements were made of the concentration of A-3′:5′-P at the top and bottom surfaces of the solution, in addition to a measurement of the concentration distribution at equilibrium.

An additional value for the molecular weight was calculated from measurements of the diffusion coefficient ($D_{20,w}$ 40 $\times 10^{-7}$) and sedimentation coefficient ($S_{20,w}$ 0.3 S.) of A-3′:5′-P (approx. 0.5% solution) in 0.2 *M* sodium chloride. The measurement of the diffusion coefficient was made from the rate of spreading of the boundary obtained in a synthetic boundary cell.

Hydrolysis in Acid and Alkaline Solutions.—An aqueous solution of A-3′:5′-P (8 mg./ml.) was made 1 *N* in hydrochloric acid. It was kept at 92° and aliquots were removed at suitable time intervals. The aliquots were spotted on filter paper and the papers were developed in solvent B. The only ultraviolet-absorbing hydrolysis product which was observed during the course of the reaction was adenine. The rate of the reaction was measured by determining the amount of cyclic phosphate remaining after various intervals and also from the amount of adenine present at various times. The data clearly indicated that the hydrolysis was first order in A-3′:5′-P with a half-life of 55 min. The reaction was followed for approximately six half-lives. Sutherland and Rall[7b] found the half-life of A-3′:5′-P in 1 *N* hydrochloric acid to be approximately 28 min. at 100°.

A portion of the same A-3′:5′-P solution as was used above was made 1 *N* in sodium hydroxide. The reaction mixture was treated as above, except that a temperature of 98° was used. Adenine was the principal ultraviolet-absorbing hydrolysis product observed. It was found, however, that during the course of the hydrolysis a small amount of adenosine appeared and then disappeared as the heating was continued. Furthermore, the total amount of material in the reaction mixture absorbing at about 260 mμ progressively decreased during the course of the heating period. The alkaline hydrolysis also was first order in A-3′:5′-P with a half-life of 36 min. The reaction was followed for approximately nine half-lives. The value for the half-life in 1 *N* sodium hydroxide given by Sutherland and Rall[7b] is approximately 11 min. at 100°.

A mixture of 5 mg. of the barium salt of A-3′:5′-P and 0.50 ml. of 0.4 *N* barium hydroxide solution was heated at 100° for 30 min. It then was cooled rapidly to room temperature and brought to *p*H 1.5 with 6 *N* sulfuric acid. The precipitate was removed by centrifugation and after thorough washing it was discarded. The combined washings and supernatant were adjusted to *p*H 6 with 5 *N* ammonium hydroxide. Paper chromatography (solvent D) of a portion of this solution showed that it contained A-3′-P and A-5′-P. Another sample of the solution was subjected to paper electrophoresis at *p*H 9.2 (borate). The ultraviolet-absorbing bands corresponding to the adenylic acids were eluted from the paper separately and the absorbance of the separate eluates determined. A-3′(2′)-P and A-5′-P were found to be present in a molar ratio of 5:1. In order to demonstrate conclusively that A-3′-P was present in the barium hydroxide digest, and that the nucleotide designated A-5′-P in the solvent D chromatogram was not A-2′-P, material in the appropriate band from a borate electrophoresis was chromatographed on paper using solvent A. The single ultraviolet-absorbing band on this paper was removed and it was shown to be pure A-3′-P by comparison with authentic A-2′-P and A-3′-P by means of paper chromatography with solvent D as the developing solvent. The fact that the barium hydroxide hydrolysis of A-3′:5′-P yields A-5′-P and A-3′-P, but no A-2′-P, also was demonstrated by means of anion exchange chromatography.

Discussion

The outstanding difference in chemical properties between A-3′:5′-P and A-2′:3′-P is their stability toward hydrolysis. The A-3′:5′-P, in which the phosphate is part of a six-membered ring, is far more stable toward both acid and alkaline hydrolysis than A-2′:3′-P. This difference in hydrolytic stability between five- and six-membered cyclic phosphates has been reported previously for a number of other compounds.[21,38] The stability of A-3′:5′-P toward hydrolysis indicates that the phosphate probably is not part of a strained structure and that the ribofuranose ring is not planar. This conclusion is in agreement with the suggestion of Furberg,[39] based on X-ray diffraction data, that the 3′-carbon atom in adenosine is 0.5 Å. out of the plane of the ribofuranose ring. The fact that A-3′:5′-P can be prepared by the action of barium hydroxide on ATP, although many competing reactions are possible to give various other products,[5] also argues for a relatively unstrained six-membered cyclic phosphate structure in A-3′:5′-P. It is worth noting, however, that under comparable conditions the half-life toward hydroxide ion-catalyzed hydrolysis of 1:2-isopropylidene-D-xylofuranose-3:5-phosphoric acid (4 hr.)[21] is about seven times the half-life of A-3′:5′-P (36 min.).

The hydrolysis of A-3′:5′-P with 1 *N* sodium hydroxide at 98° yields adenine as the principal ultraviolet-absorbing product. During the course of the hydrolysis, the only intermediate which appears is adenosine in minor amount. No adenylic acids are found at any stage of the hydrolysis. On the other hand, as mentioned previously, A-3′-P and A-5′-P in a ratio of 5:1 are the principal products of the barium hydroxide hydrolysis of A-3′:5′-P.[40] This is analogous to the behavior of α-methyl-D-glucoside-4:6-phosphoric acid[11] and T-3′:5′-P.[14] In the former case, α-methyl-D-glucoside-4-phosphoric acid and the 6-isomer are formed in a ratio of 5:1; T-3′:5′-P yields the 3′-isomer and T-5′-P in a ratio of *ca.* 4:1. In contrast to this behavior, pantothenic acid-2:4-phosphoric acid[42] and 1:2-isopropylidene-D-xylofuranose-3:5-phosphoric acid[21] hydrolyze in alkaline solution with the rupture of the phosphate

quantity of material in the pycnometer has to be adjusted to take into account water of crystallization. In doing this it also was necessary to assume that A-3′:5′-P had a composition corresponding to that of an adenylic acid minus one molecule of water. This assumption, which is borne out by the results, does not in fact necessitate a large correction.

(36) H. G. Tennant and C. F. Vilbrandt, THIS JOURNAL, **65**, 427 (1943).

(37) L. F. Cavalieri, *ibid.*, **74**, 5804 (1952); **75**, 5268 (1953).

(38) A bibliography on this subject is given in ref. 21; see also H. S. Mosher, J. Reinhart and H. C. Prosser, *ibid.*, **75**, 1899 (1953).

(39) S. Furberg, *Nature*, **164**, 22 (1949); *Acta Cryst.*, **3**, 325 (1950); *Acta Chem. Scand.*, **4**, 751 (1950).

(40) The fact that A-2′-P is not one of the hydrolysis products is further confirmation of the suggestion that the oxygen of the 2′-hydroxyl group cannot attack the phosphorus atom in A-3′:5′-P. The same conclusion was based on the results obtained in the Purdie methylation of A-3′:5′-P.

(41) P. Szabo and L. Szabo, *Compt. rend.*, **247**, 1748 (1958).

(42) J. Baddiley and E. M. Thain, *J. Chem. Soc.*, 3121 (1951).

ester bond at the secondary, rather than the primary, carbon atom.[43]

The conversion of A-5′-P to A-3′:5′-P by the action of DCC in anhydrous tri-*n*-butylamine–pyridine is a reaction of some interest. The first step in the reaction is the formation of P^1,P^2-diadenosine-5′-pyrophosphoric acid[44] (AppA). At room temperature this is the end-product. Only on heating the reaction mixture for extended periods is A-3′:5′-P obtained. It is unlikely that the DCC is involved directly in the conversion of AppA to A-3′:5′-P. This conversion is probably catalyzed by the organic bases, since it has been found that P^1,P^2-dithymidine-5′-pyrophosphoric acid is converted to T-3′:5′-P by heating in pyridine solution.[14] The formation of A-3′:5′-P is believed to be, therefore, another example of intramolecular phosphorylation,[21] like the conversion of ATP to A-3′:5′-P by means of barium hydroxide.[5] There is no evidence that the formation of A-3′:5′-P from A-5′-P plus DCC involves the formation of a neutral pyrophosphate ester as an intermediate, a reaction mechanism which has been suggested for the conversion of 4-hydroxybutylphosphoric acid to butane-1,4-diolphosphoric acid by means of DCC.[21]

It has been known for many years that O-glycosides are very rapidly decomposed by liquid, anhydrous hydrogen fluoride, while ordinary ethers are stable toward this reagent.[45] Under strictly anhydrous conditions glycosyl fluoride is the principal product of the reaction, but by varying conditions the sugar itself may be obtained. It was found that the N-glycosidic bond in nucleosides and nucleotides also is broken by the action of liquid, anhydrous hydrogen fluoride at room temperature. In the purine compounds the reaction appears to be as rapid as with O-glycosides. However, not only is the glycosidic bond attacked by liquid, anhydrous hydrogen fluoride, but with nucleotides rapid dephosphorylation also takes place.

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(43) This similarity in behavior of A-3′:5′-P, T-3′:5′-P and α-methyl-D-glucoside-4:6-phosphoric acid may indicate a similarity in conformation. It is presumed that, like in the latter, the 3′-hydroxyl group and the CH_2OH are equatorial in both A-3′:5′-P and T-3′:5′-P. The X-ray data of Furberg[39] are in agreement with this presumption. However, the alkaline hydrolysis of β-methyl-D-galactoside-4:6-phosphoric acid also yields the 4- and 6-phosphate esters in a ratio of 5:1.[41]

(44) M. Smith and H. G. Khorana, THIS JOURNAL, **80**, 1141 (1958).

(45) K. Wiechert in "Newer Methods of Preparative Organic Chemistry," Interscience Publishers, Inc., New York, 1958, pp. 346–349.

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[CONTRIBUTION FROM RESEARCH AND ENGINEERING DIVISION, MONSANTO CHEMICAL CO.]

π-Complexes of the Transition Metals. X. Acetylenic π-Complexes of Chromium in Organic Synthesis[1]

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Disubstituted acetylenes are cyclically condensed by triaryl- and trialkyl-chromium compounds to benzene derivatives, to polynuclear aromatic hydrocarbons and to aromatic π-complexes. These condensations are considered to proceed *via* acetylenic π-complexes of chromium and are useful as a general synthetic method.

The isolation of triphenylchromium(III) in the form of its tetrahydrofuranate and its rearrangement to aromatic π-complexes of chromium have been described in detail.[2] This compound and its congeners are now found to have broad potentialities in organic synthesis. Their ability to promote cyclic condensation of acetylenes is reported as the first part of our investigation in this area.

The existence of triphenylchromium as a fully coördinated chromium(III) compound, *e.g.*, $(C_6H_5)_3$-Cr·3 THF, allows the assumption that its molecular geometry is that of the octahedron such as obtains in the coördinated chromium(III) salts and also in chromium hexacarbonyl, *i.e.*, d^2sp^3 bonding to chromium. Using this geometrical representation, the reaction of phenylmagnesium bromide and chromic trichloride may be described by Fig. 1, which summarizes the literature bearing on this reaction (*cf.* paper VII, footnotes 2, 3, 4, and 5).[3]

The formation of triphenylchromium is not observed when phenylmagnesium bromide and chromic trichloride are allowed to react in diethyl ether; but in tetrahydrofuran, a solvent which is considerably more basic than ether,[4] the triarylchromium is stable and isolable. The stabilizing effect of tetrahydrofuran in the hexacoördinated organochromium compound is clearly demonstrated by the irreversible rearrangement of triphenylchromium to the π-complex structure when its crystalline tetrahydrofuranate is washed with diethyl ether. The instability of the diethyl etherate of triphenylchromium provides an explanation for past failures in isolating this substance from atttempted preparations in the latter solvent. The unstable etherate appears also to be the ephemeral intermediate in the solution of reacting phenylmagnesium bromide–chromic trichloride which Job and Cassal treated with carbon monoxide to obtain the zero valent chromium hexacarbonyl.[3,5]

(1) Preliminarily communicated to THIS JOURNAL, **80**, 2913 (1958).

(2) W. Herwig and H. Zeiss, paper VIII in this series, *ibid.*, **81**, 4798 (1959); paper IX, G. N. Schrauzer, *ibid.*, **81**, 5307 (1959).

(3) M. Tsutsui and H. Zeiss, *ibid.*, **81**, 1367 (1959).

(4) H. C. Brown and R. M. Adams, *ibid.*, **64**, 2557 (1942).

(5) The Job–Cassal reaction also leads to organic carbonyl derivatives and is a forerunner of new methods of synthesis now being ex-