The wash water was re-extracted with fresh benzene, this benzene washed three times and combined with the main sapogenin extract. Upon evaporation to dryness in a tared beaker the crude sapogenin was converted to an acetate and an analysis calculated from subsequent infrared spectral data. Results are presented in Table II as percentage of theoretical yield as determined by acid hydrolysis.

Frozen *Agave toumeyana* leaves, 2.25 kg., were ground and immediately extracted three times with a total of 8 l. boiling water. The extract was centrifuged, adjusted to *p*H 6.5 and poured into a 5-gal. carboy (method B). Into the mouth of the carboy was wired a rubber stopper previously fitted with a stirrer, sampling tube, air sparger and air vent tube. After autoclaving, *in toto*, and cooling to room temperature the substrate was inoculated with 0.5 l. of a 48-hr. culture of *Penicillium cyclopium* Westling (ERRL). As soon as the stirrer had created a uniform mixture a sample was withdrawn. Duplicate 100-ml. portions were immediately extracted with benzene–ethanol to determine zero-time free sapogenin content. Two other 100-ml. portions served as acid hydrolyzed controls for the experiment. Air was introduced into the mixture at the rate of 300–500 ml. per minute. Duplicate 50-ml. samples were withdrawn aseptically at regular intervals. After 147 hr., stirring and aeration were discontinued. Microscopic examination of the substrate at this time revealed no contaminating microörganisms, and although no alkali had been added, the *p*H of the substrate was still above 5.0. All yields were calculated as weight of sapogenin acetate per 100 ml. of substrate and are presented in Fig. 1 as percentage of theoretical yield as determined by acid hydrolysis.

The above experiment was repeated with another aqueous extract of the sample of *Agave toumeyana* with the exception that the extract was not sterilized and no special precautions were taken to prevent contamination of the substrate with other microörganisms. As a result of contamination with acid-forming organisms, there was a rapid drop in *p*H. Hence, sodium hydroxide was added as needed to maintain *p*H 5.5–6.5. Sampling was performed as before and results of analyses are depicted in the "non-sterile" curve of Fig. 1.

Solvent Extraction of Sapogenins from Dried Fermentation Solids.—While data of fungal saponase action were obtained more rapidly by direct extraction of the aqueous substrate, this procedure was not applicable to large scale runs. Recovery of sapogenins from dried fermentation solids was therefore studied.

At the conclusion of the first large scale fermentation of aqueous extract, the slurry was centrifuged. The solids were oven dried and ground to a fine powder in a Wiley mill. A representative 5-g. sample was weighed into a 125-ml. erlenmeyer flask and extracted, with continuous stirring, with 25 ml. of a selected solvent, heating on the steam-bath

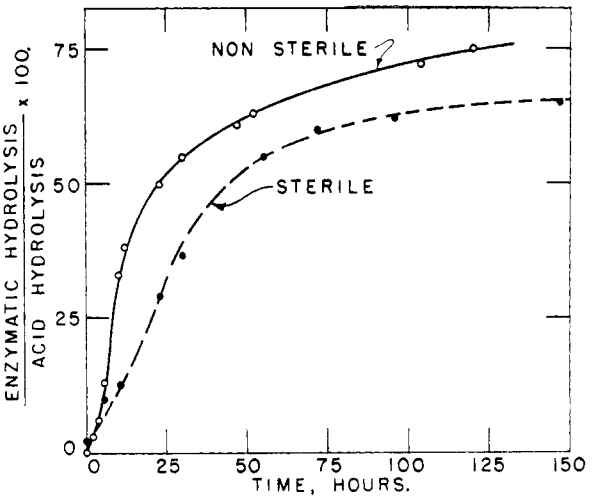


Fig. 1.—Comparison of the hydrolysis of *A. toumeyana* saponins by *P. cyclopium* when grown on "sterile" *versus* "non-sterile" extract.

for 5 minutes. The mixture was filtered. The extraction process was repeated once each with 25 ml. and 10 ml. of the same solvent. The filtrates were combined and evaporated to dryness. After chromatography the yield of sapogenin was determined from infrared spectral data of the sapogenin acetates. Maximum yields of sapogenin were obtained with chloroform or with benzene containing 10% ethanol. Methanol, benzene and ether gave incomplete extraction.

Acknowledgments.—The authors wish to express their thanks to Dr. K. B. Raper, formerly of the Northern Utilization Research Branch, for identification of microörganisms; to Janet Branaman, Theodore Perlstein and George Eppley for their technical assistance; to C. Roland Eddy and Howard Jones for infrared data; and to D. S. Correll and H. S. Gentry, Section of Plant Introduction, Horticultural Crops Research Branch, Agricultural Research Service, Beltsville, Md., for the various plant samples used.

PHILADELPHIA 18, PENNA.

[CONTRIBUTION FROM THE CHEMISTRY DIVISION OF BRITISH COLUMBIA RESEARCH COUNCIL]

Carbodiimides. Part V.[1] A Novel Synthesis of Adenosine Di- and Triphosphate and P^1,P^2-Diadenosine-5′-pyrophosphate

BY H. G. KHORANA

RECEIVED JANUARY 23, 1954

A one-step synthesis of ADP[2] and ATP[2] has been achieved in fair yield through prolonged treatment at room temperature of a mixture of AMP[2] and 85% phosphoric acid with excess of dicyclohexylcarbodiimide (DCC)[2] in aqueous pyridine. AMP alone under similar conditions gave excellent yield of P^1,P^2-diadenosine-5′-pyrophosphate.[2]

The extensive studies by Todd and collaborators[3] during the last several years have resulted in a variety of methods for synthesis in the general nucleotide field. Whilst these methods have been applied successfully to the synthesis of, among others, ADP[4,5] and ATP,[6] we wish to describe a novel, one step synthesis of these two biologically important substances.

It was shown recently[7] that dicyclohexyl- and di-

(1) Part IV. H. G. Khorana, *Can. J. Chem.*, **32**, 261 (1954).

(2) The following abbreviations are used: AMP, adenosine-5′-phosphate, muscle adenylic acid; ADP, adenosine diphosphate; ATP, adenosine triphosphate; DAPP, P^1,P^2-diadenosine-5′-pyrophosphate; DCC, dicyclohexylcarbodiimide.

(3) F. R. Atherton, H. T. Openshaw and A. R. Todd, *J. Chem. Soc.*, 382 (1945), and subsequent papers in this series.

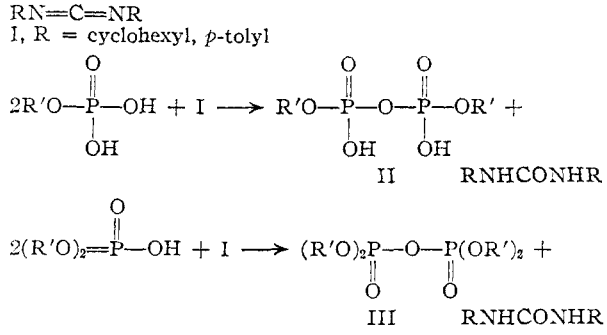
(4) G. W. Kenner, "The Chemistry of Nucleotides," in L. Zechmeister, "Progress in the Chemistry of Organic Natural Products." Vol. VIII, 1951, p. 97.

(5) J. Baddiley and A. R. Todd, *J. Chem. Soc.*, 648 (1947).

(6) J. Baddiley, A. M. Michelson and A. R. Todd, *ibid.*, 582 (1949); A. M. Michelson and A. R. Todd, *ibid.*, 2487 (1949).

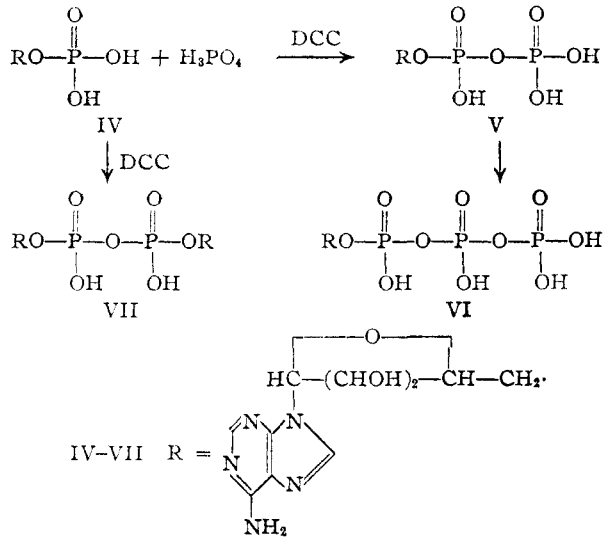
(7) H. G. Khorana and A. R. Todd, *ibid.*, 2257 (1953); this communication is regarded as Part I of the present series.

p-tolylcarbodiimides[8] (I) react instantaneously with mono- and diesters of phosphoric acid to form, respectively, the symmetrical di- and tetraesters of pyrophosphoric acid (II and III, respectively.) This new route to the synthesis of pyrophosphates had the advantage that it obviated the use of phosphorochloridates[9,10] which are frequently difficult to synthesize.



Further, as no protecting groups were necessary, the method offered for the first time the possibility of direct synthesis of the relatively stable biologically important diesters of pyrophosphoric acid.[4,10,11,12]

Seeking to extend the general scope of the synthesis in the nucleotide field through carbodiimides we investigated the direct use of 85% phosphoric acid to form polyphosphates according to the scheme IV → VI. The synthesis of ADP (V) and ATP (VI) from AMP (IV) by this method was undertaken because the biological importance[11,13] of these substances is clearly recognized and from the practical standpoint, the identification of these three adenine nucleotides in presence of one another[14] and their separation by known procedures offer little difficulty.



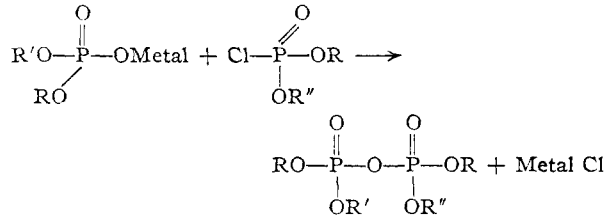
In the synthesis of unsymmetrical esters of pyrophosphoric acid (*e.g.*, V) using stoichiometric amounts of the compounds (*e.g.*, IV and phosphoric acid) the simultaneous formation of the undesirable symmetrical compounds (*e.g.*, VII, DAPP[2]) would be expected. Before seeking to establish the experimental conditions under which the formation of DAPP[2] would be eliminated[15] we proceeded to synthesize it so as to facilitate its identification in the subsequent experiments. We also learned from experiments directed toward the preparation of the symmetrical pyrophosphate about the appropriate medium and general conditions of reactions involving unprotected nucleotides and carbodiimides.[16] The synthesis of DAPP also has been accomplished by another method by Christie, *et al.*[12]

AMP had very little solubility in organic solvents including pyridine, dimethylformamide, phenol or a mixture of phenol and methyl cyanide, solvents which have been used in previous synthetic work in this field.[4-6] Although rather insoluble even in water, AMP was found to be readily soluble as pyridinium salt in pyridine containing a small proportion of water. The possibility of using partly aqueous medium in synthesis with carbodiimides has already been pointed out by Khorana and Todd.[7] In such media[17] it is clearly desirable to employ a very large excess of the carbodiimides. Table I records the yields of DAPP[18] obtained by shaking mechanically mixtures of excess of DCC

(8) The chemistry of carbodiimides has been reviewed recently; H. G. Khorana, *Chem. Revs.*, **53**, 145 (1953).

(9) New nomenclature of phosphorus compounds adopted by the International Union of Pure and Applied Chemistry; see *e.g.*, *J. Chem. Soc.*, 5122 (1952).

(10) By far the most widely used reaction for the synthesis of esters of pyrophosphoric acid has been that between a metal salt of a substituted phosphoric acid and a substituted phosphorochloridate. Thus



If the groups R are protectmg groups, *e.g.*, benzyl, hydrogenation affords diesters of pyrophosphoric acid. *Cf.* N. S. Corby, G. W. Kenner and A. R. Todd, *J. Chem. Soc.*, 1234 (1952), and the references cited therein; F. R. Atherton, *Quart. Rev. (London)*, **3**, 146 (1949).

(11) W. D. McElroy and B. Class, "Phosphorus Metabolism," Vol. 1, 1951; Vol. II, 1952, The John Hopkins Press, Baltimore, Md.

(12) The synthesis of the symmetrical P[1],P[2]-diuridine-5'-pyrophosphate through reaction of uridine-5'-phosphate with dicyclohexylcarbodiimide in pyridine also has been achieved; S. M. H. Christie, D. T. Elmore, G. W. Kenner, A. R. Todd and F. J. Weymouth, *J. Chem. Soc.*, 2947 (1953).

(13) H. M. Green and H. B. Stoner, "Biological Actions of the Adenine Nucleotides," H. K. Lewis and Co., London, 1950.

(14) (a) W. E. Cohen and C. E. Carter, THIS JOURNAL, **72**, 4273 (1950); (b) L. V. Eggleston and R. Hems, *Biochem. J.*, **52**, 156 (1952); (c) R. M. Bock and R. A. Alberty, *J. Biol. Chem.*, **193**, 435 (1951); (d) A. L. Dounce, *et al.*, *ibid.*, **174**, 361 (1948); (e) M. Johnson, M. A. G. Kaye, R. Hems and H. A. Krebs, *Biochem. J.*, **54**, 625 (1953).

(15) The unreacted phosphoric acid and pyrophosphoric acid which also are formed can be removed readily through precipitation at low *p*H of the mercury salts of adenine nucleotides with Lohmann reagent (100 g. of $Hg(NO_3)_2 \cdot 8H_2O$, 25 cc. of H_2O and 25 cc. of coned. HNO_3). In blank experiments it was shown that mercury salts of phosphoric acid and pyrophosphoric acid were not precipitated with this reagent at low *p*H.

(16) Other reactions studied in this manner will be reported separately.

(17) This very novel feature of this synthesis of pyrophosphates should be emphasized. As far as we are aware, all syntheses reported heretofore have required anhydrous media. The results reported herein further substantiate the mechanism of the formation of pyrophosphates involving carbodiimides already advanced by Khorana and Todd.[7]

(18) This is the only product of reaction in aqueous pyridine. Any possible products arising from further condensations must be unstable.

and AMP, in aqueous pyridine. The pyrophosphate was readily identified and estimated by paper chromatography using the solvent system 5% disodium hydrogen phosphate–isoamyl alcohol[14a,19]; DAPP, R_f, 0.63; AMP, R_f, 0.72. The comparative sluggishness of the reaction under conditions employed in the present work is in contrast with the instantaneous reactions of strong acids observed earlier.[7,12,20]

Although prolonged agitation of the reaction mixture gave very good yields of the pyrophosphate some unreacted AMP always contaminated the product.[21] The separation of the small proportion of AMP was achieved on an ion exchange column (Dowex 2; mesh, 200–325; chloride form *cf.*[14a]). After absorption of the mixture of pyridine salts (*p*H 5) on the column, elution with 0.003 *N* and then 0.01 *N* hydrochloric acid removed, respectively, AMP and the pyrophosphate. The yield of the desired product was confirmed from the optical density of the respective eluates.[22] The separation of the pyrophosphate as the barium salt after neutralization of the eluate with sodium hydroxide and concentration at low temperature could not be achieved because of the presence of excess of sodium chloride. In one experiment lithium hydroxide was used to neutralize the eluate and the lithium salt of DAPP was freed from lithium chloride by washing with acetone containing some alcohol in which the latter is soluble. A simple procedure which obviated the use of an ion exchange column consisted of fractional precipitation of the barium salt in aqueous alcohol. Thus, pure DAPP was obtained in 55% yield. It travelled as a single spot in disodium hydrogen phosphate (5%)–isoamyl alcohol and in 1% ammonium sulfate–isopropyl alcohol[23] (1:2, v./v.), R_f 0.21; R_f of AMP 0.33. Its purity was also checked by ion exchange analysis as described above. Elution with 0.003 *N* hydrochloric acid gave only a trace of AMP, whereas 0.01 *N* hydrochloric acid eluted practically all the material. Experiments on the hydrolysis of DAPP are recorded in the Experimental section.

Attention was now turned to the condensation of phosphoric acid with AMP through reaction with DCC.[24] The use of an excess of phosphoric acid was obviously desirable in order to minimize the formation of the symmetrical DAPP. Preliminary experiments[25] on a 10-mg. (AMP) scale showed that with the use of 5 molecular proportions of phosphoric acid only a small amount (*ca.* 10%) of DAPP was formed, ADP and ATP being the main products. Use of tenfold excess of phosphoric acid suppressed practically completely the formation of DAPP. However, using this large excess of phosphoric acid a homogeneous mixture[26] of the reactants in aqueous pyridine could not be obtained. Table II records the results obtained by vigorously[27] shaking a two-phase reaction mixture. The yields shown were those obtained fairly consistently by ion exchange analyses according to Cohen and Carter[14a] of products freed from most of the "inorganic phosphates" (*cf.* ref. 15 and see below). The isolation of ADP and ATP included precipitation of the mercury salts with Lohmann reagent[15] and separation on an ion exchange column by the well established procedure of Cohen and Carter.[14a,28] ADP and ATP precipitated as their barium salts from the concentrated eluates were purified *via* mercury salts according to the method of LePage.[29]

These preparations travelled as single spots on paper chromatograms in 5% disodium hydrogen phosphate–isoamyl alcohol system[14a] (ADP, R_f 0.72; ATP, R_f 0.81). Samples of these substances obtained from commercial sources (see Experimental) applied simultaneously on paper chromatograms had R_f values identical, respectively, with those recorded for the synthetic samples. When the purity of these preparations was checked carefully by ion exchange technique[14a] (*ca.* 5-mg. scale, the amounts in the eluates being estimated spectrophotometrically), ADP showed 2% contamination by ATP and the latter, 5% contamination by the former. The total phosphorus content and the ratios of labile phosphorus (*i.e.*, phosphorus cleaved in 15 minutes by 1 *N* hydrochloric acid at 100°) to total phosphorus, which serve as further criterion[29] of purity, were determined by the method of Allen[30] and were in accord with the molecular compositions already established for the barium salts of these substances.[6,29] The infrared spectra[31] of the synthetic samples were identical, respectively, with those of samples from natural sources. According to the enzymatic method of LePage and Potter[32] the ac-

(19) Because of the separation of DAPP, AMP, ADP and ATP (*cf.* ref. 14a) using it, this solvent system was employed throughout the present work. The bottom layer consisting of 5% disodium hydrogenphosphate was replaced frequently.

(20) H. G. Khorana, *Can. J. Chem.*, 585 (1953).

(21) This is readily understandable because the presence of *ca.* 85% of DAPP which itself is apparently inert toward DCC, only catalyzes the hydration of the latter to dicyclohexylurea; $RN{=}C{=}NR \xrightarrow{H^{\oplus}} H_2O \longrightarrow RNHCONHR$.

(22) The figure of 14,200 at *p*H 2 (see ref. 14a, footnote page 4274) has been used for the molecular extinction coefficient of AMP at 260 mμ.

(23) *Cf.* N. Anand, V. M. Clark, R. H. Hall and A. R. Todd, *J. Chem. Soc.*, 3665 (1952).

(24) This seems to be the reagent of choice. Di-*p*-tolylcarbodiimide is much less reactive. A detailed study of the reactivity of variously substituted carbodiimides toward strong acids will be published later.

(25) The reaction mixtures were analyzed throughout by paper chromatography (*cf.* 14a).

(26) The two opposing factors involved are: (1) the insolubility of DCC in pyridine containing substantial amount of water, and (2) the low solubility of phosphoric acid in pyridine containing small amounts of water. In this connection it should be pointed out that pyrophosphoric acid was practically insoluble in pyridine unless the latter was diluted considerably with water. In the few experiments carried out in attempts to condense pyrophosphoric acid with AMP in the presence of DCC large proportions of AMP remained unreacted.

(27) After extensive experimentation we emphasize the important influence of the rate of agitation on the relative proportions of the reaction products.

(28) See *e.g.*, E. Goldwasser, *Nature*, **171**, 126 (1953), and I. Green and W. H. F. M. Mommaerts, *J. Biol. Chem.*, **202**, 541 (1953); P. Siekewitz and V. R. Potter, *ibid.*, **200**, 188 (1953).

(29) G. A. LePage in H. E. Carter, "Biochemical Preparations," Vol. 1, John Wiley and Sons, Inc., New York, N. Y., 1949, p. 165.

(30) R. J. L. Allen, *Biochem. J.*, **34**, 858 (1940). The assistance of Dr. C. A. Dekker, Biochemistry and Virus Laboratory, University of California, Berkeley, with some of the phosphorus analyses is gratefully acknowledged.

(31) These were taken in a Perkin–Elmer infrared spectrophotometer model 21 with rock salt optics, using nujol mulls. One weak band at 1687 cm.$^{-1}$ in the spectrum of the commercial ADP sample was absent from that of the synthetic sample.

(32) G. A. LePage and V. R. Potter, *J. Biol. Chem.*, **179**, 1229 (1949).

tivity of the enzyme system containing the synthetic sample of ATP was equal or better than the activity when the system was supplemented with comparable amounts of the standard ATP employed routinely by these workers.

It was desirable to check the R_f values of the synthetic samples with those of the commercial samples on paper chromatograms developed in more than one suitable solvent system. In *n*-butyl alcohol–acetic acid–water (4:1:5; top phase) using descending technique and developing the chromatogram for 60 hours according to Johnson, Kaye, Hems and Krebs,[14e] R_f values checked again, but all samples gave elongated spots. A very useful solvent system has been found to be 1% aqueous ammonium sulfate solution–isopropyl alcohol[23] (1:2, v./v.), the paper being previously soaked in 1% ammonium sulfate solution and dried. R_f's using ascending technique: AMP, 0.33; ADP, 0.18; ATP, 0.10–0.11. The synthetic samples of ATP showed slight tailing in this solvent system. The mixtures of reaction products (Table II) prior to ion exchange separations showed similarly some ultraviolet absorbing material travelling slower than ATP, indicating probably the presence of higher polyphosphates. Although these impurities were largely removed during separations on ion exchange columns (see Experimental) it is likely that the synthetic sample of ATP is contaminated with a trace of another substance, possibly adenosine tetraphosphate. Further work is in progress to determine accurately whether in fact the present method can lead to the preparation of the tetraphosphate.

Although as recorded in Table II, ADP and ATP are formed in good yield, the isolation procedure particularly the ion exchange separations, involving large volumes and high salt concentrations, caused great losses of the products and the yields of final preparations of ADP and ATP were no higher than 13% and 20%, representing a total of 33% based on the amount of AMP used. Using Kalckar's[33] observations on the solubility characteristics of the barium salts of adenosine phosphates an alternative isolation procedure involving fractional precipitation has been devised which results in somewhat improved yields of ADP and ATP. The samples prepared in this way were, however, mutually contaminated and the sample of ATP retained the above mentioned impurity of another polyphosphate.

Dounce, Rothstein, Beyer, Meier and Freer[14d] described the isolation of ATP labeled with P^{32} from the rabbit after injection of radioactive phosphorus. Hems and Bartley[34] also have reported a method for the preparation of small quantities of P^{32}-labeled ATP using respiring tissue suspensions. It is clear that the present chemical synthesis affords a ready method for the preparation of ADP and ATP labeled quantitatively at the two labile phosphate bonds.

This work which constitutes the first example of the direct use of phosphoric acid[35] for the synthesis of sensitive, biologically important polyphosphates under mild conditions, is being extended to the preparation of other nucleoside polyphosphates.[36]

(33) H. M. Kalckar, *J. Biol. Chem.*, **148**, 127 (1943).

(34) R. Hems and W. Bartley, *Biochem. J.*, **55**, 434 (1953).

(35) Phosphorylation of vitamin B_1 with metaphosphoric acid to yield tri-phosphoric esters has been recorded; L. Velluz, G. Amiand, and J. Bartos, *Bull. soc. chim. France*, 871 (1948); M. Viscontini, G. Bonetti and P. Karrer, *Helv. Chim. Acta*, **32**, 1478 (1949).

(36) Uridine-5'-triphosphate has been isolated recently from yeast-derived nucleotides; S. H. Lipton, S. A. Morell, A. Frieden and R. M. Bock, THIS JOURNAL, **75**, 5449 (1953). Dr. V. R. Potter of the University of Wisconsin has kindly informed us that Schmitz, Hulbert and Potter have found the occurrence of guanosine-5'-uridine-5', and cytidine-5'-triphosphates in significant amounts in animal tissues. We are indebted to Dr. Potter for communicating these results prior to publication.

(37) J. X. Khym and W. E. Cohen, THIS JOURNAL, **75**, 1153 (1953). also have remarked on the mutual contaminations of these samples.

(38) (a) E. Schmidt, F. Hitzler and E. Lahde, *Ber.*, **71**, 1933 (1938); (b) E. Schmidt, M. Seefelder, R. G. Jennen, W. Striewsky and H. Von Marius, *Ann.*, **571**, 83 (1951). Mercuric oxide was preferred to the cheaper oxidizing agent, sodium hypochlorite, used in (b), the product using the latter being always inferior. It is interesting to observe that the addition of some anhydrous calcium chloride to the mixture of dicyclohexylthiourea and mercuric oxide inhibited almost completely the oxidation of the thiourea to the carbodiimide. Dehydrating agents have sometimes been employed (*cf.* ref. 8) to remove the water formed in the reaction $RNHCS\,NHR + HgO \rightarrow RN{=}C{=}NR + HgS + H_2O$.

(39) Prepared from the commercially available 200–400 mesh resin by removing the fines, etc., using a 325 mesh sieve.

Experimental

Materials.—AMP was a commercially available sample. It travelled as a single spot on paper chromatograms in 5% disodium hydrogen phosphate–isoamyl alcohol solvent system and 1% ammonium sulfate–isopropyl alcohol (1:2).- ADP and ATP used as reference compounds were also obtained from commercial sources. ADP[37] (barium salt) contained a small amount of AMP and a trace of ATP. The ATP[37] sample contained appreciable amounts of ADP. It was further purified by careful reprecipitation of the barium salt at *p*H 3.5.[33] Pyrophosphoric acid (solid) used in some experiments[26] was purchased from City Chemicals Corporation, New York. DCC[38] was made by the treatment of finely ground N,N'-dicyclohexylthiourea with mercuric oxide (Baker and Adamson reagent grade) in carbon disulfide. The mechanical agitation was continued for 6–7 hours.

DAPP. General Method.—AMP was dissolved in aqueous pyridine and DCC added. The resulting clear solution (or two layers) was agitated mechanically. Dicyclohexylurea began to separate usually after about one hour. After shaking for varying lengths of time the urea was filtered off and washed thrice with small amounts of water. The combined filtrate and washings were extracted exhaustively with ether to remove excess of pyridine. The aqueous solution (*p*H 3–4) was examined on paper chromatograms (5% disodium hydrogen phosphate–isoamyl alcohol). The spots were located under a suitable ultraviolet radiation source; DAPP, R_f 0.63; AMP, 0.71. The yields as estimated spectrophotometrically (optical density at 260 mμ) after elution of the spots are recorded in Table I.

TABLE I

THE REACTION OF AMP[a] WITH DCC

Pyridine,[b] cc.	0.6[c]	0.6[c]	0.6[c]	0.6	0.7[c]	0.4	0.4
DCC, mg.	200	200	300[d]	400[e]	400[e]	200[f]	200
Time, hours	1/4	3.5	8	18	42	19	26
Yield of DAPP, %	5	45–50	60–65	70–75	80–85	51	57

[a] Ten mg. used in each experiment. [b] This represents the amount of anhydrous pyridine employed. To it was added in each experiment 0.1 cc. of water. [c] Clear solution until the separation of the urea began. [d] 200 mg. at first, 100 mg. after 4 hours. [e] 300 mg. at first, 100 mg. after 11 hours. Some unreacted DCC observed on working up the reaction mixtures in most experiments. [f] 100 mg. at first, 100 mg. after 5 hours.

Isolation of DAPP. Method A.—The experiment 5 of Table I was repeated using 100 mg. of AMP and the product was absorbed on a Dowex 2 (200–325 mesh,[39] chloride form)

column 2.5 cm. diameter × 7 cm. long. After a preliminary wash with water which removed only pyridine[40] 850 cc. of 0.003 *N* hydrochloric acid was passed (flow rate 10 cc./minute) to remove AMP[14a] (corresponding to 18.7 mg.). Subsequent washing with 0.01 *N* hydrochloric acid removed DAPP (corresponding to 80% of AMP; volume *ca.* 700 cc.). The solution containing the pyrophosphate was neutralized with lithium hydroxide to *p*H 5, then concentrated at 25–30° *in vacuo* to *ca.* 10 cc. and this concentrate was further evaporated to a sirup in a vacuum over calcium chloride. Trituration with a mixture of acetone and ethyl alcohol (50 cc., 5:1 v./v.) precipitated the lithium salt of the pyrophosphate which was spun off and washed with fresh acetone; yield of the crude lithium salt 80 mg.

Method B. Isolation as Barium Salt.—The mixture of pyridine salts of AMP and DAPP (from 100 mg. scale experiment) was evaporated to dryness in a vacuum over phosphorus pentoxide. To the solution of the residue in 1 cc. of water was added 0.5 cc. of 1.78 *N* barium acetate solution followed by 1 cc. of 95% ethanol. The mixture began to deposit a gummy precipitate and was kept overnight. The solidified deposit was then thoroughly triturated, spun off and washed with another 1-cc. portion of alcohol. Paper chromatography showed the solid material (100 mg.) to be mostly the barium salt of DAPP. (The supernatants contained mainly AMP, and further dilution with 1 cc. of 95% ethyl alcohol gave 20 mg. of pure barium salt of AMP.) This was triturated thoroughly with 2 cc. of water and the aqueous extract removed by centrifugation. The insoluble barium salt was further washed with 0.5 cc. of water; yield 67 mg. (55%). *Anal.* Found: P, 7.2. Calcd. for $C_{20}H_{26}N_{10}O_{13}P_2Ba\cdot 2H_2O$: P, 7.3. Ten mg. of the barium salt was triturated with a few drops of a dilute solution of ammonium sulfate and barium sulfate removed by centrifugation. To the clear solution was added 1 cc. of *N* sodium hydroxide solution. Aliquots were removed at intervals and examined on paper chromatograms after neutralization with hydrochloric acid. DAPP was stable at room temperature for 48 hours. Heating the alkaline solution for 2 hours at 100° caused degradation to AMP, but another ultraviolet absorbing spot (R_f 0.37 in disodium hydrogen phosphate–isoamyl alcohol) presumably adenine also appeared on paper chromatograms. DAPP was found similarly to be stable to *N* hydrochloric acid at room temperature. These results are in agreement with those reported by Christie, *et al.*

The Reaction of a Mixture of AMP and Phosphoric Acid with DCC.—AMP and 85% phosphoric acid were first dissolved in a small amount of a mixture of pyridine and water and a pyridine solution of DCC then added. The mixture consisting of two phases was agitated mechanically. Dicyclohexylurea began to separate usually in *ca.* 30 minutes. At intervals, more DCC and pyridine were added. The reaction mixture was worked up as described under DAPP. The aqueous solution of pyridine salts was cooled to 0°, acidified with dilute nitric acid to *p*H < 2. The mercury salts of adenosine phosphates were precipitated by the dropwise addition of Lohmann reagent (total amount necessary *ca.* 0.5 cc. for products obtained from 100 mg. of AMP).

After being allowed to stand at 0° for several hours, the mercury salts were centrifuged off and washed with small amount of 0.2 *N* nitric acid. These were then suspended in *ca.* 4–5 cc. of water and decomposed by bubbling hydrogen sulfide at 0° for 20 minutes. After removal of the supernatant by centrifugation mercuric sulfide was resuspended in a few cc. of water and hydrogen sulfide passed again. The supernatants were combined, neutralized with alkali to *p*H 6 and freed from hydrogen sulfide by continued bubbling of air. The solution was analyzed by the ion exchange method of Cohen and Carter.[14a] A volume of the solution containing equivalent of 5 mg. of AMP was used for each run of ion exchange analysis on a bed 1.5 cm. long × 1 cm. diameter of Dowex 2. The results are recorded in Table II.

Isolation of ADP and ATP. Method A.—Experiment 5 of Table II was repeated using 400 mg. of AMP and the reaction mixture worked up as above (including careful precipitation and decomposition of the mercury salts). The neutral solution of the products (25 cc.) was stored in a frozen state at −20°. It was chromatographed on Dowex 2 column (5 cm. long × 4 cm. diameter), in three portions. The eluates[41] with 0.01 *N* hydrochloric acid, containing 0.025 *N*[42] sodium chloride, containing ADP (500–550 cc. from each run) were neutralized with sodium hydroxide to *p*H 6 and pooled.[43]

The eluates[41] with 0.01 *N* hydrochloric acid containing 0.3[42] *N* sodium chloride, containing ATP (*ca.* 600–650 cc. from each run), were similarly neutralized and pooled. The solutions were concentrated *in vacuo* (2 mm. pressure) at 0–5° (short path condensation, receiver immersed in solid carbon dioxide–acetone-bath). The concentrates (*ca.* 20 cc. each) containing ADP and ATP were further concentrated[44] to half *in vacuo* over anhydrous calcium chloride and to each of these excess (2 cc.) barium acetate solution (approximately molar) was added. The precipitation of barium salts was completed by keeping the mixtures at 0° and in the case of ADP, by the addition of 5 cc. of ethanol. The precipitates were centrifuged off and washed with small amounts of water, ethanol and ether. These samples were again converted to mercury salts and finally the barium salts prepared as described by LePage.[29] Yield of ATP, 150–200 mg. (15–20%). *Anal.* Found: labile P, 7.0, 7.2; total P, 10.5, 10.7. Calcd. for $C_{10}H_{12}N_5O_{13}P_3Ba_2\cdot 6H_2O$: P, 10.5; labile:total P, 1:1.5. Yield of ADP, 60–100 mg. (8–13%). *Anal.* Found: labile P, 4.7; total P, 8.9. Calcd. for $Ba_3(C_{10}H_{12}N_5O_{10}P_2)_2\cdot 8H_2O$: P, 8.8; labile:total P, 1:1.

Method B.—Experiment 5 of Table II was repeated using 500 mg. of AMP and the reaction mixture worked up as before, including precipitation and decomposition of mercury salts. The final aqueous solution (20 cc.) was neutralized to *p*H 4 with sodium hydroxide and freed from hydrogen sulfide. Three cc. of barium acetate solution (molar) was added and the mixture containing the precipitated barium salt was kept at 0° for ten hours, then centrifuged and the supernatant poured off. The precipitate of barium salt was washed thoroughly with cold 0.02% acetic acid and

TABLE II

THE REACTION OF A MIXTURE OF AMP AND PHOSPHORIC ACID WITH DCC[a]

AMP, mg.	H_3PO_4, mg.	Water,[b] cc.	Pyridine,[b] cc.	DCC,[b] g.	Total time, hours	Yield, % AMP	ADP	ATP[h]
200	800	0.6	9[c]	7[c]	26	35	30	35
100	400	.55[d]	4.5[e]	3.5[e]	24	19	44	37
100	400	.55[d]	7.5[f]	3.5[f]	24	16	42	42
200	800	2.1[g]	15[g]	7[g]	7.25	29	32	39
200	800	2.1[g]	15[g]	7[g]	26	11	33	56

[a] Preliminary experiments were carried out on a 10-mg. (AMP) scale. As the rate of agitation appears to influence the relative yields of ADP and ATP, only the experiments carried out on a similar scale and as far as possible under identical conditions are recorded. [b] The figures in these columns represent the total amounts of these reagents added, in individual experiments. [c] 6 cc. of pyridine + 4 g. of DCC at first, 2 cc. of pyridine + 2 g. of DCC after 5 hours, and 1 cc. of pyridine + 1 g. of DCC, added after 18 hours. [d] 0.5 cc. at first, 0.05 cc. after 18 hours. [e] 3 cc. of pyridine + 2 g. of DCC, at first; 1 cc. of pyridine + 1 g. of DCC after 5 hours, 0.5 cc. of pyridine and 0.5 g. of DCC after 18 hours. [f] 6 cc. of pyridine at first, otherwise as in *e*. [g] 12 cc. of pyridine, 4 g. of DCC and 2 cc. of water at first, 2 cc. of pyridine and 2 g. of DCC after 5 hours; 1 cc. of pyridine, 1 g. of DCC and 0.1 cc. of water after 18 hours. [h] The yields recorded in this column include small amounts of another ultraviolet absorbing material which appears to be eluted almost simultaneously with ATP, corresponding perhaps to a higher phosphate.

(40) The bases are not absorbed under these conditions (*cf.* ref. 14a).

(41) The eluates with optical density less than 0.5 at 260 mμ were discarded.

(42) These concentrations of sodium chloride are higher than those used by Cohen and Carter (ref. 14a). Much smaller volumes of these modified eluants were required.

(43) The stability of the respective products was checked at all stages during these operations, the solutions and concentrates being stored, whenever necessary, at −20° in a frozen state.

(44) Large amounts of sodium chloride, which are removed, separate from the solution containing ATP.

spun off again. Paper chromatography showed the barium salt to be of mainly ATP, with ADP and a higher phosphate (R_f 0.06, in 1% ammonium sulfate–isopropyl alcohol, 1:2, v./v.) as the impurities. It was converted to the mercury salt (precipitation with Lohmann reagent) and the mercury salt decomposed as usual. Final precipitation of the barium salt at *p*H 3.8 and repeated washing with 0.2% acetic acid gave 350 mg. of a sample which was slightly contaminated by ADP and a small amount of the slower travelling higher phosphate.

The supernatant from the precipitation at *p*H 4 was brought to *p*H 8 with alkali and the precipitated barium salt, mostly of ADP, was collected by centrifugation after keeping the mixture at 0° for some hours, and washed with water; yield 120 mg. This sample of ADP was slightly contaminated by AMP and ATP.

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Carbodiimides. VI. The Reaction of Dicyclohexylcarbodiimide with Yeast Adenylic Acid. A New Method for the Preparation of Monoesters of Ribonucleoside 2′- and 3′-Phosphates

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The reaction of adenylic acids *a* and *b* and their mixture (yeast adenylic acid) with dicyclohexylcarbodiimide in aqueous pyridine at room temperature has been studied. The corresponding 2′,3′-cyclic phosphate which in every case is the initial product reacts further to give two products, presumably the 2′- and the 3′-isomers, having the novel structures III and IV. Although stable to aqueous ammonia, III and IV yield adenylic acids *a* and *b* and dicyclohexylurea as the ultimate products when treated with either dilute sodium hydroxide or dilute hydrochloric acid. The action of sodium benzoxide on either III or IV gives in good yield a mixture of the monobenzyl esters of the isomeric adenylic acids *a* and *b*. These results demonstrate that the cyclic phosphate is an intermediate in all of these reactions. The significance of these findings in the problem of synthesis of dinucleoside phosphates containing 2′,5′- and 3′,5′-phosphodiester linkages has been pointed out.

It has been reported quite recently[3] that the prolonged treatment of adenosine-5′-phosphate (muscle adenylic acid) in aqueous pyridine with excess of dicyclohexylcarbodiimide (DCC) affords in very good yield the symmetrical P^1,P^2-diadenosine-5′-pyrophosphate. The reaction conditions[4] employed are particularly suitable for work in the general nucleotide field and the one-step synthesis of adenosine di- and triphosphate (ADP and ATP[3]) demonstrates the practical application of this new and attractive approach to the synthesis of nucleotide-derived coenzymes. In seeking to extend the study of the reactions of carbodiimides with nucleotides we have investigated the reaction of DCC with the isomeric adenylic acids *a* and *b*[5] (I) which, from the known reactions of carbodiimides,[6] could lead either to pyrophosphate formation or by virtue of the adjacent free hydroxyl group to internal diester (cyclic phosphate) formation. A preliminary study[7] of the reaction of DCC with yeast uridylic acid in dimethylformamide had indeed suggested the formation of the corresponding 2′,3′-cyclic phosphate. Compounds of the latter type, since their discovery as intermediates in the alkaline and ribonuclease catalyzed hydrolysis of ribonucleic acid,[8–10] have been the subject of extensive chemical and enzymatic investigation.[11–16]

By the treatment of yeast adenylic acid[17] (I) (a mixture of adenosine-2′- and 3′-phosphate), adenylic acid *a*[17] or adenylic *b*[17] with an excess of DCC in aqueous pyridine, it was found that the course of reaction was identical irrespective of the particular isomer or mixture of isomers employed. After a reaction period of about 30 minutes, adenosine 2′,3′-cyclic phosphate (II) was the major product (70–75%) contaminated by starting material. The identification of this expected reaction product was readily accomplished by synthesis by an independent route[11] and comparison of physical properties. However, with a slightly prolonged

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(4) No protecting groups are required. Anhydrous conditions are not mandatory. The reagents are stable and easy to handle.

(5) The initial separation of two isomeric adenylic acids from alkaline hydrolysates of yeast ribonucleic acid was accomplished jointly by C. E. Carter, THIS JOURNAL, **72**, 1466 (1950), and W. E. Cohn, *ibid.*, **72**, 1471 (1950). These compounds, termed adenylic acids *a* and *b*, were later shown to be the 2′-, and 3′-phosphates but not necessarily respectively (D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 44 (1952)). Recent evidence (J. X. Khym, D. G. Doherty, E. Volkin and W. E. Cohn, THIS JOURNAL, **75**, 1262 (1953), and the references cited therein; L. F. Cavalieri, *ibid.*, **75**, 5268 (1953); and D. M. Brown, G. D. Fasman, D. I. Magrath, A. R. Todd, W. Cochran and M. M. Woolfson, *Nature*, **172**, 1184 (1953)) has firmly established the *a*-isomer as the 2′-phosphate and the *b*-isomer as the 3′-phosphate. For the sake of convenience we have used throughout this paper the original terms: adenylic acids *a* and *b*.

(6) H. G. Khorana, *Chem. Revs.*, **53**, 145 (1953).

(7) Unpublished work of D. M. Brown, referred to in ref. 6.

(8) R. Markham and J. D. Smith, *Nature*, **168**, 406 (1951).

(9) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552 (1952).

(10) D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 52 (1952).

(11) D. M. Brown, D. I. Magrath and A. R. Todd, *ibid.*, 2708 (1952).

(12) D. M. Brown, C. A. Dekker and A. R. Todd, *ibid.*, 2715 (1952).

(13) R. Markham and L. A. Heppel, *Nature*, **171**, 1152 (1953).

(14) D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 2040 (1953).

(15) L. A. Heppel, P. R. Whitfield and R. Markham, Abstract of a paper presented at a meeting of the Faraday Society, October, 1953.

(16) C. A. Dekker, *Federation Proc.*, **13**, 197 (1954).

(17) Like muscle adenylic acid, these substances are practically insoluble in anhydrous organic solvents including dimethylformamide and pyridine. Although difficultly soluble even in water, they dissolve readily in aqueous pyridine.