Dismutation Reactions of Nucleoside Polyphosphates. VI. Chemical Synthesis of [γ-^{32}P]Nucleoside 5′-Triphosphates*

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ABSTRACT: A completely general chemical synthesis of γ-^{32}P-labeled nucleoside 5′-triphosphates has been developed.

The method consists of the reaction of a nucleoside or deoxynucleoside 5′-diphosphate with morpholine and dicyclohexylcarbodiimide to form the corresponding P^1-(nucleoside-5′),P^2-(4-morpholine) pyrophosphate (VI) which can be isolated in pure form and high yield. Reaction of these terminally activated nucleoside 5′-diphosphates with tributylammonium [^{32}P]orthophosphate in anhydrous dimethyl sulfoxide leads to formation of the corresponding [γ-^{32}P]nucleoside 5′-triphosphate in 66–74% yields. By partial hydrolysis with *E. coli* alkaline phosphatase followed by chromatographic separation of the resulting products it was possible to show that at least 99.8% of the isotope was present in the desired γ position. Preparations of the γ-^{32}P-labeled triphosphates of uridine, cytidine, guanosine, and thymidine are described in detail and the method should be directly applicable to unnatural nucleoside triphosphates that would not be amenable to enzymatic synthesis.

In recent years much detailed knowledge has accrued concerning the mechanism of biological reactions. Many of these reactions involve the participation of nucleoside 5′-triphosphates either as direct substrates or as precursors of phosphorylated intermediates or cofactors. Frequently the role of the nucleoside triphosphate has been elucidated through the use of these compounds labeled specifically with ^{32}P in one or more of their phosphorus atoms, and the requirement for such specifically labeled compounds has led to the development of a variety of enzymatic methods for their synthesis. In particular, the preparation of [γ-^{32}P]-nucleoside 5′-triphosphates has been accomplished using a variety of enzymatic techniques. These methods frequently involve the phosphorylation of a nucleoside 5′-diphosphate by an appropriate ^{32}P-labeled phosphate donor such as acetyl phosphate (Kornberg *et al.*, 1956), carbamyl phosphate (Tanaka, 1960; Mokrasch *et al.*, 1960), phosphoenolpyruvate (Tanaka, 1960), diphosphoglyceric acid (Glynn and Chappell, 1964), or polyphosphoric acid (Kornberg, 1957) in the presence of an appropriate kinase. If products containing isotope located exclusively in one phosphorus atom are desired, it is imperative to use carefully purified enzymes. Due to the specificities of the enzymes involved, these methods have been primarily restricted to the synthesis of γ-^{32}P-labeled adenosine 5′-triphosphate ([γ-^{32}P]ATP).[1]

Preparations of a variety of [γ-^{32}P]nucleoside 5′-triphosphates have also been accomplished with reasonable selectivity using photophosphorylation of the corresponding 5′-diphosphates in the presence of chloroplasts (Avron, 1961; Littauer *et al.*, 1964).

In the course of our studies on the mechanism of the dismutation reactions undergone by nucleoside 5′-triphosphates in anhydrous pyridine (Wehrli and Moffatt, 1965; Verheyden *et al.*, 1965), we recently described the development of purely chemical methods for the synthesis of ATP labeled with ^{32}P in exclusively the α, β, or γ positions (Wehrli *et al.*, 1965). These methods permitted the synthesis of substantial amounts of these compounds in which greater than 99% of the isotope was located in the desired position. The synthesis of [γ-^{32}P]ATP involved the reaction of ADP (I) with morpholine and dicyclohexylcarbodiimide (DCC) to form P^1-(adenosine-5′),P^2-(4-morpholine) pyrophosphate (II, ADP-morpholidate) which could be isolated in pure form. The latter compound is a derivative of ADP in which only the terminal phosphate is activated. Reaction of II with a threefold excess of the soluble tributylammonium salt of [^{32}P]orthophosphoric acid in rigorously anhydrous dimethyl sulfoxide led to the formation of [γ-^{32}P]ATP which was isolated in pure form in 65% yield.

Both the preparation of nucleoside 5′-phosphoromorpholidates (Moffatt and Khorana, 1961) and the use of these compounds in the synthesis of various pyrophosphate derivatives (Roseman *et al.*, 1961; Moffatt,

* Contribution No. 49 from the Institute of Molecular Biology, Syntex Research, Palo Alto, California. *Received November 9, 1967.*

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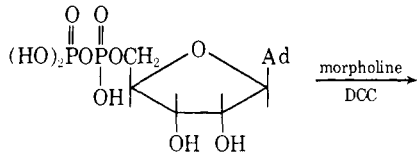
[1] The abbreviations AMP, ADP, ATP, UMP, UDP, UTP, GMP, GDP, GTP, TMP, TDP, and TTP refer to the 5′-mono-, -di-, and -triphosphates of adenosine, uridine, guanosine, and thymidine, respectively. DMSO and DCC refer to dimethyl sulfoxide and dicyclohexylcarbodiimide. Optical density units (OD units) refer to the product of volume and optical density of a solution and is always measured at the λ_{max} of the specified compound.



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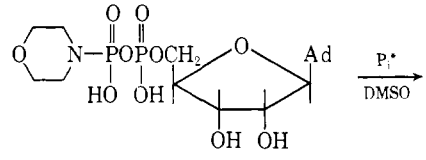

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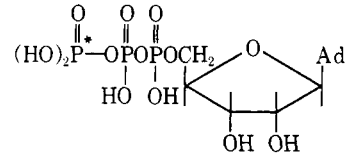
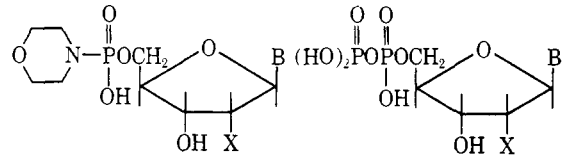


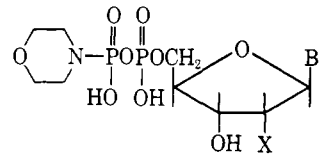
III

1964) are known to be equally applicable to any of the common ribo- or deoxyribonucleotides or to a wide range of nucleotide analogs (Moffatt, 1966). Accordingly, it was to be expected that the method we developed for the synthesis of [γ-^{32}P]ATP could also be used for other terminally labeled nucleoside 5′-triphosphates. In this paper the generality of this synthetic method is verified through the synthesis of the γ-^{32}P-labeled triphosphates of cytidine, guanosine, uridine, and thymidine.

In each case the appropriate nucleoside 5′-diphosphate was prepared through the reaction of the nucleoside 5′-phosphoromorpholidate (IV) with an excess of tributylammonium orthophosphate in anhydrous dimethyl sulfoxide. The use of dimethyl sulfoxide as the solvent offers considerable advantage when dealing with the morpholidates of GMP and CMP which have only limited solubilities in anhydrous pyridine, the solvent originally used in this type of reaction (Moffatt and Khorana, 1961). In each case the nucleoside 5′-diphosphate (V) was isolated by ion-exchange chromatography, the yields varying between 62 and 77%. The products were identified by total and labile phosphorus analysis and by paper chromatographic and electrophoretic comparison with authentic nucleoside 5′-diphosphates. Each diphosphate was then allowed to react with morpholine and dicyclohexylcarbodiimide in refluxing aqueous *t*-butyl alcohol, the reactions being followed by paper chromatography and electrophoresis. In each case only traces of starting material remained after a 4–5-hr reaction and the principal product was the desired P^1-(nucleoside-5′),P^2-(4-morpholine) pyrophosphate (VI) together with minor amounts of the corresponding nucleoside 5′-phosphoromorpholidate arising from some hydrolysis of the diphosphate to the monophosphate under the reaction conditions. The products could be readily purified by ion-exchange chromatography on DEAE-cellulose (HCO_3^-) using a linear gradient of triethylammonium bicarbonate. In the case of the uridine and guanosine derivatives the product peak was contaminated with a minor amount of orthophosphate resulting from the above-mentioned hydrolysis of perhaps 10% of the starting diphosphate. For analytical purposes this contaminant could be removed by rechromatography (GDP-morpholidate) or by charcoal adsorption (UDP-morpholidate), but for practical purposes this step is unnecessary since excess orthophosphate is used in the subsequent reaction. The nucleoside diphosphate morpholidates were obtained, following chromatography, in yields of 53–76% and were isolated as their analytically pure 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine salts, which show excellent solubility properties and can be used directly in the next step. As would be expected, these compounds were completely resistant to attack by *E. coli* alkaline phosphatase but were rapidly cleaved by the purified phosphodiesterase from *Crotalus adamanteus* venom to the corresponding nucleoside 5′-phosphate and phosphoromorpholidic acid which decomposes under the incubation conditions to orthophosphate (Wehrli *et al.*, 1965).



IV V



VI

B = cytosine, guanine, uracil, thymine
X = OH or H

The reaction of the various nucleoside diphosphate morpholidates (VI) with 4 equiv of [^{32}P]orthophosphate was carried out in rigorously anhydrous dimethyl sulfoxide at 36° for 45 hr as previously described (Wehrli *et al.*, 1965). Providing that adequate care has been taken to remove all residual pyridine from the reaction mixture, the resulting γ-^{32}P-labeled nucleoside 5′-triphosphate is stable in this solvent and no dismutation, accompanied by labeling of the β phosphorus, is observed. The products of the reactions were readily purified by ion-exchange chromatography on DEAE-cellulose (HCO_3^-) giving chromatographically homogeneous [γ-^{32}P]nucleoside 5′-triphosphates in yields of 66–74%. In each case radioactivity was located almost exclusively in the triphosphate peak and in excess orthophosphate which immediately preceded, or partially overlapped, the peak of unreacted morpholidate. The elution profiles closely resembled that previously published for the synthesis of [γ-^{32}P]ATP (Wehrli *et al.*, 1965) and are not further reproduced here. The labeled triphosphates were isolated as their sodium salts by treatment of the triethylammonium salts directly obtained from

ion-exchange purification with sodium iodide in acetone (Moffatt, 1964). Losses of radioactivity in this procedure are less than 1% and the resulting sodium salts are nonhygroscopic and suitable for direct use in biological experiments.

It was of particular importance to demonstrate that the isotope was, as desired, located in exclusively the γ position. This was accomplished using the technique of partial enzymatic hydrolysis that was previously developed for the determination of ^{32}P distribution in labeled adenosine polyphosphates (Wehrli and Moffatt, 1965). Thus 1–2 μmoles of each labeled triphosphate was subjected to controlled partial hydrolysis by *E. coli* alkaline phosphatase under conditions leading to a mixture of the corresponding nucleoside 5′-di- and -monophosphates together with unreacted triphosphate, some nucleoside, and released orthophosphate. These products were then separated by ion-exchange chromatography on a small column of DEAE-cellulose (HCO_3^-) and the specific activities of the di- and triphosphate peaks were determined. If, as desired, only the γ-phosphate group was labeled, only the released orthophosphate and undegraded triphosphate should contain isotope. In each case the triphosphate remaining had, within experimental error, the same specific activity as the untreated material. The pooled and concentrated diphosphate peaks, however, each contained some radioactivity and the specific activities (counts per minute per optical density unit) were 3.5–5.9% that of the triphosphate. Each diphosphate peak was then concentrated and examined by paper chromatography. In each case the resulting purified nucleoside diphosphate was eluted and shown to contain negligible radioactivity in excess of background. The observed specific activities were then roughly 0.1% those of the triphosphates, indicating that 99.9% of the isotope was located in the desired γ position. Autoradiography of the paper chromatograms from purification of the enzymatically released nucleoside diphosphates showed that the unexplained radioactivity in each sample appeared as a single, isotope-containing spot which was identical with orthophosphate. Since the bulk of the radioactive orthophosphate appeared as a sharp peak just before, or superimposed upon, the nucleoside monophosphate and was thus well separated from the diphosphate peak, we can offer no suitable explanation for the presence of this contaminant. One possible explanation is that the alkaline phosphatase used in the partial hydrolysis step had not been completely denatured by vigorous agitation with chloroform prior to ion-exchange separation of the products. If this were the case then some further release of labeled phosphate could occur during the chromatography leading to the appearance of labeled orthophosphate at an unexpectedly late position. If this were the case, however, one would expect the leading edge of the triphosphate peak to be somewhat skewed, and this did not appear to be the case even in the most extreme case (see Figure 1). Since only 5% of the labeled orthophosphate appeared with the diphosphate peak, however, this skewing could perhaps be masked. Whatever the explanation of this effect may

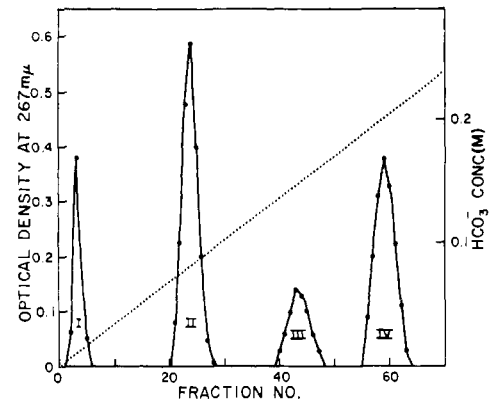


FIGURE 1: Ion-exchange separation of the products from partial hydrolysis of [γ-^{32}P]TTP by *E. coli* alkaline phosphatase. For the conditions of the enzymatic reaction and chromatography see Experimental Section. Peak I (1.8 OD units) is thymidine, peak II (7.1 OD units) is TMP, peak III (2.3 OD units) is TDP, and peak IV (6.0 OD units) is TTP. [^{32}P]Orthophosphate was almost exclusively in tubes 20–25.

be, we are convinced that the diphosphate (and hence also the monophosphate) obtained by partial hydrolysis is, within experimental error, free of isotope (less than 0.2%), indicating exclusive labeling in the desired γ position.

While we have not had occasion to prepare isotopically labeled triphosphates derived from unnatural nucleosides, we are confident that the general method described in this paper can be successfully applied to a wide range of such compounds, many of which would probably be resistant to enzymatic methods of preparation.

Experimental Section

General Methods and Materials. Nucleoside 5′-phosphates were obtained from Schwarz Bioresearch or from P. L. Biochemicals and nucleoside 5′-phosphoromorpholidates were prepared as previously described (Moffatt and Khorana, 1961). Free acid ([^{32}P]orthophosphoric acid with a specific activity of 57 mCi/mg) was obtained from Abbott Laboratories. Pyridine was dried by distillation from, and storage over, calcium hydride, and dimethyl sulfoxide was dried by distillation and storage over Linde Molecular Sieve Type 4A. Ultraviolet measurements were obtained using Cary Model 15 and Zeiss PMQ-II spectrophotometers and ^{32}P was measured using a Nuclear-Chicago gas-flow counter. Total phosphorus was determined by the method of King (King, 1932), and acid-labile phosphate by the same method except that digestion with perchloric acid was replaced by treatment with 1 N hydrochloric acid at 100° for 8 min. Other elemental analyses were obtained from Dr. A. Bernhardt, Mülheim, Germany. *E. coli* alkaline phosphatase was a chromatographically purified sample from Worthington

Biochemical Corp. It was initially dialyzed first against 0.05 M ammonium bicarbonate and then against water at 0° for 15 hr and made up to a concentration of 100 μg/ml in 0.05 M ammonium bicarbonate buffer at pH 8. Phosphodiesterase-I from *Crotalus adamanteus* was obtained from Worthington Biochemical Corp. and was made up to a concentration of 500 μg/ml in 0.05 M Tris buffer, pH 9. This preparation showed negligible 5'-nucleotidase activity. Paper chromatography was done on Schleicher & Schuell No. 589 orange ribbon paper using the following systems: solvent I, isobutyric acid–1 M ammonium hydroxide–0.1 M tetrasodium ethylenediaminetetraacetic acid (100:60:1.6); solvent II, 1-propanol–28% ammonium hydroxide–water (6:3:1). Paper electrophoresis was carried out on the same paper impregnated with either 0.1 M ammonium acetate buffer, pH 3.8, or 0.05 M ammonium bicarbonate, pH 7.6, and using a potential difference of 1500–2000 V for roughly 30 min. Phosphorus-containing products were located using a molybdate spray (Hanes and Isherwood, 1949) followed by ultraviolet irradiation (Bandurski and Axelrod, 1951). Autoradiography was conducted using Kodak Blue Brand medical X-ray film. All evaporations of solvent were done at a pressure of roughly 1 mm and a bath temperature of 30° using a rotary evaporator with the condensing bulb cooled with circulating glycol at −15°.

Uridine 5'-Diphosphate (V, B = Uracil, X = OH). The 4-morpholine-*N,N'*-dicyclohexylcarboxamidine salt of uridine 5'-phosphoromorpholidate (Moffatt and Khorana, 1961) (1.99 g, 2 mmoles) was dissolved in anhydrous pyridine (15 ml) and evaporated to dryness *in vacuo*. This procedure was repeated three times and followed by two evaporations with 10-ml portions of benzene. Separately a solution of the pyridine salt of orthophosphoric acid (10 mmoles) in 20 ml of 80% pyridine was mixed with 4.8 ml (20 mmoles) of tributylamine and evaporated to dryness. The residue was rendered anhydrous by four evaporations of its solutions in 10-ml portions of pyridine followed by two evaporations with benzene as above. The final glassy residue was dissolved in anhydrous dimethyl sulfoxide (20 ml) and quickly added to the dried morpholidate. The resulting clear solution was kept at room temperature for 4 days. It was then diluted with water (50 ml) and directly applied to a 3.2 × 33 cm column of DEAE-cellulose (HCO_3^-), and the column was thoroughly washed with water until no ultraviolet-absorbing material (240 mμ) appeared. The column was then eluted with a linear gradient (0.005–0.4 M) of triethylammonium bicarbonate (6 l.). Unreacted morpholidate (1800 OD units at 262 mμ) and UMP (1250 OD units) were eluted together with excess orthophosphate at 0.02–0.06 M salt, and a large peak containing 15,450 OD units (77%) of UDP appeared at roughly 0.15 M salt. This peak was pooled and evaporated to dryness *in vacuo*. Residual bicarbonate was removed by repeated evaporations with 20-ml portions of methanol. The glassy triethylammonium salt so obtained was chromatographically homogeneous (solvent I and electrophoresis) and showed a ratio of uridine:total P:labile P of 1.00:2.06:1.05. The product was used directly in the next step or could be isolated as the dry sodium salt by the sodium iodide method (Moffatt, 1964).

Thymidine 5'-Diphosphate (V, B = Thymine, X = H). The reaction between hydrated 4-morpholine-*N,N'*-dicyclohexylcarboxamidinium thymidine 5'-phosphoromorpholidate (1.64 g, 2 mmoles) and tributylammonium orthophosphate (10 mmoles) was carried out in dimethyl sulfoxide as above. The products were directly chromatographed on a 3.2 × 33 cm column of DEAE-cellulose (HCO_3^-) using a 6-l. gradient (0.005–0.4 M) of triethylammonium bicarbonate. Overlapping peaks of TMP-morpholidate (4650 OD units at 267 mμ) and TMP (or orthophosphate) were first obtained followed by a large peak containing 12,450 OD units (65%) of TDP. Evaporation of the solvent followed by repeated evaporation with methanol left the chromatographically homogeneous triethylamine salt of TDP with a ratio of thymidine:total P:labile P of 1.00:1.97:0.99.

Cytidine 5'-Diphosphate (V, B = Cytosine, X = OH). The reaction between CMP-morpholidate (1.50 g, 2 mmoles) and tributylammonium orthophosphate (10 mmoles) was carried out as above except that the morpholidate was dried by three evaporations of its solutions in a 1:1 mixture of dimethylformamide and pyridine due to its low solubility in the latter solvent alone. Chromatography on a 3.2 × 32 cm column of DEAE-cellulose (HCO_3^-) using a 6-l. gradient (0.005–0.35 M) of triethylammonium bicarbonate gave unreacted CMP-morpholidate (3000 OD units at 272 mμ, 16.5%) and CMP (1100 OD units, 6%) followed by a large peak containing 11,300 OD units (62%) of CDP. After evaporation of the solvent and removal of excess bicarbonate by evaporation with methanol, the chromatographically homogeneous CDP had a ratio of cytidine:total P:labile P of 1.00:1.96:0.96 and was used directly in the next step.

Guanosine 5'-Diphosphate (V, B = Guanine, X = OH). The reaction between GMP-morpholidate (2 mmoles) and tributylammonium orthophosphate was carried out as above using a mixture of pyridine and dimethylformamide to dry the morpholidate. Chromatography on a 3.2 × 30 cm column of DEAE-cellulose (HCO_3^-) using a 6-l. gradient (0.005–0.3 M) of triethylammonium bicarbonate gave a mixture of unreacted GMP-morpholidate, GMP, and orthophosphate (3000 OD units at 252 mμ, 11%), followed by a peak containing 21,000 OD units (77%) of GDP. Evaporation of the solvent and removal of bicarbonate left the homogeneous triethylammonium salt of GDP with ratios of guanosine:total P:labile P of 1.00:1.92:0.94.

P^1-(Cytidine-5'),P^2-(4-morpholine) Pyrophosphate (VI, B = Cytosine, X = OH; CDP-morpholidate). A solution of triethylammonium CDP as directly obtained above (11,250 OD units at 272 mμ, 1.24 mmoles) was passed through a column containing 20 ml of Dowex 50 resin in the morpholine form. The eluate and washings were then concentrated to a volume of 15 ml and added to a solution of morpholine (0.33 ml, 3.75 mmoles) in *t*-butyl alcohol (15 ml). This mixture was

then heated under reflux while a solution of dicyclohexylcarbodiimide (1.55 g, 7.5 mmoles) in *t*-butyl alcohol (15 ml) was added dropwise over 2 hr. After a further 2-hr heating paper electrophoresis at pH 7.5 showed essentially complete disappearance of CDP and formation of one major, slower moving product. The mixture was then cooled, filtered, and evaporated to roughly one-third its volume. The concentrate was extracted twice with ether and the aqueous solution was chromatographed on a 2.2 × 40 cm column of DEAE-cellulose (HCO_3^-) using a 3.6-l. gradient (0.005–0.25 M) of triethylammonium bicarbonate. The first product eluted was CMP-morpholidate (1000 OD units at 272 mμ, 9%) and was followed by a large peak containing 8650 OD units (77%) of the desired CDP-morpholidate at roughly 0.08 M salt. A small amount (2%) of CDP appeared later. The pooled peak was evaporated *in vacuo* to dryness and freed from residual bicarbonate by repeated evaporation with 20-ml portions of methanol. The final residue was dissolved in methanol (10 ml) together with free base 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine (830 mg, 2.85 mmoles) (Moffatt and Khorana, 1961), and the mixture was evaporated to dryness. The carefully dried residue was then dissolved in methanol (5 ml), and ether (25 ml) was added. The resulting white precipitate was washed three times with ether and dried *in vacuo*, giving 984 mg (70%) of the di-(4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine) salt of P¹-(cytidine-5′),P²-(4-morpholine) pyrophosphate as the tetrahydrate.

Anal. Calcd for $C_{47}H_{84}N_{10}O_{13}P_2 \cdot 4H_2O$: C, 49.89; H, 8.19; N, 12.38; cytidine:P, 1:00:2:00. Found: C, 49.96; H, 8.18; N, 12.54; cytidine:P, 1.00:1.99.

The ammonium salt of this product (1 μmole) was completely degraded to CMP by 10 μl of venom phosphodiesterase-I within 1 hr at pH 9 but remained completely unchanged after incubation with 20 μl of *E. coli* alkaline phosphatase at pH 9 for 4 hr.

P¹-(Thymidine-5′),P²-(4-morpholine) Pyrophosphate (VI, B = Thymine, X = H; TDP-morpholidate). A solution of dicyclohexylcarbodiimide (1.24 g, 6 mmoles) in *t*-butyl alcohol (15 ml) was added dropwise over 2 hr to a refluxing solution of thymidine 5′-diphosphate (1 mmole) and morpholine (6 mmoles) in 20 ml of 50% aqueous *t*-butyl alcohol. After a further 2 hr under reflux the reaction was worked up as above and chromatographed on a 2.2 × 40 cm column of DEAE-cellulose (HCO_3^-) using a linear gradient of 4 l. of triethylammonium bicarbonate (0.005–0.3 M). The first product eluted (roughly 0.04 M salt) consisted of 700 OD units (7%) of TMP-morpholidate while the major peak at roughly 0.09 M salt contained 7200 OD units (75%) of the desired TDP-morpholidate. A small amount (5%) of unreacted TDP appeared at roughly 0.15 M salt. The major peak was evaporated and isolated as its di(4-morpholine-*N*,*N*-dicyclohexylcarboxamidine) salt as described above.

Anal. Calcd for $C_{49}H_{85}N_9O_{15}P_2 \cdot 5H_2O$: C, 50.20; H, 8.34; N, 10.98; thymidine:P, 1.00:2.00. Found: C, 50.46; H, 8.43; N, 11.21; thymidine:P, 1.00:2.05.

Under the conditions described above this product was completely resistant to the action of *E. coli* alkaline phosphatase but was degraded to TMP by venom phosphodiesterase.

P¹-(Guanosine-5′),P²-(4-morpholine) Pyrophosphate (VI, B = Guanine, X = OH; GDP-morpholidate). A solution of dicyclohexylcarbodiimide (1.55 g, 7.5 mmoles) in *t*-butyl alcohol (30 ml) was added dropwise over 4 hr to a refluxing solution of GDP (1.25 mmoles) and morpholine (7.5 mmoles) in 40 ml of 50% aqueous *t*-butyl alcohol. After 1 hr further under reflux the reaction was worked up as above and chromatographed on a 2.2 × 42 cm column of DEAE-cellulose (HCO_3^-) using a linear gradient of 4 l. of triethylammonium bicarbonate (0.005–0.2 M). The first peak, at roughly 0.04 M salt, contained 3600 OD units at 252 mμ (21%) of GMP-morpholidate, while the major peak at 0.09 M salt contained 11,500 OD units (67%) of GDP-morpholidate. Two other very small peaks were obtained at higher salt concentrations. The early tubes in the main peak contained a little orthophosphate and were rechromatographed as above. The resulting chromatographically homogeneous product was isolated as its di(4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine) salt as described for CDP-morpholidate, giving 850 mg (58%) of the tetrahydrate.

Anal. Calcd for $C_{48}H_{84}N_{12}O_{13}P_2 \cdot 4H_2O$: C, 49.21; H, 7.91; N, 14.35; guanosine:P, 1.00:2.00. Found: C, 49.07; H, 7.79; N, 14.25; guanosine:P, 1.00:1.95.

The behavior of the product toward *E. coli* alkaline phosphatase and venom phosphodiesterase was the same as described above for CMP-morpholidate.

P¹-(Uridine-5′),P²-(4-morpholine) Pyrophosphate (VI, B = Uracil, X = OH; UDP-morpholidate). The reaction between uridine 5′-diphosphate (1.4 mmoles), morpholine (8.5 mmoles), and dicyclohexylcarbodiimide (1.75 g, 8.5 mmoles) was carried out as described above for CDP-morpholidate. After the usual work-up the mixture was chromatographed on a 2.2 × 40 cm column of DEAE-cellulose (HCO_3^-) using a linear gradient of 3.6 l. of triethylammonium bicarbonate (0.005–0.25 M). The first peak contained 1800 OD units at 262 mμ (13%) of UMP-morpholidate while the main peak (at 0.08 M salt) contained 10,100 OD units (72%) of the desired UDP-morpholidate. The product was, however, contaminated by a small amount of orthophosphate, and, in order to obtain an analytical sample, a portion of the evaporated peak (2000 OD units) was adjusted to pH 4 and adsorbed on 5 g of acid-washed Norit A. After a thorough water wash the nucleotide was eluted with 50% ethanol containing 5% ammonium hydroxide. The eluate was essentially free of orthophosphate but was slightly yellow. It was accordingly chromatographed on a small column of DEAE-cellulose (HCO_3^-), as above, and the 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine salt was isolated in the usual way.

Anal. Calcd for $C_{47}H_{83}N_9O_{14}P_2 \cdot 2H_2O$: C, 51.49; H, 8.00; N, 11.50; uridine:P, 1.00:2.00. Found: C, 51.74; H, 8.23; N, 11.44; uridine:P, 1.00:1.97.

For practical use the carboxamidine salt of the morpholidate isolated from the initial chromatography was

entirely suitable. As in the other cases the product was completely resistant to *E. coli* alkaline phosphatase but was rapidly degraded to UMP by venom phosphodiesterase.

[γ-^{32}P]*Cytidine 5'-Triphosphate.* The tetrahydrate of the 4-morpholine-*N,N'*-dicyclohexylcarboxamidine salt of CDP-morpholidate (113 mg, 0.1 mmole) was dissolved in a 2:1 mixture of pyridine and dimethylformamide (3 ml) and evaporated to dryness. This procedure was then repeated twice and final traces of pyridine were carefully removed by three evaporations with 10-ml portions of benzene. Separately orthophosphoric acid (0.4 mmole) containing 1 mCi of ^{32}P was dissolved in 3 ml of 75% aqueous pyridine containing 0.10 ml (0.4 mmole) of tributylamine. The clear solution was evaporated to dryness and then rendered anhydrous by three evaporations of solutions of the residue in 3-ml portions of pyridine. After three further evaporations with benzene (3-ml each) the residue was dissolved in anhydrous dimethyl sulfoxide (2 ml) and added to the dried morpholidate. The clear solution was kept at 37° for 45 hr, then diluted with water (10 ml) and applied directly to a 1.7 × 25 cm column of DEAE-cellulose (HCO_3^-). After a thorough water wash the column was eluted with a linear gradient of 2 l. of triethylammonium bicarbonate (0.005–0.25 M), the elution being followed by optical density at 271 mμ and by the radioactivity of 10-μl aliquots. Three ultraviolet-absorbing peaks resulted. The first consisted of 40 OD units (4%) of unreacted CDP-morpholidate and was superimposed upon the peak of excess orthophosphate. The second peak contained 150 OD units (17%) of CDP and virtually no radioactivity. The third peak (650 OD units, 72%) was chromatographically homogeneous [γ-^{32}P]-CTP. The pooled peak was evaporated to dryness *in vacuo*, and residual bicarbonate was removed by repeated evaporations with 10-ml portions of methanol. The final residue was dissolved in methanol (2 ml) and mixed with a 1 M solution of sodium iodide in acetone (0.5 ml). Precipitation was completed by the addition of a further 10 ml of acetone, and the resulting white precipitate was washed four times with 10-ml portions of acetone. After drying *in vacuo* 44 mg (70%) of the chromatographically homogeneous sodium salt of [γ-^{32}P]-CTP was obtained as the trihydrate with a specific activity of 2.74 × 10^6 cpm/μmole;[2] λ_{max}^{pH2} 280 mμ (ϵ 12,950); cytidine:total P:labile P, 1.00:3.02:1.96.

Incubation of 12 OD units (1.32 μmoles) of this product with 25 μl of *E. coli* alkaline phosphatase and 25 μl of 1 M ammonium bicarbonate, pH 8, was carried out for 1 hr at 36°. The mixture was then diluted with water (1 ml) and chloroform (1 ml) and vigorously agitated for 2 min on a Vortex mixer. The resulting aqueous phase was filtered through glass wool directly onto a 1 × 21 cm column of DEAE-cellulose (HCO_3^-) and the column was eluted with a linear gradient of 120 ml of triethylammonium bicarbonate (0.005–0.25 M), 3-ml fractions being collected. The first ultraviolet-absorbing peak contained cytidine (2 OD units) and was followed by a peak containing 1 OD unit of CMP mixed with [^{32}P]orthophosphate. Electrophoresis at pH 5 of this entire evaporated peak showed all radioactivity to be present as orthophosphate. The CDP peak contained 2.2 OD units and had an apparent specific activity of 3050 cpm/OD unit. Paper chromatography in solvent II of the entire pooled peak, however, showed the ^{32}P to be present solely as orthophosphate, elution of the purified CDP showing it to have a specific activity of only 40 cpm/OD unit. The undegraded CTP peak contained 7 OD units and had a specific activity of 56,200 cpm/OD unit, whereas the original solution prior to phosphatase action showed 58,000 cpm/OD unit at the same time, indicating 99.9% of the label to be in the γ position.

[γ-^{32}P]*Uridine 5'-Triphosphate.* The di(4-morpholine-*N,N'*-dicyclohexycarboxamidine) salt of UDP-morpholidate·$2H_2O$ (110 mg, 0.1 mmole) was allowed to react with 0.4 mmole of tributylammonium orthophosphate containing 1 mCi of ^{32}P in 2 ml of anhydrous dimethyl sulfoxide for 45 hr at 36°. The mixture was worked up as above by chromatography on a 1.7 × 25 cm column of DEAE-cellulose (HCO_3^-) using a gradient of triethylammonium bicarbonate. The first peak contained 50 OD units (5%) of UMP admixed with excess orthophosphate, the second peak 150 OD units (15%) of UDP with only a trace of isotope, and the major peak 680 OD units (68%) of [γ-^{32}P]UTP. The pooled triphosphate peak was evaporated and isolated as the tetrasodium salt trihydrate (41 mg, 66%) by treatment with sodium iodide in acetone as above. This material was chromatographically homogeneous in solvent I and had a specific activity of 2.72 × 10^6 cpm/μmole;[2] $\lambda_{max}^{H_2O}$ 262 mμ (ϵ 10,000); uridine:total P:labile P, 1.00:2.90:1.97.

A sample of 20 OD units at 262 mμ of this material was incubated with 50 μl of *E. coli* alkaline phosphatase and 25 μl of ammonium bicarbonate, pH 8, for 1 hr at 37°. Following denaturation of the enzyme by vigorous agitation with chloroform the products were separated on a 1 × 32 cm column of DEAE-cellulose (HCO_3^-) using a linear gradient of 200 ml of triethylammonium bicarbonate (0.005–0.3 M). Uridine (2 OD units) was followed by completely nonradioactive UMP (6 OD units) admixed with [^{32}P]orthophosphate. The UDP peak (2 OD units) had an apparent specific activity of 2000 cpm/OD unit while the peak of unhydrolyzed UTP (10 OD units) had a specific activity of 55,600 cpm/OD unit. Paper chromatography of the entire UDP peak using solvent I showed the radioactivity to be present as orthophosphate, the purified UDP having a specific activity of only 66 cpm/OD unit, indicating 99.9% γ labeling. Untreated [γ-^{32}P]UTP had a specific activity of 57,000 cpm/OD unit at the same time.

[γ-^{32}P]*Guanosine 5'-Triphosphate.* The carboxamidine salt of GDP-morpholidate (117 mg, 0.1 mmole) was

[2] The syntheses of [α-^{32}P]CTP and -UTP were made using a common stock solution of [^{32}P]orthophosphate with a specific activity of 2.75 × 10^6 cpm/μmole, while those of [γ-^{32}P]GTP and -TTP used a stock solution with 2.51 × 10^6 cpm/μmole. The reported specific activities of the isolated sodium salts of the triphosphates are corrected as of the beginning of each experiment for purposes of comparison.

allowed to react with 0.4 mmole of tributylammonium orthophosphate containing 1 mCi of ^{32}P in 2 ml of anhydrous dimethyl sulfoxide at 36° for 45 hr as above. After addition of water (10 ml) the mixture was chromatographed on a 1.7 × 25 cm column of DEAE-cellulose (HCO_3^-) using a linear gradient of 2 l. of triethylammonium bicarbonate (0.005–0.3 M). A large peak of excess orthophosphate immediately preceded a small amount (48 OD units at 252 mμ, 3.5%) of unreacted GDP-morpholidate. GDP (220 OD units, 16%) was eluted at roughly 0.17 M salt and was immediately preceded by a small, unidentified radioactive peak (perhaps pyrophosphate). The major peak of [γ-^{32}P]GTP (1040 OD units, 76%) was eluted at roughly 0.25 M salt and the chromatographically homogeneous sodium salt was isolated as its dihydrate (48 mg, 74%) by treatment with sodium iodide in the usual way. This material had a specific activity of 2.32 × 10^6 cpm/μmole;[2] λ_{max}^{pH7} 252 mμ (ε 13,600); guanosine:total P:labile P, 1.00:2.90:2.04.

Incubation of 20 OD units of this compound with 50 μl of *E. coli* alkaline phosphatase and 25 μl of ammonium bicarbonate, pH 8, was terminated after 70 min at 37°. Chromatography on a 1 × 25 cm column of DEAE-cellulose (HCO_3^-) using a linear gradient of 150 ml of triethylammonium bicarbonate (0.005–0.35 M) gave an initial peak of guanosine (5.3 OD units) followed by 2.80 OD units of GMP which slightly overlapped the end of the radioactive orthophosphate peak. The peak of GDP (2.3 OD units) at roughly 0.2 M salt had an apparent specific activity of 1830 cpm/OD unit, while the GTP (7.2 OD units) eluted at 0.28 M salt had a specific activity of 30,400 cpm/OD unit. A sample of the original, untreated GTP had a specific activity of 30,200 cpm/OD unit at the same time. Following purification of the GDP peak by paper chromatography in solvent I the specific activity was found to be only 68 cpm/OD unit, indicating 99.8% γ labeling, the remaining isotope being chromatographically identical with orthophosphate.

[γ-^{32}P]Thymidine 5′-Triphosphate. The reaction between the carboxamidine salt of TDP-morpholidate (115 mg, 0.1 mmole) and 0.4 mmole of tributylammonium orthophosphate containing 1 mCi of ^{32}P was carried out as above in 2 ml of dimethyl sulfoxide at 36° for 45 hr. Chromatography on DEAE-cellulose using a linear gradient of 2 l. of triethylammonium bicarbonate (0.005–0.25 M) gave TMP-morpholidate (70 OD units at 267 mμ, 7%) superimposed upon the peak of excess orthophosphate, TDP (180 OD units, 19%) at roughly 0.12 M salt, and a major peak of TTP (660 OD units, 69%) at 0.16 M salt. Isolation in the usual way with sodium iodide gave 41 mg (66%) of the chromatographically homogeneous tetrasodium salt of TTP as the trihydrate with a specific activity of 2.52 × 10^6 cpm/μmole;[2] λ_{max}^{pH7} 267 mμ (ε 9700); thymidine:total P:labile P, 1.00:2.90:1.93.

Incubation of 20 OD units of the isolated sodium salt with 50 μl of *E. coli* alkaline phosphatase and 25 μl of 1 M ammonium bicarbonate, pH 8, was terminated after 60 min at 37°. Chromatography (Figure 1) on DEAE-cellulose (HCO_3^-) using a linear gradient of 300 ml of triethylammonium bicarbonate (0.005–0.3 M) gave 1.8 OD units of thymidine, 7.1 OD units of TMP admixed with orthophosphate, 2.3 OD units of TDP at 0.15 M salt, and 6.0 OD units of undegraded TTP at 0.2 M salt. The apparent specific activity of the TDP was 4900 cpm/OD unit while that of the TTP was 78,000 cpm/OD unit and a sample of untreated TTP showed 77,000 cpm/OD unit at the same time. Purification of the isolated TDP by paper electrophoresis at pH 3.5 clearly showed the radioactivity to be present as orthophosphate, the eluted diphosphate then having a specific activity of only 40 cpm/OD unit and indicating 99.9% γ labeling.

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