TABLE II

Each tube contained 0.2 ml. of freshly prepared rabbit bone marrow suspension,[15] 0.2 μg. of 2-C^{14}-labeled thymidine,[16] plus 1 μmole of neutralized compound. The tubes were shaken two hours at 37° in a Dubnoff shaker. The DNA was isolated according to Friedkin's procedure[15] and its radioactivity was measured in a Tracerlab windowless flow counter. Each value represents a separate tube.

Compound	C.p.m. of DNA fraction
Saline (10 λ)	2020;1950
6-Methyl-*asym*-triazine-3,5(2,4)-dione	2010;1980
2-(2'-Deoxy-D-ribofuranosyl)-6-methyl-*asym*-triazine-3,5(2,4)-dione	1100; 900
The 3'-phosphate	1700;1400
The 5'-phosphate	1550;1370
The 3',5'-diphosphate	1560;1400

Action of *Crotalus adamanteus* Venom on 2-(2'-Deoxy-D-ribofuranosyl)-6-methyl-*asym*-triazine-3,5(2,4)-dione-3'- and 5'-phosphates.—In a test-tube containing 0.1 cc. of 1 *M* glycine buffer (*p*H 8.5), 0.1 cc. of 0.1 *M* magnesium chloride and 0.5 cc. of enzyme solution[23] was added 10 μmoles of nucleotide. The tubes were incubated for one hour at 37° then analyzed for orthophosphate by Gomori's procedure.[24] At the end of this time the tube containing the second eluted mononucleotide gave a blank reading (3'-phosphate). The first eluted mononucleotide gave a reading corresponding to 50% of the theoretical amount of orthophosphate, therefore this was the -5'-nucleotide.

Acknowledgment.—The authors wish to thank Dr. T. H. Jukes for many helpful suggestions concerning this problem. They are particularly indebted to Drs. A. D. Welch and W. H. Prusoff of Yale University for many discussions and help with the transdeoxyribosidation enzyme system of Dr Prusoff. They also wish to thank Dr. J. Clark and staff for the preparation of azathymine and Mr. L. Brancone and staff for the microanalyses.

(23) The enzyme solution was prepared by dissolving 10 mg. of crude lyophilized venom in 1 cc. of water.

(24) G. Gomori, *J. Lab. Clin. Med.*, **27**, 955 (1942).

PEARL RIVER, N. Y.

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

Nucleoside Polyphosphates. VI.[1] An Improved and General Method for the Synthesis of Ribo- and Deoxyribonucleoside 5'-Triphosphates

BY MICHAEL SMITH AND H. G. KHORANA

RECEIVED SEPTEMBER 5, 1957

A generally satisfactory procedure for the synthesis of ribo- and deoxyribonucleoside 5'-triphosphates is described. The method involves the reaction of tri-*n*-butylammonium salts of nucleoside 5'-monophosphates and orthophosphoric acid with dicyclohexylcarbodiimide.

The carbodiimide method[2] for the condensation of unprotected phosphate esters has been used successfully for the synthesis of a number of nucleoside 5'-polyphosphates,[3,4] nucleotide coenzymes[5] and related compounds of biological interest.[6] However, the experimental conditions for effecting condensation have varied greatly with different nucleotides[7] and the yields of the desired products have often been unsatisfactory. Clearly, the usefulness of the method would be considerably increased if generally applicable procedures for the condensation reactions could be found. The present communication describes one such procedure which leads to improved syntheses of the ribo- and deoxyribonucleoside 5'-triphosphates.

Aqueous pyridine is the only solvent system which has proved satisfactory in the previous synthetic work. Homogeneous solutions were not obtained in this medium because of the contrasting solubility properties, on the one hand, of phosphate esters and, on the other, of DCC,[8a] the reagent which has been used exclusively in the past. The varying degree of partition of different nucleotides in the two phase systems employed precluded standardization of reaction conditions and led in some cases[1] to unsatisfactory yields of the desired products. In seeking to devise a general procedure for nucleoside polyphosphate synthesis, our aim was to develop a one phase reaction system.

In the present work, attempts first were made to obtain homogeneous solutions[8b] in aqueous pyridine by the use of a more polar carbodiimide. The water-soluble reagent IV[9] was prepared according to the route I → IV, and its use in the self-condensa-

(1) Paper V. R. W. Chambers and H. G. Khorana, THIS JOURNAL, **79**, 3752 (1957).

(2) H. G. Khorana, *ibid.*, **76**, 3517 (1954).

(3) R. H. Hall and H. G. Khorana, *ibid.*, **76**, 3056 (1954).

(4) (a) R. L. Potter, S. Schlesinger, V. Buettner-Janusch and L. Thompson *J. Biol. Chem.*, **226**, 381 (1957); (b) A. Kornberg, unpublished work on the synthesis of thymidine 5'-triphosphate; (c) B. L. Horecker, J. Hurwitz and L. A. Heppel, THIS JOURNAL, **79**, 701 (1957).

(5) (a) E. P. Kennedy, *J. Biol. Chem.*, **222**, 185 (1956). (b) F. M. Huennekens and G. L. Kilgour, THIS JOURNAL, **77**, 6716 (1955). (c) G. W. Kenner, A. R. Todd and R. F. Webb, *J. Chem. Soc.*, 2843 (1954). (d) P. Reichard and N. R. Ringertz, THIS JOURNAL, **79**, 2025 (1957).

(6) (a) P. T. Talbert and F. M. Huennekens, *ibid.*, **78**, 4671 (1956). (b) P. Berg, *Federation Proc.*, **16**, 152 (1957).

(7) Compare, for example, the syntheses of uridine and guanosine 5'-polyphosphates.[1]

(8) (a) Abbreviations used in this paper are: dicyclohexyl carbodiimide, DCC; adenosine 5'-monophosphate, AMP; adenosine 5'-diphosphate, ADP; adenosine 5'-triphosphate, ATP; uridine 5'-monophosphate, UMP; uridine 5'-diphosphate, UDP; uridine 5'-triphosphate, UTP; cytidine 5'-phosphate, CMP; cytidine 5'-diphosphate, CDP; cytidine 5'-triphosphate, CTP; guanosine 5'-phosphate, GMP; guanosine 5'-diphosphate, GDP; guanosine 5'-triphosphate, GTP; 2'-deoxynucleoside 5'-phosphates are indicated by adding the prefix "deoxy-" to the above abbreviations. (b) A one-phase system was obtained in one previous case by using free acids in dimethylformamide. The unsatisfactory results then obtained indicated that the presence of a base such as pyridine in the reaction mixture was desirable.

(9) Water-soluble aliphatic carbodiimides of the general type IV were first prepared by E. Schmidt and W. Striewsky, *Ber.*, **74**, 1285 (1941). See also E. Schmidt, W. Striewsky and F. Hitzler, *Ann.*, **560**, 222 (1948). The preparation of some members of this group by completely analogous procedures has recently been recorded by J. C. Sheehan and J. J. Hlavka, *J. Org. Chem.*, **21**, 439 (1956).

tion of AMP to P[1]P[2]-diadenosine 5′-pyrophosphate and in the synthesis of adenosine 5′-polyphosphates from AMP and orthophosphoric acid was investigated. While the reactions in homogeneous aqueous pyridine solutions occurred rapidly, the presence of water necessitated very large (*ca.* 100 or over molar excess) amounts of the reagent. Furthermore, the recoveries of the nucleotide material from the clear solutions containing large quantities of the quaternary ammonium salts were unsatisfactory (50–70%). This approach was not pursued further.

$$\underset{\text{I}}{(C_2H_5)_2N(CH_2)_3NCS} + C_6H_{11}NH_2 \longrightarrow \underset{\text{II}}{(C_2H_5)_2N(CH_2)_3NHCSNHC_6H_{11}}$$

$$\text{II} \xrightarrow[+\ \text{NaOCl}]{\text{NaOH}} \underset{\text{III}}{(C_2H_5)_2N(CH_2)_3N{=}C{=}NC_6H_{11}}$$

$$\text{III} + CH_3I \longrightarrow \underset{\text{IV}}{(C_2H_5)_2\overset{+}{N}(CH_3)(CH_2)_3N{=}C{=}NC_6H_{11}\ \ I^-}$$

Attention was next turned to the use of trialkylammonium salts in order to enhance solubilities of phosphoric acid esters in anhydrous solvents. The only previously reported attempt on the use of such salts in reactions involving carbodiimides and phosphate esters is that with triethylammonium dibenzyl phosphate and DCC in benzene.[10] No reaction occurred and this result has led to the general impression that trialkylammonium salts of phosphate esters cannot be employed in carbodiimide promoted condensations. The present experiments and detailed studies to be reported later have revealed important differences in reactivity toward carbodiimides of trialkylammonium salts of diesters on the one hand and the corresponding salts of unsubstituted phosphoric acid and monoesters on the other.

In the first experiments, uridine 5′-phosphate reacted with DCC in a mixture of pyridine and tri-*n*-butylamine to form quantitatively P[1]P[2]-diuridine 5′-pyrophosphate. Although the condensation occurs more slowly than in pyridine alone, the present procedure for the preparation of diesters of pyrophosphoric acid is preferred to the older one using mixtures of water and pyridine.[2] The reaction goes to completion without the repeated additions of the reagent which were necessary before and the resulting diester of pyrophosphoric acid does not undergo the subsequent reactions which occur when anhydrous pyridine alone is the solvent.[11]

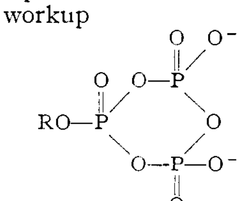
Orthophosphoric acid (85%) was also soluble in anhydrous pyridine in the presence of two equivalents of tri-*n*-butylamine and the solution remained clear on the subsequent addition of DCC. Reaction to form a polymeric anhydride occurred under these conditions, although the exact structure of the product remains to be investigated. Experiments on the synthesis of nucleoside 5′-polyphosphates were carried out using a tenfold excess of orthophosphoric acid over the nucleoside monophosphate. The homogeneous reaction mixtures permitted for the first time a more precise study of the nucleoside 5′-polyphosphate synthesis using varying amounts of the carbodiimide reagent. UMP was used in these experiments and the products of the reaction were analyzed routinely by ion-exchange chromatography using a linear gradient elution apparatus similar to that described by Paar.[12] The results are shown in Table I (see also Fig. 1). It will be seen that the amount of unreacted UMP decreased, as expected, with increase in the amount of DCC, although this starting material never completely disappeared from the final product. The presence of UDP as the major product in the experiments using smaller amounts (5 and 10 moles) of DCC may also be expected. However, the most interesting finding in these experiments was the formation of substantial amounts of, presumably, the tetra- and the pentaphosphate with ten moles of DCC (Fig. 1a) and their decrease with increasing amounts of the reagent (Fig. 1b). UTP emerged ultimately as the major product and this procedure, therefore, enables a highly improved and selective synthesis of the triphosphate.

TABLE I

REACTION OF UMP AND PHOSPHORIC ACID WITH DCC

Expt. no.	DCC (moles)	Products (%) UMP	UDP	UTP	Higher
1	5	67	19	14	0
2	10	33	30	18	19
3	15	10	27	38	25
4	20	6	27	54	13
5	50	5	21.5	64.5	9

It is difficult to offer any simple or unique explanation for the increase of the triphosphate at the expense of all other initially formed products. The kinetic situation must be complex due to the large number of allowable reactions between the various reacting species. However, one attractive hypothesis to explain the accumulation of the triphosphate seems to be that it may exist in the reaction medium as a stable entity, for example, as the cyclic metaphosphate V. On treatment with water during the workup



V, R = uridine

V would be expected to hydrolyze instantaneously to give the linear triphosphate. Another observation based on unpublished experiments seems pertinent. This bears on the role of the "dismutation" reactions of nucleoside 5′-polyphosphates. Thus, it was observed that ADP on prolonged reaction with DCC in aqueous pyridine gave AMP and ATP. These products probably arise from the breakdown of the initial condensation product of ADP, presumably, P[1]P[4] diadenosine 5′-tetraphosphate.

(10) H. G. Khorana and A. R. Todd, *J. Chem. Soc.*, 2257 (1953).

(11) H. G. Khorana, W. E. Razzell, P. T. Gilham, G. M. Tener and E. H. Pol, THIS JOURNAL, **79**, 1002 (1957), and unpublished work from this Laboratory.

(12) C. W. Paar, *Biochem. J.*, **56**, xxvii (1954).

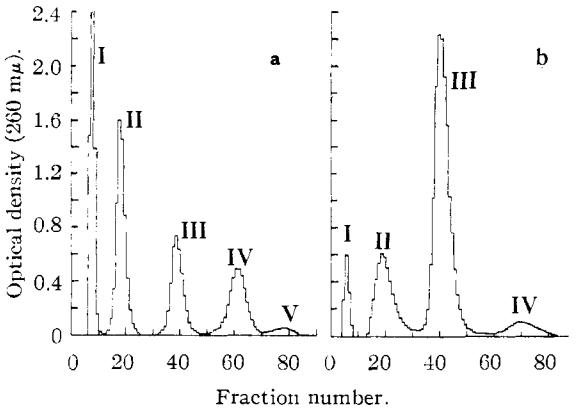


Fig. 1.—Ion-exchange analysis of the products of the reaction of UMP and phosphoric acid with different amounts of DCC: a, 10 moles; b, 50 moles. Peaks from left: I, UMP; II, UDP; III, UTP; IV, uridine tetraphosphate; V, higher phosphates.

Extension of the synthesis using the most favorable reaction conditions for the triphosphate formation (Fig. 1d) to adenosine, guanosine and deoxyguanosine 5'-triphosphates was straightforward. The monophosphates were all soluble in the pyridine–tri-*n*-butylamine solution of orthophosphoric acid and the yields of the triphosphates were consistently high (60–79.5%) (Fig. 2, Table II). No variation from this general pattern is expected in the case of deoxyadenosine and thymidine 5'-monophosphates, since these correspond, in their solubility properties, to AMP and UMP, respectively. In experiments with cytosine nucleotides, the remaining members, it was found that although both CMP and deoxy-CMP dissolved in the phosphoric acid–pyridine–tri-*n*-butylamine mixture, partial precipitation occurred on the addition of DCC. The corresponding triphosphates which were still the major products, were formed in 39 and 43% yields (Fig. 2b), respectively. While these yields are not as satisfactory as those recorded above, they represent substantial improvement over those obtained earlier in these series.[4a,13]

That the major products in the present experiments are indeed the triphosphates follows from the elution patterns obtained above. In each series the triphosphates emerged as would be expected from the known chromatographic properties of these substances. Further evidence was obtained by paper chromatography of the total reaction mixtures in suitable solvent systems. In all but the cytosine series the major products could thus be shown to be the triphosphates by direct comparison with well-characterized samples. Finally, as an illustration, the experiment on the synthesis of ATP was carried out on a preparative (1 mmole) scale. After a two-day reaction period, the nucleotides were freed from inorganic polyphosphates by adsorption on charcoal. ATP was separated by ion-exchange chromatography and finally isolated as the lithium salt (36%). Its identity was confirmed by paper chromatography, total and labile phosphorus analyses and an enzymatic assay.

(13) Unpublished experiments from this Laboratory.

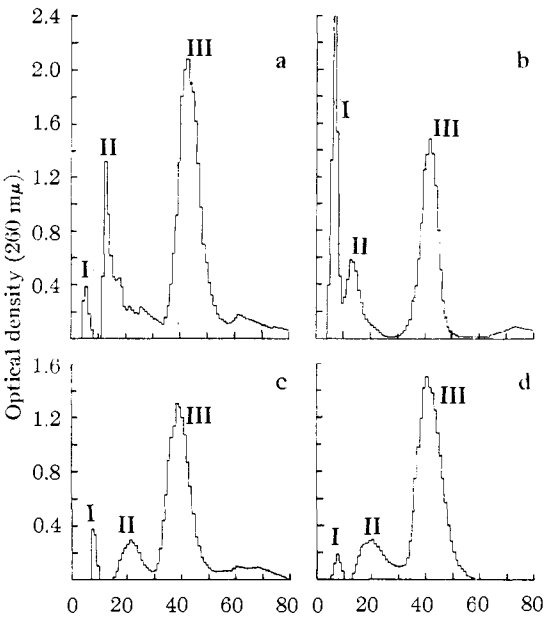


Fig. 2.—Ion-exchange analysis of the nucleoside polyphosphates from the reactions of various nucleoside 5'-monophosphates and orthophosphoric acid with DCC (50 moles): a, adenosine; b, cytidine; c, guanosine; d, deoxyguanosine. Peaks: I, monophosphates; II, diphosphates; III, triphosphates.

Experimental

Paper Chromatography.—Descending technique was employed throughout. The two solvent systems which proved suitable in the present work were isopropyl alcohol–1% aqueous ammonium sulfate (2:1)[14] (solvent A) and isobutyric acid (100 cc.)–1 *N* ammonia (60 cc.)–0.1 *M* ethylenediaminetetraacetic acid (1.6 cc.) disodium salt[15] (solvent B). For chromatography in solvent A, the filter papers were previously soaked in 1% ammonium sulfate solution and dried.

P^{1}P^2-Diuridine 5'-Pyrophosphate.—(a) To a solution of pyridinium UMP[16] (0.3 m*M*) in pyridine (6 ml.) were added tri-*n*-butylamine (1.5 ml.) and DCC (3.09 g., 15 m*M*) and the mixture kept at room temperature for 48 hr. The mixture was then filtered from dicyclohexylurea and the urea washed with water. The combined filtrate and washings were diluted with water, extracted with ether twice and then evaporated under reduced pressure to a small volume (*ca.* 2 ml.). Paper chromatography in solvent systems A and B showed the presence of a single ultraviolet absorbing spot, corresponding to diuridine 5'-pyrophosphate. (R_f's: In solvent A, 0.28 (UMP 0.42); solvent B, 0.11 (UMP, 0.24)). The solution was adjusted to *p*H 4 with acetic acid and barium iodide (0.6 m*M*) added. The barium salt which separated on the addition of ethyl alcohol was collected by centrifugation, was washed successively with ethyl alcohol, acetone and ether and then dried *in vacuo* at room temperature; yield 105 mg. The following tests were performed to confirm the identity of the product. (1) It gave a positive reaction when the chromatograms were sprayed with the periodate–benzidine spray.[17] (2) An electrometric titration of a solution of the free acid, prepared by passing 21 mg. of the barium salt through a Dowex 50(H^+) column, showed no buffering in the *p*H range 4–8, thus indicating the absence of

(14) N. Anand, U. M. Clarke, R. H. Hall and A. R. Todd, *J. Chem. Soc.*, 3665 (1952).

(15) H. A. Krebs and R. Hems, *Biochem. Biophys. Acta*, **12**, 172 (1953). *Cf.* R. Zetterström and M. Ljunggren, *Acta Chem. Scand.*, **5**, 291 (1951).

(16) Prepared by passing the sodium salt (R. H. Hall and H. G. Khorana, THIS JOURNAL, **77**, 1871 (1955)) through a pyridinium Dowex-50 column.

(17) M. Viscontini, D. Hoch and P. Karrer, *Helv. Chim. Acta*, **38**, 642 (1955).

a secondary phosphoryl dissociation. (3) It was rapidly degraded by the snake venom diesterase fraction[18] to give UMP quantitatively.[19]

(b) A mixture of pyridinium UMP (0.025 mmole), tri-*n*-butylamine (0.02 ml., 0.085 mmole) and pyridine (0.5 ml.) was treated with a solution of DCC (0.25 mmole) in pyridine (0.25 ml.) at room temperature. The progress of the reaction was followed by paper chromatography in solvent A. Conversion to diuridine pyrophosphate was almost complete after 5 hr.

The Reaction of UMP and Orthophosphoric Acid with DCC.—A solution of 85% orthophosphoric acid (116 mg., 1 m*M*), tri-*n*-butylamine (0.5 ml., 2.1 m*M*) and UMP (0.1 m*M*) in pyridine (2 ml.) was treated with DCC (for amounts see Table I) at 20° for 48 hr. Dicyclohexylurea was then filtered off and washed thoroughly with water. The combined washings and filtrate were further diluted with water (*ca.* 10 ml.), extracted with ether and evaporated to a sirup under reduced pressure. The residue was made up to 5 ml. with water. One milliliter of this solution was adjusted to *p*H 8.5 with 0.1 *N* sodium hydroxide (2 ml. required). The solution which became turbid due to separation of tri-*n*-butylamine, was applied to the top of a Dowex 2 (chloride form, 200–325 mesh) column (1.5 cm. × 1 cm. diameter). After a water wash (~10 ml.) elution was carried out using the linear gradient elution technique.[12] The mixing flask contained 0.003 *N* hydrochloric acid (500 ml.) and the reservoir an equal volume of 0.5 *M* sodium chloride solution in 0.003 *N* HCl. The average flow rate was 1 ml./min. and 10-ml. fractions were collected. The optical density of each fraction was determined at 260 mμ. Uridine polyphosphates not eluted by this system were subsequently washed off the column with 2 *N* hydrochloric acid. The recovery of the total optical density was >95%. The results obtained using different amounts of DCC are recorded in Table I (see also Fig. 1).

Ribo- and Deoxyribonucleoside 5'-Polyphosphate Syntheses.—The conditions used in experiment No. 5 of Tabie I for the uridine nucleotides were used for the synthesis of polyphosphates from AMP, GMP, deoxy-GMP, deoxy-CMP- and CMP. The ion-exchange analyses of the products were carried out as above except for these several changes in the reservoir solutions: CMP and deoxy-CMP, 0.15 *M* sodium chloride solution in 0.003 *M* hydrochloric acid; AMP, 0.3 *M* sodium chloride in 0.003 *M* hydrochloric acid. The results are recorded in Table II (see also Fig. 2). The recoveries of optical density were greater than 95% in the case of the cytosine and the adenine nucleotides and 70% for guanine nucleotides.

TABLE II

YIELDS OF NUCLEOSIDE 5'-POLYPHOSPHATES FROM MONOPHOSPHATES

Nucleotide	Monophosphate, %	Diphosphate, %	Triphosphate, %	Higher phosphates, %
AMP	2	28[a]	60	10
CMP	26	14	39	21
GMP	5	15	71	9
UMP	5	21.5	64.5	9
deoxy-CMP	34	14	43	9
deoxy-GMP	3.3	17.2	79.5	..

[a] This is the total material eluted after AMP and before the major ATP peak. Figure 2a indicates two minor peaks in this region.

Isolation of Adenosine 5'-Triphosphate.—AMP (766 mg. of the dihydrate; 2 mmoles) and 85% phosphoric acid (2.32 g., 20 mmoles) were dissolved in pyridine (40 ml.) containing tri-*n*-butylamine (10 ml., 21 mmoles) and treated with DCC (20.6 g., 100 mmoles) at 20° for 46 hr. The precipitated dicyclohexylurea was filtered off and washed thoroughly with water. The combined filtrate and washings were further diluted with water (total volume, 100 ml.) and extracted three times with ether (total volume, 200 ml.). The ether solution was washed with a small amount of water and the combined aqueous solutions concentrated in a flash evaporator at a temperature not exceeding 20°. Water was added to the concentrate (~20 ml.) and the solution re-evaporated. The resulting concentrate, which did not smell of pyridine and was neutral, was made up to 50 ml. An analytical ion-exchange column run on the material at this stage indicated that the recovery of the nucleotidic material was quantitative and that 60% of this was present as the 5'-triphosphate.

Twenty-five milliliters of the above solution (≡ 1 mmole AMP) was made up to 175 ml. with water. Acid-washed Norite A[20] (10 g.) was added with stirring to the solution over a 2-minute period. The charcoal was spun down and a further amount of Norite A (1 g.) added to adsorb some ultraviolet absorbing material remaining in the supernatant liquid. After collection on a Celite filter-bed the total charcoal was washed several times with water (total volume of water used, 1 liter). The nucleotides were then eluted with 50% aqueous ethyl alcohol containing 2% of concentrated ammonia solution (S.G. 0.9) (total volume of eluent 800 ml.). Concentration of the eluate in a flash evaporator (bath temperature, 30–35°) gave a neutral solution which was freed from a trace of charcoal by filtration through Celite. The solution was made up to 40 ml. and adjusted to *p*H 8 with 1.0 *N* sodium hydroxide solution. The recovery of nucleotides at this stage, as estimated spectrophotometrically, was 86% of theoretical.

The total solution was adsorbed onto a Dowex-2 (chloride) column (7 × 2.2 cm.). After a water wash, elution was begun with 0.003 *N* hydrochloric acid containing the following amounts of lithium chloride. (An average flow rate of 4 ml./min. was maintained.) (1) 0.003 *N* HCl + 0.02 *M* LiCl (1465 ml.). (2) 0.003 *N* HCl + 0.05 *M* LiCl (790 ml.). (3) 0.003 *N* HCl + 0.075 *M* LiCl (2 liters). These eluents removed a total of 4,463 O.D. units (at 260 mμ), which were discarded. Subsequent elution with 0.003 *N* HCl + 0.15 *M* LiCl gave 6,160 optical density units contained in 1200 ml. of solution. The solution was neutralized with 2 *N* lithium hydroxide and stored at 0°. Four hundred milliliters of this solution was concentrated to a sirup using a flash evaporator and a bath temperature 30–40°. The sirup was diluted with an equal volume of methyl alcohol (*ca.* 10 ml.) and then with acetone (200 ml.) to precipitate the tetralithium salt of ATP. The precipitate was collected by centrifugation, washed with acetone containing some methyl alcohol, then with ether and dried over phosphorus pentoxide at 20° (1 mm.) to give the salt as the octahydrate (82.3 mg., 36.5%). *Anal.* Calcd. for $C_{10}H_{12}N_5O_{13}P_3Li_4 \cdot 8H_2O$: total P, 13.77; labile P, 9.17.[21] Found: total P, 13.52; labile P, 9.01. The ratio of adenine, as estimated spectrophotometrically/total P/labile P was found to be 1/2.97/2.00. An enzymatic assay[22,23] showed this sample to be 97% pure. Paper chromatography in solvent B showed a single spot corresponding to ATP.

1-Cyclohexyl-3-(γ-diethylaminopropyl)-thiourea.—Cyclohexylamine (99 g., 1 mole) was added slowly to a stirred solution of γ-diethylaminopropyl isothiocyanate[24] (172 g., 1 mole) in light petroleum (b.p. 65–110°, 200 ml.), the temperature being kept below 50° with an ice-water bath. After cooling to 0°, the crystalline thiourea separated (266 g., 98%), m.p. 74–75°. The analytical sample crystallized from light petroleum–benzene as prisms, m.p. 76–78°. A sample was dried in a high vacuum at room temperature over phosphorus pentoxide. *Anal.* Calcd. for $C_{14}H_{29}N_3S$: C, 61.95; H, 10.77; N, 15.48. Found: C, 61.77; H, 10.84; N, 15.68.

1-Cyclohexyl-3-(γ-diethylaminopropyl) Carbodiimide.—A solution of 1-cyclohexyl-3-(γ-diethylaminopropyl)-thiourea (180 g.) in methylene dichloride (500 ml.) was placed in a 5-l. three-necked flask equipped with stirrer, reflux condenser and dropping funnel. The flask was cooled in an

(18) Unpublished work of Dr. R. L. Sinsheimer.

(19) We are grateful to Dr. W. E. Razzell of this Laboratory for this experiment.

(20) D. Lipkin, P. T. Talbert and M. Cohn, THIS JOURNAL, **76**, 2871 (1954).

(21) After hydrolysis in 1 *N* hydrochloric acid at 100° for seven minutes.

(22) A. Kornberg, *J. Biol. Chem.*, **182**, 779 (1950).

(23) We are grateful to Dr. W. E. Razzell of this Laboratory for this assay.

(24) E. Schmidt, E. Kammerl, D. Ross and F. Zoller, *Ann.*, **594**, 233 (1955).

ice-water bath and alkaline sodium hypochlorite solution[25] (14.9%, 1550 ml.) added dropwise with stirring so that the mixture refluxed gently. After addition of the sodium hypochlorite solution (45 minutes) the mixture was stirred at 20° for 12 hr. The organic layer was then separated, the aqueous layer extracted with methylene chloride and the combined organic solutions washed with brine and dried over Drierite. Removal of the solvent gave a brown oil (170 g.) which was dissolved in light petroleum (b.p. 20–40°, 500 ml.) and kept at 0° overnight. Unchanged thiourea (23 g.) which separated was removed by filtration and the clear solution was concentrated to an oil which was distilled in a short path molecular still at 45–50° (5×10^{-4} mm.) to give the carbodiimide as a pale yellow oil (104.9 g., 67%) $n^{19}D$ 1.4861. An analytical sample was redistilled at 45° (5×10^{-4} mm.); $n^{22.5}D$ 1.4862. *Anal.* Calcd. for $C_{14}H_{27}N_3$: C, 70.80; H, 11.47; N, 17.71. Found: C, 70.64; H, 11.46; N, 17.63.

The methiodide (IV) was obtained by dissolving the carbodiimide (104 g.) in light petroleum (b.p. 20–40°, 500 ml.) and treating with methyl iodide (100 ml.) at 0° for 7 days. The methiodide separated as an oil which was redissolved in acetone and reprecipitated with light petroleum. This process was repeated until an aqueous solution of the heavy oil was neutral.

Reaction of AMP with 1-Cyclohexyl-3-(γ-diethylaminopropyl) Carbodiimide Methiodide (IV).—AMP (10 mg., 0.0275 m*M*) in a mixture of water (0.1 ml.) and pyridine (0.6 ml.) was treated with the diimide (379 mg., 1.0 m*M*) at 20°. The reaction in the homogeneous mixture was followed chromatographically in solvent A, nucleotide containing spots being eluted with dilute hydrochloric acid (0.1 *M*) and estimated spectrophotometrically. Reaction was complete in 1–2 hr., P¹P²-diadenosine 5'-pyrophosphate (62%) being the only new product (R_f, 0.2, AMP, 0.3). Under otherwise identical conditions the reaction in water (0.35 ml.)–pyridine (0.35 ml.) and water (0.6 ml.)–pyridine (0.1 ml.) gave 25 and 18%, respectively, of the pyrophosphate.

The Reaction of AMP and Orthophosphoric Acid with IV.—AMP (36.5 mg. of the monohydrate, 0.1 m*M*) and 85% orthophosphoric acid (116 mg., 1.0 m*M*) in water (0.42 ml.)–pyridine (2.52 ml.) was treated with the diimide (3.79 g., 10.0 m*M*) at 20° (cooling required, initially) for 1 hr. Solvent was removed *in vacuo* at 20° and the residual gum taken up in acetone (100 ml.) containing barium iodide (2.136 g. of the dihydrate, 5.0 m*M*). After keeping the mixture at 0° overnight, the precipitate which separated was spun down, washed with acetone and dried. The dry white powder was suspended in water and stirred with I.R.-120 ion-exchange resin (sodium form). The resulting solution was made up to 10 ml. with water and an aliquot (2 ml.) chromatographed on an analytical ion-exchange column as described before. The recovery of optical density was 50%; AMP, 28; ADP, 15; and ATP, 56%.

Acknowledgment.—This work was carried out under Defence Research Board of Canada grant number 2020-23 Project D52-20-20-23.

VANCOUVER 8, B. C., CANADA

(25) E. Schmidt, F. Zoller, F. Moosmüller and E. Kammerl, *Ann.*, **585**, 230 (1954).

[CONTRIBUTION FROM THE NUTRITION AND PHYSIOLOGY DEPARTMENT, RESEARCH DIVISION, AMERICAN CYANAMID CO.]

Riboside Derivatives of 6-Methyl-*asym*-triazine-3,5(2,4)-dione[1]

BY ROSS H. HALL

RECEIVED AUGUST 23, 1957

The chemical synthesis of 2-D-ribofuranosyl-6-methyl-*asym*-triazine-3,5(2,4)-dione is described. The synthesis of the corresponding 4-ribosyl and 2,4-diribosyl derivatives and their identification is also reported. A facile ring opening of the 4-substituted derivatives is described.

Various investigators have synthesized analogs of the naturally occurring pyrimidines for use as experimental anti-tumor compounds. The suggestion has been made recently that the nucleosides of such compounds may be more effective than the free bases.[2] A publication[3] supporting this concept demonstrated the value of the deoxyriboside of 6 - methyl - *asym* - triazine - 3,5(2,4) - dione (azathymidine) as an inhibitor of deoxyribonucleic acid (DNA) synthesis in contrast to the inertness of the free base. The deoxyriboside is obtainable at the present time only by enzymic means,[3] but in view of the evidence of Roll, *et al.*,[4] and Rose and Schweigert[5] that facile conversion of ribosides to deoxyribosides occurs within living cells it was decided to synthesize 6-methyl-*asym*-triazine-3,5-(2,4)-dione riboside *via* chemical means in order to evaluate its use as an anti-cancer agent. This paper describes the synthesis of a mixture of ribosides, their separation and identification.

A mercury salt of 6-methyl-*asym*-triazine-3,5-(2,4)-dione (VIII) was prepared and this was condensed with 1-chloro-2,3,5-tri-*O*-benzoyl-D-ribose,[6] according to the procedure of Davoll and Lowy.[7] The resulting mixture of products (65–70% yield) was separated on alumina and silicic acid columns to yield three products, IV, V and VI.[8] Each of these compounds was catalytically debenzoylated in anhydrous media by sodium methoxide to yield the three riboside derivatives I, II and III. These three derivatives also were obtained by first debenzoylating the condensation mixture, then separating the free nucleosides by partition chromatography on Celite. The analytical data indicated that two of the compounds were monoribosyl derivatives of 6-methyl-*asym*-triazine-3,5(2,4)-dione, while the third was a diribosyl derivative;

(1) This base is often referred to as 6-azathymine.

(2) Examples of the effectiveness of nucleosides over the corresponding free bases as antimetabolites are: 5-bromodeoxyuridine *versus* 5-bromouracil, T. J. Bardos, G. M. Levine, R. R. Herr and H. L. Gordon, THIS JOURNAL, **77**, 4279 (1955); 6-azauridine *versus* 6-azauracil, R. E. Handschumacher, *Federation Proc.*, **16**, 191 (1957); A. D. Welch and R. Schindler, *Science*, **125**, 548 (1957); azathymidine *versus* azathymine, W. H. Prusoff and A. D. Welch, *J. Biol. Chem.*, **218**, 929 (1956), and W. H. Prusoff, L. G. Lajtha and A. D. Welch, *Biochim. et Biophys. Acta*, **20**, 209 (1956); W. H. Prusoff, *J. Biol. Chem.*, **226**, 901 (1957).

(3) Ross H. Hall and R. Haselkorn, THIS JOURNAL, **80**, 1138 (1958).

(4) P. Roll, H. Weinfeld, E. Carroll and G. B. Brown, *J. Biol. Chem.*, **220**, 439 (1956).

(5) I. A. Rose and B. S. Schweigert, *ibid.*, **202**, 635 (1953).

(6) R. K. Ness, H. W. Diehl and H. G. Fletcher, THIS JOURNAL, **76**, 763 (1954).

(7) J. Davoll and B. A. Lowy, *ibid.*, **74**, 1563 (1952).

(8) The glycosidic linkages of all formulas are written as if they were in the β-configuration, although there is no evidence to support this.