XXXII. THE LABELING OF END GROUPS IN POLYNUCLEOTIDE CHAINS: THE SELECTIVE PHOSPHORYLATION OF PHOSPHOMONOESTER GROUPS IN AMINO ACID ACCEPTOR RIBONUCLEIC ACIDS¹

U. L. RAJBHANDARY, R. J. YOUNG,† and H. G. KHORANA

From the Institute for Enzyme Research, The University of Wisconsin, Madison 6, Wisconsin

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The methods which are currently available for the sequential analysis of the nucleic acids broadly fall into two groups. The first group consists of the stepwise degradation of polynucleotide chains either by chemical methods¹ (2-4) or by the use of specific exonucleases (5, 6). The second group involves initial fragmentation of the polynucleotide chains by the use of chemical (7, 8) or enzymic (endonucleases) reagents (9) followed by separation and structural analysis of the fragments.

In attempting to devise new approaches to the end group and sequential analysis of polynucleotides, we have adopted as a guide the principle which involves as the first step the labeling of the end groups of the polynucleotide chains with specific agents, and as the second step the fragmentation of the polynucleotide chains and determination of the sequences in those fragments which contain the labeled end group.

Recently a method was reported for the labeling of the phosphomonoester groups in amino acid acceptor RNA,² which involved the conversion of the 5' phosphomonoester end groups to phosphomonoester groups in amino acid acceptor RNA, which in involves the reaction of the latter with 14C-methyl phosphoro-morpholidate (Formula I) as shown in Fig. 1. The method which is considered to be superior to that developed previously is shown to be specific for the labeling of the phosphomonoester end group and promises to be of general use for the detection and labeling of 5'-phosphate groups in DNA's and 3'- or 5'-phosphomonoester groups in DNA's. Preliminary reports of parts of this work have already appeared (11-13).

An accompanying paper reports on an approach to the complementary problem of the labeling of hydroxyl end groups in deoxyribopolynucleotides (14).

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† Present address, Department of Biochemistry, Monash University, Clayton, Victoria, Australia.

² That is, by the use of periodate oxidation of the 3', 3'-vinyl diol groups followed by base-catalyzed elimination (2-4).

The abbreviations used are: acceptor RNA, amino acid acceptor RNA; MeppX, P₁ methyl, P₂ ribonucleoside 5'-pyrophosphate. One optical density unit is defined as that amount of material per milliliter of solution which produces an absorbance of 1 in a 1-cm light path cell at 260 mp.

EXPERIMENTAL PROCEDURE

Dimethylsulfoxide was dried by two distillations and then stored over Type 4XA molecular sieve beads (Linde Chemical Company) for at least 2 weeks before use. The reagent was left inside a dry box, a positive pressure of dry nitrogen being maintained in the dry box. Pyridine was distilled from chlorosulfonic acid and stored over molecular sieve beads. Dioxane was purified by refluxing in the presence of an excess of sodium and distilled before use. Di-p-nitrophenyl phosphoric acid (Aldrich Chemical Company) and di-p-tolyl carbodiimide were purified by recrystallization. ¹C-Methanol (specific activity, 5 mc per mmole) was purchased from New England Nuclear Corporation.

Paper Chromatography—The descending technique was used at room temperature with Whatman No. 1 and No. 40 and the diethylaminoethyl cellulose ion exchange (Whatman DE-20) papers. The solvent systems used were: Solvent A, 2-propanol-concentrated ammonium hydroxide-water (7:1:2, v/v); Solvent B, isobutyric acid-concentrated ammonium hydroxide-water (66:1:33, v/v); Solvent C, ethanol-concentrated ammonium bicarbonate; Solvent D, 0.2 M ammmonium sulfate; Solvent E, l-propanol-concentrated ammonium hydroxide-water (55:10:35, v/v); and Solvent F, ammonium sulfate (60% w/v in 0.1 M phosphate buffer)-l-propanol (100:2, v/v).

Column chromatography was carried out at 2°C.

Paper Electrophoresis—This was performed in an apparatus similar to that described by Markham and Smith (16). The buffer used was freshly prepared 0.05 M ammonium acetate (pH 5.15), and all electrophoretic runs were carried out at 2°C on Whatman No. 31 papers.

Elution of ultraviolet-absorbing or radioactive compounds from paper was mostly carried out with water adjusted to pH 9 with ammonia except for guanosine compounds, in which case 0.2 M ammonia was used. To ensure quantitative recovery, elution was carried out by the descending technique until no more ultraviolet-absorbing compounds or radioactivity, or both, was eluted. Blanks prepared by eluting appropriate strips of paper were used at all times. Paper strips previously used for counting in the liquid scintillation counter were washed alternately with ether and ethyl alcohol.

Optical density units, as used in this paper, were usually measured in dilute Tris buffer, pH 7.5, against appropriate blanks at 260 mp in a Zeiss PMQ II spectrophotometer.

Counting and Scanning of Paper Chromatograms for Radioac-
enzyme preparation for the assay of amino acid acceptor activity

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of Dr. L. A. Heppel. The preparation of diesterase was purchased from Worthington Biochemical Corporation which was treated in order to inactivate nucleases provided by Dr. L. A. Heppel. T1 RNase was a gift from Drs. Egami and Takahashi. Pancreatic RNase and spleen phosphodiesterase were purchased from Worthington Biochemical Corporation (16) which was treated in order to inactivate nucleases provided by Dr. R. M. Hoskinson of this laboratory. For scanning of paper chromatograms and location of radioactivity, a Baird 4π gas flow windowless paper scanner was used.

Enzymes—The Escherichia coli alkaline phosphatase preparation was a commercial product (Worthington Biochemical Corporation) which was treated in order to inactivate nucleases as described previously and had a protein content of 1 mg per ml. Another sample of the purified enzyme was kindly provided by Dr. L. A. Heppel. T1 RNase was a gift from Drs. Egami and Takahashi. Pancreatic RNase and spleen phosphodiesterase were purchased from Worthington Biochemical Corporation. Pork liver endonuclease was prepared in the laboratory of Dr. L. A. Heppel. The preparation of Lactobacillus acidophilus exonuclease has been described earlier (17). The crude enzyme preparation for the assay of amino acid acceptor activity was kindly supplied by Dr. R. M. Hoskinson of this laboratory.

Analytical—Estimation of inorganic phosphate was carried out with the method of Chen, Toribara, and Warner (18), as modified by Ames and Dubin (19). Estimation of organic phosphate was done by alkaline hydrolysis of RNA before treatment with alkaline phosphatase. Phosphate esters were detected on paper strips the scintillation medium consisted of 2,5-diphenyloxazole, 4 g, and dimethyl p-bis-2'(5'-phenyloxazolyl)benzene, 0.1 g, in reagent grade toluene (1 liter). Aliquots of column fractions or aqueous solutions were usually mixed with a scintillation medium consisting of napthalene, 56 g, 2,5-diphenyloxazole, 7 g, and dimethyl p-bis-2'(5'-phenyloxazolyl)benzene, 0.35 g, in xylene, 100 ml, dioxane, 300 ml, and methyl Cellosolve, 300 ml (16). The efficiency of 14C counting with an aqueous content of 2% was around 65%.

For scanning of paper chromatograms and location of radioactivity, a Baird 4π gas flow windowless paper scanner was used. Enzymes—The Escherichia coli alkaline phosphatase preparation was a commercial product (Worthington Biochemical Corporation) which was treated in order to inactivate nucleases as described previously (17) and had a protein content of 1 mg per ml. Another sample of the purified enzyme was kindly provided by Dr. L. A. Heppel. T1 RNase was a gift from Drs. Egami and Takahashi. Pancreatic RNase and spleen phosphodiesterase were purchased from Worthington Biochemical Corporation. Pork liver endonuclease was prepared in the laboratory of Dr. L. A. Heppel. The preparation of Lactobacillus acidophilus exonuclease has been described earlier (17). The crude enzyme preparation for the assay of amino acid acceptor activity was kindly supplied by Dr. R. M. Hoskinson of this laboratory.

Analytical—Estimation of inorganic phosphate was carried out with the method of Chen, Toribara, and Warner (18), as modified by Ames and Dubin (19). Estimation of organic phosphate was done by alkaline hydrolysis of RNA before treatment with alkaline phosphatase. Phosphate esters were detected on paper chromatograms with the ammonium molybdate-perchloric acid spray (20).

RESULTS

14C-Methyl Phosphate—This compound was prepared by phosphorylation of 14C-methanol either with a mixture of β-cyanoethylpyridinium phosphate and dicyclohexylcarbodimide (21) or by the use of tetra p-nitrophenyl pyrophosphate as described previously (22). The steps are shown in Fig. 2. The intermediate esters in both cases were hydrolyzed and the product, methyl phosphate, in both cases was isolated as the crystalline barium salt in yields of 65 to 70%. The phosphorylation reaction was complete in a shorter time in the latter method and hence was preferred for phosphorylation of 14C-methanol of high specific activity (5 mc per mmole). The details were as follows.

Di-p-nitrophenyl phosphoric acid (1.36 g, 4 mmoles) was dissolved in anhydrous dioxane by warming and then cooled to room temperature. To this solution was added di-p-tolyl carbodiimide (440 mg; ≈ 2 mmoles), and then the sealed mixture was shaken intermittently for 10 minutes. 14C-Methanol (1 mmole) was dissolved in anhydrous dioxane and the two solutions were mixed and allowed to stand at room temperature. After 36 hours the precipitate of di-p-tolyl urea was filtered off and washed with small volumes of dioxane. The filtrate and washings were evaporated to a gum, dissolved in chloroform (2 ml), and the chloroform solution extracted eight times with water (25 ml each time) until the aqueous extract was neutral. The chloroform layer was filtered to remove some fine particles and the filtrate was evaporated to a gum. The gum was dissolved in a mixture of 1 ml lithium hydroxide (12 ml) and dioxane (10 ml). The mixture was shaken occasionally for 30 minutes and the clear solution was then evaporated. Water (12 ml) was added to the residue and the solution was heated at 100° for 5 hours in a polyethylene bottle. The solid precipitate of trilithium phosphate was removed by centrifugation and the supernatant was carefully adjusted to pH 5. The clear yellow solution was extracted six times with ether (80 ml each time) until an ethereal extract gave no yellow color with alkali. The aqueous solution was then passed through a column (1.6 × 10 cm) of Dowex 50 (H+ form) resin and the acid eluate and washings evaporated to a small volume and neutralized to pH 7.5 with saturated barium hydroxide. Barium phosphate was removed by centrifugation and the supernatant was carefully adjusted to pH 5. The clear yellow solution was extracted six times with ether (80 ml each time) until an ethereal extract gave no yellow color with alkali. The aqueous solution was then passed through a column (1.6 × 10 cm) of Dowex 50 (H+ form) resin and the acid eluate and washings evaporated to a small volume and neutralized to pH 7.5 with saturated barium hydroxide. Barium phosphate was removed by centrifugation and the supernatant was carefully adjusted to pH 5. The clear yellow solution was extracted six times with ether (80 ml each time) until an ethereal extract gave no yellow color with alkali. The aqueous solution was then passed through a column (1.6 × 10 cm) of Dowex 50 (H+ form) resin and the acid eluate and washings evaporated to a small volume and neutralized to pH 7.5 with saturated barium hydroxide. Barium phosphate was removed by centrifugation and the supernatant was carefully adjusted to pH 5. The clear yellow solution was extracted six times with ether (80 ml each time) until an ethereal extract gave no yellow color with alkali. The aqueous solution was then passed through a column (1.6 × 10 cm) of Dowex 50 (H+ form) resin and the acid eluate and washings evaporated to a small volume and neutralized to pH 7.5 with saturated barium hydroxide. Barium phosphate was removed by centrifugation and the supernatant was carefully adjusted to pH 5.
The following tests were performed with the solution. (a) An aliquot was directly chromatographed in Solvent A. (b) An procedure was repeated twice with additions of pyridine; the aliquot (50 μl) was evaporated to dryness under vacuum and the removed by extraction with ether, and the aqueous alkaline temperature for 30 minutes. The guanidine (Formula III) was hydroxide (2 ml), and the homogeneous solution was left at room temperature for many months. Treatment of the compound had taken place. The product was stable in aqueous Organic Bases—Pyridine solution of 14C-methyl phosphoromorpholidate, was stored in 0.2 N sod.ium hydroxide at -15°. The specific activity of W-methyl phosphate to the desired products (for RF values see Table I) were prepared either by condensation of a nucleoside 5'-phosphate with methyl phosphoromorpholidate. The typical method of preparation was as follows. Ribonucleoside 5'-phosphoromorpholidate (25 μmoles) as the guanidinium (Formula III) salt was rendered anhydrous by repeated evaporation of anhydrous pyridine solution (3 x 5 ml) and to this was added the pyridinium salt of methyl phosphate (50 μmoles). The reagents were dried by several evaporation of anhydrous pyridine solution and, finally, the residues was dissolved in a small volume (0.5 ml) of pyridine and the solution was left sealed at room temperature. In the case of cytidine and guanosine derivatives, small amounts of dimethylsulfoxide (0.2 ml) were added to obtain clear solutions. After 48 hours the individual reaction mixtures were chromatographed with Solvent B and C. The latter on dephosphorylation would yield compounds of the general structure 14C-MeppXp (X = nucleoside) (e.g. Formula II, Fig. 1). The four synthetic markers of MeppX (X = A, C, U, and G) could easily separated from each other by two-dimensional chromatography with Solvents B and C.

Stability of W-Methyl Phosphoromorpholidate as Salts of Various Organic Bases—Pyridine solution of 14C-methyl phosphoromorpholidate as its guanidine (Formula III) salt (65 μmoles; specific activity, 0.1 μc per μmole) was mixed with 0.5 N sodium hydroxide (2 ml), and the homogeneous solution was left at room temperature for 30 minutes. The guanidine (Formula III) was removed by extraction with ether, and the aqueous alkaline solution was passed slowly through a column (0.8 x 5 cm) of Dowex 50 (triethylammonium form) resin and the column washed with water (the total volume of eluate and washings was 10 ml). The following tests were performed with the solution. (a) An aliquot was directly chromatographed in Solvent A. (b) An aliquot (50 μl) was evaporated to dryness under vacuum and the procedure was repeated twice with additions of pyridine; the residue was sucked under vacuum for a further period of 20 minutes, and the final residue was dissolved in water (20 μl). (c) Another aliquot (50 μl) was evaporated in the presence of tri-n-hexylamine as above, and the residue was dissolved in aqueous pyridine.

The above solutions were chromatographed in Solvent A and the chromatograms were scanned for radioactivity. The column eluate, (a) above, showed a single peak of 14C-methyl phosphoromorpholidate; the aliquot evaporated in the presence of pyridine, (b) above, showed complete decomposition to 14C-methyl phosphate; whereas the aliquot evaporated in the presence of added tri-n-hexylamine, (c) above, was unchanged 14C methyl phosphoromorpholidate.

Synthesis of P1-Methyl, P2-Ribonucleoside 5'-Pyrophosphates (MeppX)—The radioactive products of alkaline hydrolysis of end-labeled acceptor RNA would have the general structure 14C-MeppXp (X = nucleoside) (e.g. Formula II, Fig. 1). The latter on dephosphorylation would yield compounds of the general structure 14C-MeppX. Nonradioactive markers of MeppX (X = A, C, U, and G) were prepared either by condensation of a nucleoside 5'-phosphoromorpholidate with methyl phosphate or by the reaction of a nucleoside 5'-phosphate with methyl phosphoromorpholidate. The four synthetic markers of MeppX (X = A, C, U, and G) could easily separated from each other by two-dimensional chromatography with Solvents B and C.

The typical method of preparation was as follows. Ribonucleoside 5'-phosphoromorpholidate (25 μmoles) as the guanidinium (Formula III) salt was rendered anhydrous by repeated evaporation of anhydrous pyridine solution (3 x 5 ml) and to this was added the pyridinium salt of methyl phosphate (50 μmoles). The reagents were dried by several evaporation of anhydrous pyridine solution and, finally, the residues was dissolved in a small volume (0.5 ml) of pyridine and the solution was left sealed at room temperature. In the case of cytidine and guanosine derivatives, small amounts of dimethylsulfoxide (0.2 ml) were added to obtain clear solutions. After 48 hours the individual reaction mixtures were chromatographed with Solvent F and the desired products (for RF values see Table I) were eluted from the paper and stored at -15°. Chromatography in Solvents A, B, C, and F showed that the products were homogeneous.

**Table I**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>RF in Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Methyl phosphate</td>
<td>0.26</td>
</tr>
<tr>
<td>Dimethyl pyrophosphate</td>
<td>0.37</td>
</tr>
<tr>
<td>Methyl phosphoromorpholidate</td>
<td>0.6</td>
</tr>
<tr>
<td>MeppA</td>
<td>0.24</td>
</tr>
<tr>
<td>MeppC</td>
<td>0.15</td>
</tr>
<tr>
<td>MeppG</td>
<td>0.2</td>
</tr>
</tbody>
</table>

The loss of triethylamine during evaporation would cause a lowering of the pH value, thus accelerating the decomposition of the phosphoromorpholidate.
the amount of \(^{14}C\)-incorporation into acceptor RNA provided an accurate estimation of 5'-phosphomonoester end group content is available. Various estimates of the chain length of acceptor RNA have been previously made and a wide range of 5'-phosphomonoester end group content has been reported (24, 25). Furthermore there may be the possibility of variation in the phosphomonoester end group content in different preparations of acceptor RNA. The previous determinations of the 5'-phosphomonoester end groups have been based on the amounts of the guanosine-2'-(or 3'),5'-diphosphates isolated after alkaline hydrolysis of the acceptor RNA.\(^{1}\) A determination of the amount of 5'-phosphomonoester end groups in the acceptor RNA preparation used in the present work was carried out as follows.

Acceptor RNA, 246.6 optical density units, was hydrolyzed with 1 N sodium hydroxide, 6 ml, at room temperature for 48 hours and excess alkali was then neutralized carefully to pH 7.5 by gradual addition of Dowex 50 (H\(^+\) form) resin. The resin was filtered off and washed exhaustively with small amounts of dilute ammonia. The filtrate and washings were evaporated and chromatographed on three strips (7 inches wide) of Whatman No. 40 papers with Solvent A for 48 hours. The slower moving bands of nucleoside diphosphate and alkali-resistant dinucleotides were eluted until no more ultraviolet-absorbing compounds could be detected in the eluate. The total eluate was evaporated and applied as a 3-inch wide band on Whatman No. 31 paper. A good separation of nucleoside diphosphates from the alkali-resistant dinucleotides (mobility 15 cm from the origin) was achieved by paper electrophoresis. The nucleoside diphosphate bands located by the use of markers (faint faster band and main band with 19.3-cm mobility) were eluted, evaporated, and made up to a known volume. The total amount of nucleoside diphosphates produced from 246.6 optical density units of acceptor RNA was 0.2878 μmole, and hence 1 μmole of 5'-phosphomonoester group was present in 857 optical density units of acceptor RNA.

\[
\text{Assay for Incorporation of } ^{14}C \text{ Radioactivity into Acceptor RNA and Rate of Reaction.} \]

The rate of reaction of \(^{14}C\)-methyl phosphoromorpholidate with acceptor RNA was followed by a method similar to that described previously (27). Aliquots of the reaction mixture removed under anhydrous conditions were spotted on Whatman DE-20 papers and chromatographed in Solvent D for 9 to 11 hours. The chromatograms were scanned for location of radioactivity (Fig. 3), and the ultraviolet-absorbing material at the origin corresponding to the acceptor RNA was cut out and counted in the liquid scintillation counter. It was made certain that no radioactive compound was trapped in the acceptor RNA band and that the assay measured only the radioactivity covalently linked to the acceptor RNA (Table II).

From a reaction mixture of acceptor RNA (400 optical density units) with \(^{14}C\)-methyl phosphoromorpholidate (30 μmoles), aliquots were taken out at intervals and assayed as described above. The results given in Fig. 4 show that the incorporation of \(^{14}C\)-radioactivity into acceptor RNA reaches a maximum and levels off in about 140 hours. At this point, at least 40% of the original \(^{14}C\)-methyl phosphoromorpholidate was undegraded. It is clear from Fig. 4 that the reaction had already proceeded to a fair extent before the addition of dimethylsulfoxide. This is understandable because of the time required (2 to 3 hours) for repeated evaporations from anhydrous pyridine after the reactants have been mixed together (see below for the general method of reaction).

\[
\text{Reaction of } ^{14}C\text{-Methyl Phosphoromorpholidate with Acceptor RNA.} \]

\(\text{Table II}\)

<table>
<thead>
<tr>
<th>Reaction components</th>
<th>Radioactivity at origin of chromatogram</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete reaction mixture</td>
<td>13,485</td>
</tr>
<tr>
<td>Control ((^{14}C)-methyl phosphoromorpholidate, (I))</td>
<td>148</td>
</tr>
<tr>
<td>(I) + acceptor RNA sodium salt</td>
<td>125</td>
</tr>
<tr>
<td>(I) + acceptor RNA cetyltrimethylammonium salt</td>
<td>160</td>
</tr>
</tbody>
</table>

Fig. 3. Chromatography of the reaction mixture for end group labeling of acceptor RNA and scan for location of radioactivity. The only ultraviolet-absorbing spot was at the origin.
RNA—The general method developed previously for the formation of the pyrophosphate linkage in nucleotide coenzymes involved the condensation between the trialkylammonium salt of the component bearing the phosphomonooester group and the guanidinium (Formula III) salt of the phosphoramidates in an anhydrous medium (28). In the first experiments carried out for end group labeling of acceptor RNA, the guanidinium (Formula III) salt of \(^{14}C\)-methyl phosphoromorpholidate was brought into reaction with tri-\(n\)-hexylammonium salt of acceptor RNA in a mixture of dimethylformamide and pyridine (11, 12). Pyridinium Dowex 50 resin (2% cross-linked) was added to remove any dimethylamine produced by decomposition of dimethylformamide. After continuous shaking for periods up to 1 week at room temperature, the extent of end group labeling was in the range of 30 to 50\%. In these experiments a homogenous reaction mixture was never obtained.

The use of quaternary ammonium compounds containing long chain alkyl groups has been described for the solubilization of the nucleic acids in organic solvents (29). In the present work the cetyltrimethylammonium acceptor RNA and the guanidinium (Formula III) salt of \(^{14}C\)-methyl phosphoromorpholidate gave a completely homogeneous solution in anhydrous dimethylsulfoxide, but little reaction was noted over a period of several days. The result is consistent with the previous findings in which the protonation of the nitrogen in the phosphoromorpholidate was established to be the first step in the pyrophosphate bond formation, the reaction rate decreasing with the increase in the strength of the amines present in the medium (23). The general method developed now consists of the reaction of cetyltrimethylammonium salt of acceptor RNA with about 40- to 50-fold excess of guanidine (Formula III) salt of K-methyl phosphoromorpholidate. The reaction mixture was then diluted with cold ethanol (4 ml) and 3 \(m\) sodium chloride (5 ml) was added. After 15 minutes at \(0^\circ\), the sodium salt of acceptor RNA was collected by centrifugation and washed five times with cold ethanol (3 \(m\) sodium chloride (5 ml)) and finally dissolved in 0.1 \(M\) Tris buffer, pH 7.5, in a small volume (50 \(ml\)).

**General Method and Isolation of Product—**Yeast acceptor RNA was isolated by chromatography on a DEAE-cellulose column as described previously (10). A solution (7 \(ml\)) of the sodium salt (600 optical density units) in 0.005 \(M\) Tris buffer, pH 7.5, was mixed with a solution (7 \(ml\)) of cetyltrimethylammonium bromide (35 \(mg\)) in the cold. The precipitate obtained was allowed to settle at \(0^\circ\) for 15 minutes and then was collected by centrifugation and washed five times with cold water (10 \(ml\) each time). The precipitate was then suspended in water (3 \(ml\)) and the suspension lyophilized.

A solution of sodium \(^{14}C\)-methyl phosphoromorpholidate (45 \(\mu\)moles) in 0.2 \(N\) sodium hydroxide (6 \(ml\)) was slowly passed through a column (0.5 \(X\) 6 \(cm\)) of Dowex 50 (triethylammonium form) resin and the column was washed with water. To the combined eluate and washings a solution (3.5 \(ml\)) of tri-\(n\)-hexylamine in pyridine (12 \(\mu\)moles per \(ml\)) was added, followed by more pyridine if a homogeneous solution was not obtained. The solution was evaporated under vacuum with frequent additions of pyridine. The clear residue was evaporated five times from anhydrous pyridine (5 \(ml\) each time) and finally mixed as a pyridine solution with the cetyltrimethylammonium salt of acceptor RNA. After five more coevaporations from pyridine, the last traces of pyridine were removed by continued suction under vacuum, the flask was opened to atmosphere inside a dry box and anhydrous dimethylsulfoxide (1 \(ml\)) was added. The clear solution obtained was left inside the dry box at room temperature. After 96 hours, an aliquot (10 \(\mu\)l) was chromatographed on Whatman DE-20 paper in Solvent D and the chromatogram scanned for radioactivity. A representative scan is shown on Fig. 3. The sharp radioactive peak at the origin indicated incorporation of radioactivity into acceptor RNA, the other products in the order of increasing mobility being \(^{14}C\)-methyl phosphate, \(^{14}C\)-P, \(^{14}C\)-dimethyl pyrophosphate, and about 40\% of undegraded \(^{14}C\)-methyl phosphoromorpholidate. The reaction mixture was then diluted with cold ethanol (4 \(ml\)) and 3 \(m\) sodium chloride (5 \(ml\)) was added. After 15 minutes at \(0^\circ\), the sodium salt of acceptor RNA was collected by centrifugation and washed twice with ethanol-3 \(m\) sodium chloride (1:1, v/v) and finally with ethanol. (Most of the excess of radioactivity used is found in the supernatant and can be recovered.) The precipitate was dissolved in 0.01 \(M\) Tris buffer, pH 7.5 (6.7 \(ml\)), and the solution was absorbed on top of a column (1.8 \(X\) 9 \(cm\)) of DEAE-cellulose (bicarbonate form). The column was eluted with 0.2 \(m\) ammonium bicarbonate until no more radioactivity could be detected in the eluates. (This step removes all the radioactivity in the column except that bound to the acceptor RNA.) The acceptor RNA was eluted off the column with 1 \(m\) sodium chloride in 0.1 \(M\) Tris buffer, pH 7.5, in a small volume (50 \(ml\)), the recovery in terms of optical density units being around 80\%. Appropriate fractions were pooled and the acceptor RNA precipitated by addition of cold ethanol (2.5 \(vol\)), the precipitation being completed overnight. The precipitate was collected by centrifugation, washed with ethanol, and finally dissolved in 0.02 \(m\) Tris buffer, pH 7.5, and dialyzed extensively against the same buffer. An aliquot of the acceptor RNA was plated, and then counted with a thin window gas flow counter. The specific activity of the labelled acceptor RNA was 2069 c.p.m. per optical density unit; under similar conditions the specific activity of \(^{14}C\)-methyl phosphate and \(^{14}C\)-methyl phosphoromorpholidate was 2.07 \(X\) 10\(^4\) c.p.m. per \(mmole\). The extent of \(^{14}C\)-incorporation into acceptor RNA has varied between 1600 and 2100 c.p.m. per optical density unit, in the different experiments preformed with \(^{14}C\)-methyl phosphoromorpholidate of the same specific activity. Thus, on the basis of 5'-phosphonomonoester end group determination in this preparation of acceptor RNA, the extent of end group labeling varied between 70 and 85\%.

![Figure 4](http://www.jbc.org/)

**Fig. 4.** Rate of \(^{14}C\)-incorporation into phosphonomonoester end groups of acceptor RNA.
FIG. 5. Chromatography of an alkaline hydrolysate of end group-labeled acceptor RNA in Solvent A and scan of the chromatogram for radioactivity. The shaded areas correspond to the ultraviolet-absorbing spots. Spots 1 and 2 were faint and are probably alkali-resistant dinucleotides. Spot 3, guanosine 2(3')-phosphate; Spot 4, adenosine 2(3')-phosphate, uridine 2(3')-phosphate, cytidine 2(3')-phosphate; Spots 5 and 6 were faint and are probably methylated nucleotides.

Alkaline Hydrolysis of Labeled Acceptor RNA and Location of Radioactivity—End-labeled acceptor RNA (containing 16,400 total c.p.m.) was hydrolyzed with alkali and the total hydrolysate freed from alkali as already described above. The counting of an aliquot (10%) of the neutral solution of the hydrolysate showed that the recovery of radioactivity at this stage was quantitative. The bulk (90%) of the hydrolysate (14,470 c.p.m.) was chromatographed in Solvent A for 40 hours and the chromatogram scanned for radioactivity (Fig. 5). A strong band (Band I) close to the origin and a small band (Band II) traveling between guanosine 3'-phosphate and uridine 3'-phosphate was observed. The radioactive bands were eluted with 0.2 N ammonia and aliquots were plated and counted. Band I contained 13,150 c.p.m. (90.9%) and Band II contained 840 c.p.m. (5.8%) (the recovery of radioactivity from the paper chromatogram was 96.7%).

Identification of Nucleotides Bearing 5'-Phosphate End Groups in Acceptor RNA—The eluate of Band I (preceding experiment) was evaporated, and the presumed products of Type II were dephosphorylated with E. coli alkaline phosphatase at 37° for 21/2 hours at pH 8. The synthetic nonradioactive markers (MeppX, X = A, C, U, and G) were added to the incubation mixture and the total chromatographed on a sheet (18 x 22 inches) of Whatman No. 1 paper with Solvent B in one direction for 60 hours and Solvent C in the second direction for 27 hours. Autoradiography (Fig. 6) showed five radioactive spots, four of them coincident with the added markers (MeppX, X = A, C, U, and G), and the fifth spot having mobility between MeppU and MeppG in Solvent C. To ensure that the fifth radioactive spot did not arise as a consequence of splitting of one of the other spots during chromatography, the following tests were carried out. (a) The unknown radioactive spot was cut out and eluted from the paper and could be clearly separated again from MeppU and MeppG by cochromatography in Solvent C. (b) The very strong radioactive spot of 14C-MeppG was eluted and rechromatographed with an added marker of MeppU in Solvent C. Strips (1 inch) were cut out and counted in the liquid scintillation counter; negligible counts were obtained in the region of the unknown radioactive spot. Amounts of radioactivity associated with all the radioactive spots were estimated by cutting out the spots and counting in the liquid scintillation counter (Table III). The estimation of counts associated with 14C-MeppG and 14C-MeppX (X = unidentified) might be slightly low as these spots had gone through an additional transfer and rechromatography.

Nature of Radioactive Band II—Radioactive Band II obtained by alkaline hydrolysis and paper chromatography of end group-labeled acceptor RNA was next studied (Fig. 5). The leveling off of 14C-incorporation into the end group of acceptor RNA combined with the evidence that 91% of the 14C counts incorporated into the acceptor RNA could be recovered in the end group would seem to exclude any non-specific labeling by 14C-methyl phosphoromorpholinate. We have previously observed small amounts of polyphosphate contamination in yeast acceptor RNA and this small radioactive band could be due to
the labeling of the polyphosphate chain. Band II could not be hydrolyzed with E. coli alkaline phosphatase and hence could not be a linear polyphosphate compound with $^{14}$C-methyl group at one end. Such a compound would be dephosphorylated to $^{14}$C-methanol by this enzyme (30) which would have been lost during evaporation as described below.

Radioactive Band II was eluted from a chromatogram and divided into two equal portions. One portion was incubated in the presence of E. coli alkaline phosphatase at 37° for 20 hours. The incubation mixture was then evaporated, dissolved in water, and reevaporated. Finally, water (0.5 ml) was added, mixed with the scintillation medium (20 ml), and the radioactivity was counted in the liquid scintillation counter. The second portion was directly evaporated and prepared for counting. The portion which was not treated with enzyme contained 1065 c.p.m. and the one incubated in the presence of enzyme, 944 c.p.m.

**TABLE III**

**Terminal nucleotides bearing 5'-phosphomonoester groups in acceptor RNA**

End group-labeled acceptor RNA was hydrolyzed with alkali, and the radioactive compounds containing the end groups were dephosphorylated to $^{14}$C-MeppX (X = nucleoside) and separated by two-dimensional chromatography after addition of the four nonradioactive markers of MeppX (X = A, C, U, and G). Radioactive spots were located by autoradiography (Fig. 6). These were cut out and counted in the liquid scintillation counter.

<table>
<thead>
<tr>
<th>Compound detected</th>
<th>Radioactivity</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeppA</td>
<td>608</td>
<td>4.1</td>
</tr>
<tr>
<td>MeppC</td>
<td>604</td>
<td>4.1</td>
</tr>
<tr>
<td>MeppU</td>
<td>1,501</td>
<td>10.1</td>
</tr>
<tr>
<td>MeppG</td>
<td>11,189</td>
<td>75.5</td>
</tr>
<tr>
<td>MeppX*</td>
<td>916</td>
<td>6.2</td>
</tr>
</tbody>
</table>

* In this case X = the unidentified nucleotide.

**Chromatography of End Group-labeled Acceptor RNA**—We next attempted to separate, if possible, this radioactive polyphosphate from end-labeled acceptor RNA by paper or column chromatography. No clear separation was obtained on a Sephadex G-50 column, but on a DEAE-cellulose column a partial separation was detected as judged by the specific activity at various areas of the acceptor RNA curve. Paper chromatography in Solvent A for a prolonged period did not separate any radioactive material from end-labeled acceptor RNA. Ebel et al. (31) have recently examined the nucleic acid polyphosphate complexes in nucleic acid preparations and were able to separate the complex by paper chromatography with 2-propanol-trichloroacetic acid. In our experiments, when end-labeled acceptor RNA was used, slight streaking of the acceptor RNA from the origin was observed, but in addition to a strong peak of radioactivity at the origin, some traveling of radioactive material beyond the streak of acceptor RNA was observed. The chromatography experiments are described below.

1. Chromatography on Sephadex G-50: End group-labeled acceptor RNA (32 optical density units) was passed through a column (1 x 50 cm) of Sephadex G-50 and the column was then eluted with water which had been adjusted to pH 7 with ammonia. Fractions (5.5 ml) were collected every 15 minutes. Aliquots (0.5 ml) were used for counting in the liquid scintillation counter. Only one sharp peak of radioactivity coincident with the peak of optical density (Fractions 3 to 6) was obtained.

2. Chromatography on DEAE-cellulose column: End group-labeled acceptor RNA (37 optical density units) was absorbed on a column (1 x 45 cm) of DEAE-cellulose (chloride form) equilibrated with 0.05 M Tris, pH 7.5. The column was washed twice with the same buffer (5 ml each time), and the polynucleotide was then eluted with a linear gradient of sodium chloride. Fractions of 8-ml volume were collected at 10-minute intervals and aliquots used for determination of optical density and radioactivity (Fig. 7). The specific activity of acceptor RNA was almost constant except toward the beginning of the peak.

**Fig. 7.** DEAE-cellulose column chromatography of end group-labeled acceptor RNA with a linear sodium chloride gradient. The dotted lines indicate the specific activity (counts per minute per optical density unit at 260 mµ) at various points along the peak of acceptor RNA. Mixing vessel contained 0.05 M Tris, pH 7.5 (500 ml), and the reservoir contained 1 M sodium chloride (500 ml) in the same buffer.
For details of the incubation mixture, see the text. The amount of \(^{14}C\)-leucine incorporated was determined by precipitation of \(^{14}C\)-leucyl acceptor RNA, incubation at alkaline pH and acid precipitation of the RNA. Aliquots of supernatant were used for counting.*

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Radioactivity incorporated</th>
<th>Corrected value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete + acceptor RNA</td>
<td>1110</td>
<td>962</td>
</tr>
<tr>
<td>Complete - acceptor RNA</td>
<td>1031</td>
<td>883</td>
</tr>
<tr>
<td>Complete + end group-labeled acceptor RNA</td>
<td>1420</td>
<td>855</td>
</tr>
<tr>
<td>Complete + end group-labeled acceptor RNA - enzyme</td>
<td>1461</td>
<td>896</td>
</tr>
<tr>
<td>Complete + end group-labeled acceptor RNA - enzyme</td>
<td>565</td>
<td></td>
</tr>
</tbody>
</table>

* The assay system used measures the incorporation of \(^{14}C\)-leucine into unlabeled and 5'-phosphate end-labeled acceptor RNA. These experiments have been repeated many times with similar results. Small amounts of acceptor RNA become acid-soluble in the process of incubation at alkaline pH, giving rise to a substantial but consistent level of background in the case of 5'-phosphate end-labeled RNA.

DISCUSSION

By analogy with the classical work on the sequential analysis of proteins (33), the principle of the labeling of the end groups may be expected to make an important contribution to the structural analysis of the nucleic acids. Because the polynucleotide chains in naturally occurring nucleic acids are very long and because the amounts of the polynucleotidic material available for structural work will continue to be small, the aim in the present and related work (10, 14) has been to achieve high analytical sensitivity by the introduction of a radioactive labeled group at the end of the polynucleotide chains. The introduction of such a label would simplify the identification of the end group itself and furthermore, by following up the labeling reaction with controlled chemical or enzymatic degradation of the polynucleotide chain, the determination of sequences adjoining the labeled end group would be facilitated.

The unique features of end groups of polynucleotide chains are likely to be either free primary or secondary hydroxyl groups or phosphomonoester groups. Methods for the labeling of both these types of end groups have been under investigation in this laboratory. For the labeling of the phosphomonoester end groups, the extremely high nucleophilicity of such anions relative to the phosphodiester anions has been utilized. The method previously developed consisted of the conversion of the phosphomonoester group to the corresponding phosphoroanilidate by condensation with \(^{14}C\)-aniline (10). The present method has involved the selective phosphorylation of the phosphomonoester group by reaction with \(^{14}C\)-methyl phosphoromorpholidate.

The principal requirements of a satisfactory method for end group labeling of a polynucleotide chain are (a) specificity of reaction with the end group, (b) mildness of the conditions under which reaction is carried out, and (c) adequate stability of the labeled group introduced at the terminus so as to permit subsequent fragmentation of the polynucleotide chain and characterization of the fragments containing the label. As discussed below, the present method meets all these requirements.

Under the conditions developed for the phosphorylation reaction, the incorporation of radioactive label usually leveled off after 140 hours (Fig. 4). The fact that no further incorporation of the label occurred even under prolonged reaction conditions is the first evidence for the specificity of the reaction for the phosphomonoester end group. (It should be emphasized that in these experiments about 40% of the reagent methyl phosphoromorpholidate was still intact at the end of the reaction time.) After isolation and alkaline hydrolysis of the labeled acceptor RNA (Fig. 1), out of a total recovery of radioactivity of 96.7%, 99.9% of the radioactivity incorporated was accounted for by pyrophosphates of the type II. A small part (5.8%) of the

* The requirement of specificity is absolute in that any side reaction even to the fraction of 1% with any one of the functional groups occurring repetitively in the long polynucleotide chain would cause serious complication.
radioactivity was present in a product (or products) (Fig. 5, Band II) which remains unidentified. It seems highly improbable that this incorporation is due to some nonspecific labeling of polynucleotide material. We conclude tentatively that this fraction of radioactivity was incorporated into some inorganic polyphosphate type of material which contaminated the preparation of yeast acceptor RNA used in the present work. The presence of large amounts of polyphosphates is well established in yeast and recently Ebel et al. (31) have reported on the complexing of such material with nucleic acids. These authors experienced difficulty in the removal of polyphosphates from the nucleic acids and their observation that the precipitation of acceptor RNA as cetyltrimethylammonium salt removed most but not all of the polyphosphate material is noteworthy. In our experiments, although the polyphosphate contamination could not be separated easily from the end group-labeled acceptor RNA, indication of partial separation on a DEAE-cellulose column (Fig. 7) was obtained.

Ribo- and deoxyribopolynucleotides would be expected to be indefinitely stable as cetyltrimethylammonium salts and indeed acceptor RNA has been shown to retain full amino acid acceptor activity on precipitation as the quaternary salt and subsequent conversion to the sodium salt (29). The mild conditions used in the present work for the labeling reaction should not cause any fragmentation of the sensitive polynucleotide chains and experiments carried out with the end group-labeled acceptor RNA support this belief. The labeled RNA could be chromatographed on DEAE-cellulose columns with linear salt gradient and the elution pattern resembled that of the original RNA. Furthermore, the capacity to accept amino acids, as determined with $^{14}$C-leucine, was unimpaired after the labeling reaction. The result is interesting in connection with the acceptor function of this RNA. Harkness and Hilmoe (34) have previously noted that amino acid acceptor and transfer activity of E. coli acceptor RNA was unchanged after removal of the 5'-phosphate group and our present results show that the replacement of the latter group by a pyrophosphate group similarly is without effect on acceptor activity. However, the effect of this modification on the transfer reaction resulting in polypeptide synthesis is unknown.

It is essential that the pyrophosphate linkage introduced at the polynucleotide terminus be stable to acidic, neutral, or alkaline conditions, any or all of which may be used during subsequent work on the labeled polynucleotide chains. The stability of pyrophosphates of this type to acid and neutral conditions is established from earlier work (35, 36). Possible degradation of the pyrophosphate linkage during extensive treatment with alkali was considered and MeppGP was further verified to be stable to 1 N sodium hydroxide for prolonged periods. It has also been found that the pyrophosphate linkage is stable to nucleases which are most likely to be used in the controlled degradation of the labeled ribopolynucleotides. Thus pancreatic RNase, T1-RNase, and alkaline phosphatase cause no hydrolysis of the pyrophosphate bond, while pork liver endonuclease, calf spleen phosphodiesterase (37), and Lactobacillus acidophilus exonuclease (17) caused negligible hydrolysis (<5%) of the pyrophosphate linkage.

The leveling off of the incorporation of radioactive label (Fig. 4) into acceptor RNA presumably implies the completion of the labeling reaction. Determination of the extent of labeling of the acceptor RNA requires a precise estimate of the phosphomonoester group content. Various laboratories (24) have reported on the chain length of acceptor RNA, the estimates varying between 65 and 98 nucleotide units per polynucleotide chain, although it has still to be established that all acceptor RNA chains terminate in a 5'-phosphomonoester group. Our present determination of the phosphomonoester group content has yielded the figure of 857 optical density units (260 mp, pH 7.5) of acceptor RNA as containing 1 pmole of 5'-phosphomonoester group and this result appears to be close to that of Lagerkvist and Berg (38) who arrived at the value of an average of 80 nucleotide units for the bacterial RNA. As based on these estimates the extent of labeling in the present experiments has been as high as 86%. The possibility that a fraction of the 5'-phosphomonoester groups are unavailable for the labeling reaction must be kept open at this time.

Evidence for guanosine being at the phosphorylated end of acceptor RNA was provided by various workers (39, 40) by isolation of pGp from an alkaline hydrolysis. By the use of the $^{14}$C-anilidate method (10) it was shown that in yeast acceptor RNA while most of the phosphomonoester end group contained guanosine, some adenosine and uridine were also detected at the 5'-phosphate end. These results were soon verified by Bell, Tomlinson, and Tener (41), who isolated some pGp from a large scale alkali hydrolysis or pancreatic RNase digest of yeast acceptor RNA. In the present work, by alkali hydrolysis of end-labeled acceptor RNA followed by dephosphorylation of the radioactive end groups to $^{14}$C-MeppX (X = nucleoside), we have verified these observations and obtained evidence for an additional radioactive spot, a fifth base at the 5'-phosphorylated end. Although further work is required to confirm the identity of this base, the position (Fig. 6) of the new radioactive pyrophosphate relative to those derived from the other standard bases would indicate the nucleoside portion to be methylated guanosine.

Experiments to be reported separately demonstrate that the technique developed is generally applicable, the cetyltrimethylammonium salts of yeast lactic dehydrogenase DNA7 (42) of tobacco mosaic virus RNA and of R17 virus RNA being completely soluble in the solvent system used in the present work. The main requirement is the attainment of a completely unhydrolyzed medium. In experiments when as little as 1 to 2 amoles of the labeled morpholidate reagent was used in an individual reaction mixture, 30 to 40% of the reagent was demonstrated to be present intact at the termination of the reaction. Thus, it is hoped that the scale of the labeling experiments can be reduced greatly and it can be calculated that the use of a few milligrams of nucleic acids of molecular weight in the range of 2 $\times$ 10$^6$ should give adequate incorporation of the radioactive reagent possessing the specific activity used in the present work.

While the reagent used in the present work contained $^{14}$C as the radioactive label, the possibility of using $^3$H or $^{32}$P as the label does exist. This choice of label may enable the use of a different label at the opposite terminus of the polynucleotide chain. Thus as mentioned below, one may label one terminus with $^3$H reagent and the phosphomonoester terminus with the $^{32}$P-labeled reagent. A further advantage of the present method is that the excess of reagent used in the actual reaction mixture

7 Unpublished experiments in collaboration with the late Prof. R. K. Morton.

8 Unpublished experiments. We thank Dr. P. Kaesberg for a sample of R17 virus RNA and Dr. Prenkel-Conrat for a sample of tobacco mosaic virus RNA.
can be conveniently recovered during the subsequent isolation of the labeled nucleic acid, and also the recovered methyl phosphate or the dimethyl pyrophosphate can be converted to the morpholinate by the standard procedure (23). When high molecular weight RNA’s were used, the end-labeled nucleic acid and the reagent or side products could be separated and recovered quantitatively from a Sephadex G-200 column.9

It should be pointed out that, while the method could be applied to the labeling of both 3'- and 5'-phosphomonooester end groups in DNA, in the case of RNA the method is limited to the labeling of 5'-phosphomonooester groups only. The pyrophosphate derivative is in fact formed from the 3'-phosphate groups as well, but the presence of the adjacent 2'-hydroxyl group labilizes the linkage so as to cause the formation of a 2', 3'-cyclic phosphate group (43). An experiment carried out between uridine-3' phosphate and methyl phosphoromorpholinate did indeed yield uridine 2', 3'-cyclic phosphate as the major product.

The principles for work on sequential analysis subsequent to the labeling of polynucleotide chains have been outlined elsewhere previously (10). An important feature of this scheme is the labeling of that terminus of the ribooligonucleotide chain which bears a 2', 3'-diol group.9 The use of this method in conjunction with the one described in the present paper should greatly facilitate the sequential analysis of the nucleic acids and in conjunction with the one described in the present paper should indeed yield uridine 2', 3'-cyclic phosphate as the major product.

The reaction conditions are mild and the reaction proceeds to the extent of more than 80%.

The end group-labeled acceptor RNA retained full amino acceptor activity as estimated by its leucine acceptor activity.

By this method the phosphomonooester end groups in yeast acceptor RNA have been shown to be guanosine, uridine, adenosine, and cytidine and a fifth nucleoside tentatively assumed to be a methylated guanosine derivative.

This method is general and can be used for end group labeling of 5'-phosphomonooester groups in RNA and 3'- and 5'-phosphomonooester groups in DNA. The use of this type of approach towards an absolute sequential analysis of nucleic acids is discussed.

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* Unpublished experiments.
Studies on Polynucleotides: XXXII. THE LABELING OF END GROUPS IN POLYNUCLEOTIDE CHAINS: THE SELECTIVE PHOSPHORYLATION OF PHOSPHOMONOESTER GROUPS IN AMINO ACID ACCEPTOR RIBONUCLEIC ACIDS

U. L. RajBhandary, R. J. Young and H. G. Khorana