The synthesis of oligodeoxyribonucleotides using RNA ligase

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ABSTRACT

T4 RNA ligase catalyzes the addition of a single deoxyribonucleoside-3',5'-bisphosphate to the 3'-hydroxyl of oligodeoxyribonucleotides (Hinton et al. (1978) Biochemistry 17, 5091). We have determined improved conditions for this reaction which give yields equal to or greater than 85% when any of five common deoxyribonucleoside bisphosphates (pdAp, pdCp, pdGp, pdTp, or pdUp) are added to dA(pdA)₄. A low ATP concentration, which is constantly maintained by a regeneration system composed of phosphocreatine, creatine kinase, and myokinase, contributes to the attainment of high yields. The addition of RNase A and spermine also enhances the rates and yields of the reactions. These conditions facilitate the use of RNA ligase as a reagent for the stepwise synthesis of DNA of defined sequence.

INTRODUCTION

RNA ligase isolated from T4-infected E. coli catalyzes the ATP-dependent formation of a 3'→5' phosphodiester bond between an acceptor nucleic acid, containing a 3'-hydroxyl group, and a donor molecule, containing a 5'-phosphate (1). The reaction has been used with RNA substrates for the synthesis of oligoribonucleotides of defined sequence (2-5). We have reported conditions for the addition of single 2'-deoxyribonucleoside-3',5'-bisphosphate donors to oligodeoxyribonucleoside acceptors thereby extending the synthetic utility of RNA ligase to DNA substrates (6). We have further improved reaction conditions for this single addition reaction with DNA substrates and here report methods which allow greater than 85% yields using any common deoxyribonucleoside bisphosphate and the acceptor dA(pdA)₄. Since oligodeoxyadenylate acceptors are utilized in the single nucleotide addition reaction with average efficiency with respect to acceptors of other base compositions (6), these results suggest that similar yields should be obtainable with other acceptors. The optimized reaction conditions reported here and the low level of DNase activity in the enzyme preparation demonstrate that this reaction can...
be used for the practical syntheses of oligodeoxyribonucleotides.

MATERIALS AND METHODS

Materials. Unless otherwise stated, all nucleotides and oligonucleotides were purchased from PL Biochemicals, Inc. Phosphocreatine and ATP were purchased from Sigma Chemical Co., and [γ-32P]ATP was prepared by the method of Glynn and Chapell (7). Polynucleotide kinase lacking 3'-phosphatase activity was isolated from E. coli infected with PseT 1 T4 (8). Creatine kinase (type I, rabbit muscle) was purchased from Sigma Chemical Co. as a lyophilized powder and was dissolved in 100 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 8.0, 1 mM dithiothreitol (DTT), and 10 mM phosphocreatine and stored at 1750 U/ml at -20°. Myokinase (grade V, pig muscle) was purchased from Sigma Chemical Co. as an (NH₄)₂SO₄ suspension. It was dialyzed against a buffer containing 50 mM HEPES, pH 8.0, 1 mM DTT, and 10 mM NaCl and stored at 1700 U/ml in 50% glycerol at -20°. Bacterial alkaline phosphatase (BAP) was obtained from Worthington Biochemical Co. Pyruvate kinase (type III, rabbit muscle) from Sigma Chemical Co. was stored in 50 mM HEPES, pH 7.9, at 1750 U/ml at -20°. Manufacturer's specifications of activity were used. RNase A (type I-A, bovine pancreas) from Sigma Chemical Co. was dissolved in 50 mM HEPES, pH 7.9, at 37 mg/ml and heated at 90° for 10 min to destroy DNases.

Phosphocreatine, phosphoenolpyruvate (PEP), spermine, spermidine, putrescine, DTT, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. The phosphocreatine was stored as a 200 mM solution at pH 8.3 and -20°. Polypropylene tubes (1.5 and 0.75 ml) used for reactions were purchased from Walter Sarstedt, Inc. DE 81, Whatman No. 1 and 3 MM papers were obtained from Whatman, Inc., and washed as described (6). DEAE Sephadex A-25 was purchased from Pharmacia, Inc.

Purification of RNA Ligase. T4 RNA ligase was purified from E. coli cells infected with T4 am E4314 (gene 43 mutant) by the procedure of McCoy et al. employing the polymin P precipitation and two Affi-Gel Blue columns (9). The enzyme was greater than 90% pure. Acid precipitable protein was determined by the method of Lowry (10) using lysozyme as a standard (11).

Paper Chromatography of Nucleotides and Oligonucleotides. The following solvents were used to develop paper chromatograms: I, 1.89 g NH₄HCO₃ and 1.23 ml HCO₃ per 100 ml H₂O; and II, 1-C₃H₇OH:conc. NH₃:water::55:10:35.

Synthesis of Nucleotides and Oligonucleotides. Deoxyribonucleoside
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bisphosphates were prepared from the appropriate 2'-deoxyribonucleosides and pyrophosphoryl chloride and purified as described (12). [5'-32P]pdNps were synthesized in a 100 µl reaction mixture containing 50 mM Tris-HCl, pH 7.5, 0.05 mg/ml BSA, 10 mM DTT, 10 mM MgCl₂, 1 mM dNp, 10 µM [γ-32P]ATP (200 Ci/mmol), and 30 U/ml PseT 1 polynucleotide kinase. The reaction mixture was incubated for 30 min at 37° and the product purified by paper chromatography on Whatman 3 MM paper in solvent II.

The 32P-labeled acceptor dA(pdA),[3'-»-5'- P]pdA was synthesized in a 20 µl reaction mixture of 50 mM HEPES, pH 8.3, 10 µg/ml BSA, 20 mM DTT, 10 mM MnCl₂, 0.05 mM [5'-32P]pdAp (20 Ci/mmol), 0.125 mM dA(pdA), 0.50 mM ATP, and 40 µM RNA ligase. The reaction mixture was incubated at 17° for 150 hr and boiled for 2 min. The mixture was then diluted to 120 µl by adding Tris-HCl, pH 8.0, MgCl₂, and BAP to get final concentrations of 100 mM, 50 mM, and 10 U/ml, respectively. After incubation at 65° for 4 hr, the products were separated by elution from a DEAE Sephadex A-25 bicarbonate column (0.9 x 13.5 cm) with a 150 ml gradient of 0.1 to 1.5 M triethylammonium bicarbonate, pH 7.5. The fractions containing product were pooled and repeatedly dried under vacuum with the addition of methanol between dryings. Chromatography on DE 81 in solvent I resulted in greater than 98% of the radioactivity comigrating with dA(pdA),.

The commercial oligodeoxyribonucleotide dA(pdA), was further purified and desalted by chromatography on Whatman 3 MM paper in solvent II and elution with H₂O.

Reaction Conditions. Unless otherwise specified, RNA ligase reactions between a single nucleoside bisphosphate donor, pdNp, and the labeled oligodeoxyribonucleotide acceptor, dA(pdA),[3'-»-5'-32P]pdA were performed as follows: the donor, the acceptor, ATP, phosphocreatine (if used), and the polyamine (if used) were dried together under vacuum. The reaction cocktail (5-12 µl), containing 50 mM buffer at the specified pH, 10 mM MnCl₂, 20 mM DTT, 10 µg/ml BSA, and RNA ligase, was added to initiate the reaction. In addition, reactions using the ATP regenerating system also contained phosphocreatine, myokinase, and creatine kinase. (The addition of the phosphagen resulted in no change in buffer pH.) Exact concentrations are given in the figure legends and the text. The reactions were incubated at 17° and 1 or 2 µl samples were removed for assay. Aliquots were either applied to DE 81 paper and developed in solvent I for 6 hr or to Whatman I and developed in solvent II for 24 hr. The chromatograms were scanned for radioactivity in a Packard 701B strip scanner and the radioactivity quantified using a scintil-
lation counter. The yields of product were calculated as the ratio of radioactivity in the product peak to the total radioactivity and were based upon the limiting substrate present in the reaction mixture. Products migrated more slowly than acceptor in the solvent systems used, and nuclease activity was monitored by the appearance of rapidly migrating material, [5'-$^{32}$P]pdA, on the chromatograms.

RESULTS AND DISCUSSION

We have shown that T4 RNA ligase catalyzes the addition of a single 2'-deoxyribonucleoside-3',5'-bisphosphate to the 3'-hydroxyl group of an oligodeoxyribonucleotide (6). Although ATP is a required substrate in this reaction, lowering its concentration, while maintaining a concentration excess of donor deoxyribonucleoside bisphosphate over acceptor, increased the initial velocity of the reaction (6). This effect can be explained in terms of the multistep mechanism of the reaction:

\[ \begin{align*}
(1) & \quad \text{E} + \text{ATP} \rightleftharpoons \text{EpA} + \text{PPi} \\
(2) & \quad \text{EpA} + \text{pdNp} \rightleftharpoons \text{A-5'pp5'-dNp} + \text{E} \\
(3) & \quad \text{A-5'pp5'-dNp} + \text{dN OH} \rightleftharpoons \text{dN pdNp} + \text{AMP},
\end{align*} \]

where E represents RNA ligase; EpA, adenylylated ligase (13); and dN OH, the acceptor molecule. The final step of the sequence in which the 3'-hydroxyl group of the acceptor displaces AMP from the adenylylated donor (A-5'pp5'-dNp) to form the product is probably rate-limiting since large quantities of the adenylylated donor accumulate in the reaction mixture (6). The final step requires free enzyme (14) which, in the presence of high ATP concentrations, would be decreased in concentration by conversion to its adenylylated form. Thus, high ATP concentrations would inhibit the overall reaction. Conversely, high concentrations of ATP would promote the rate-limiting step by maintaining a high concentration of one of the reactants, the activated donor. We have used a donor:ATP:acceptor ratio of 8:2:1 to balance these ATP concentration effects (6). Table 1.A shows the results of the addition of either pdAp, pdCp, pdGp, pdTp, or pdUp to the labeled acceptor dA(pdA)$_3$[3'-$^{32}$P]pdA using this ratio of reactants. All the donors were added with yields greater than 50% after 192 hr with the exception of pdGp which gave 32% product. These results are similar to those we reported for the addition of these donors to the slightly less efficient acceptor (dT)$_5$ (6).

ATP and donor variation and the use of an ATP regenerating system. In an attempt to enhance yields, ATP and pdGp concentrations were varied in reaction
Table 1. Yields of dA(pdA)$_4$pdNp under various reaction conditions

<table>
<thead>
<tr>
<th>Donor</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>pdAp</td>
<td>75(8)$^a$</td>
<td>93(10)</td>
<td>89(6)</td>
</tr>
<tr>
<td>pdCp</td>
<td>75(1)</td>
<td>95(5)</td>
<td>95(1)</td>
</tr>
<tr>
<td>pdGp</td>
<td>32(8)</td>
<td>93(10)</td>
<td>81(6)</td>
</tr>
<tr>
<td>pdTp</td>
<td>64(8)</td>
<td>75(10)</td>
<td>92(7)</td>
</tr>
<tr>
<td>pdUp</td>
<td>60(8)</td>
<td>85(10)</td>
<td>83(6)</td>
</tr>
</tbody>
</table>

All reactions were performed as described in Materials and Methods with 2.0 mM donor and 0.25 mM $[^{32}P]$dA(pdA)$_4$.

Column A describes reactions in 50 mM HEPES, pH 8.3 using 40 μM RNA ligase and 0.5 mM ATP (no regeneration); Column B lists reactions in 50 mM HEPES, pH 7.9 using 30 μM RNA ligase, 0.10 mM ATP, and a regeneration system of 175 U/ml creatine kinase, 170 U/ml myokinase, and 40 mM phosphocreatine; Column C describes reactions as in B except 1 mM phosphocreatine was used for all donors other than pdGp and 2.0 mM spermine was added to all reactions.

$^a$Numbers in parentheses represent reaction time in days.

mixtures containing HEPES buffer, pH 8.3, 0.25 mM labeled dA(pdA)$_4$, and 40 μM RNA ligase as described in Methods. Although doubling the ATP concentration from 0.5 to 1.0 mM with pdGp at 2.0 mM increased the 228 hr yield of pA(pdA)$_4$pdGp from 32% to 47%, increasing the concentration of pdGp from 2.0 to 10.0 mM with ATP at 0.5 mM decreased both the initial rates and yields of the reaction. The product yields at 228 hr were 33%, 23%, and 15% for pdGp concentrations of 2.0, 5.0, and 10.0 mM. Therefore, a simple increase in reactant concentrations did not suffice to satisfactorily increase yields.

We reasoned that a low but constant ATP concentration might improve both the rate and yield of the reaction by maintaining a higher relative concentration of free enzyme with respect to its adenylylated form. As a result, we investigated the effect of low ATP concentrations in the presence of an ATP regenerating system on the reaction of pdGp with dA(pdA)$_4$. Both maximum yields and initial velocities of dA(pdA)$_4$pdGp formation were achieved using an ATP concentration of 0.08 mM or one-twentieth of the donor concentration (Fig. 1). After 215 hr, 72% of the acceptor had been converted to product at this ratio. Product formed in the absence of added ATP resulted from the
Figure 1. Effect of ATP concentration on the pdGp + dA(pdA)$_4$ reaction using an ATP Regeneration System. Reaction mixtures contained 50 mM HEPES, pH 8.3, 1.67 mM pdGp, 0.21 mM labeled dA(pdA)$_4$, ATP, 33 mM phosphocreatine, 146 U/ml creatine kinase, 142 U/ml myokinase, and 25 μM RNA ligase. A. Effect of ATP concentration on the per cent yield of dA(pdA)$_4$pGp at 143 hr (●) and 215 hr (□). B. Effect of ATP concentration on the initial velocity, $V_i$, of the reaction.

The presence of adenylylated enzyme in this RNA ligase preparation (estimated to be less than 20% of the total enzyme by spectrophotometry). Control tubes containing either no creatine kinase or no myokinase resulted in 240 hr product yields of 6-8%, an amount consistent with the limiting ATP concentration in these reactions. Furthermore, the regeneration system at these concentrations added no detectable DNase activities. These results show that maintaining an ATP concentration at values lower than those necessary for stoichiometric joining of acceptor to donor can increase the yield of the reaction. The yield of 72% product under these conditions contrasts with those of less than 47% obtained in the absence of the regeneration system (Table 1.A and above).

In another experiment the effect of the phosphocreatine concentration was examined. In the reaction of pdNp with labeled dA(pdA)$_4$ as described in Table 1.B, variation of the phosphocreatine concentrations from 1 to 40 mM resulted in little effect on either the initial velocities or final yields of
reactions with the donors pdAp, pd Cp, pdTp, and pdUp. In contrast, the yield with pdGp was strongly dependent upon the phosphocreatine concentration. Up to 40 hr of incubation, the per cent dA(pdA)_pdGp formed varied from only 35% to 44% for phosphocreatine concentrations of 1, 10, and 40 mM. Subsequently, however, the yields of the mixtures with concentrations of 10 and 40 mM increased to 75% and 86% at 200 hr, respectively, while the yield of the mixture containing 1 mM phosphocreatine was less than 50% at this time. The specific stimulatory effect of phosphocreatine for the pdGp addition reaction was investigated in a reaction mixture without the regeneration system. Reaction mixtures containing labeled dA_5 and pdGp as described in Table 1.A, except at pH 7.9, were incubated with and without the addition of 40 mM phosphocreatine. Addition of the phosphagen doubled both the initial velocity and the final yield. This result eliminates the possibility that the phosphocreatine effect was an indirect one acting through the ATP concentration.

An ATP regeneration system with pyruvate kinase (175 U/ml) and PEP (1.0 mM) in place of the phosphocreatine and creatine kinase was also examined in a reaction of labeled dA_5 and pdTp at a ratio of ATP to donor of 0.05. Up to 100 hr this system mimicked the one containing phosphocreatine and creatine kinase but at longer times it did not continue to function as effectively. Furthermore, increasing PEP concentrations to 40 mM had little effect upon the reaction with pdTp but increased yields markedly in a reaction with pdGp as the donor. This result is analogous to the effect observed with increasing phosphocreatine concentrations.

To determine if the stimulatory effect of increasing phosphocreatine or PEP concentration were simply due to increased ionic strength, we added 10 and 40 mM potassium phosphate (pH 7.9) to reaction mixtures of pdGp and dA_5 containing the regeneration system with 1 mM phosphocreatine. These additions resulted in no increase in reaction velocity or product yield, indicating that the stimulatory effect of the phosphagen and PEP could not be replaced by phosphate alone. We have no explanation for this stimulatory effect upon the addition of pdGp to the acceptor.

The ability of the two different ATP regenerating systems to promote the reactions suggests that the primary effect is mediated by the low ATP concentration and not by the added enzymes. Controls in which myokinase or creatine kinase alone were added excluded either as the sole cause of stimulation. Because the phosphocreatine-creatine kinase system gave better sustained stimulations, we have used it in all subsequent reactions.

Table 1.B shows the effect of using the ATP regeneration system on the addition of each of the common deoxyribonucleoside 3',5'-bisphosphate donors
to labeled dA(pdA)$_4$. The final yields were improved with respect to those without the regeneration system (Table 1.A) particularly for pdGp which gave 93% product in 10 days vs. 32%. Under these reaction conditions, pdTp is the poorest donor.

**Optimizing for other variables.** The pH dependence of the reaction of pdTp with dA(pdA)$_4$ in the absence of the ATP regeneration system was determined. Both yields and initial velocities showed a broad optimum centered about pH 8.0 with HEPES being a better buffer than either Tris·HCl or glycylglycine (Fig. 2). Similarly, the use of HEPES, pH 7.9 rather than HEPES, pH 8.3 with the ATP regeneration system resulted in a higher reaction rate and product yield (data not shown).

The effects of Mg$^{++}$ and Mn$^{++}$ concentrations on the reaction of pdTp with labeled dA(pdA)$_4$ in the absence of ATP regeneration were examined. Reaction mixtures were incubated as described in the legend of Table 1.A. using HEPES, pH 7.9 and either 10 mM MgCl$_2$ or 10 mM MnCl$_2$. After 280 hr a yield of 65% was obtained using Mn$^{++}$ and 16% with Mg$^{++}$. These results are similar to those reported previously (6). In addition, only 6% of the acceptor or product was degraded using Mn$^{++}$ while 20% had broken down in the presence of Mg$^{++}$. Metal

![Figure 2. pH dependence of the pdTp + dA(pdA)$_4$ reaction. Reactions were performed as described in Table 1.A. using 30 μM RNA ligase and 50 mM buffer: HEPES (●), TRIS·HCl (■), glycylglycine (▲) and incubated for 186 hr.](image-url)
cation variations done in the presence of the ATP regeneration system gave similar synthetic results with lesser degradation. These findings indicate that the ATP regeneration system can function in the presence of Mn^{++} alone. Since the yields are substantially greater and oligomer degradation is decreased with Mn^{++}, this cation has been used subsequently. One complication with the use of Mn^{++} as the cation arises from the insolubility of manganese pyrophosphate after a few hours at 17°. Its precipitation results in the progressive removal of both the reaction product PPi and the cation. Preliminary results indicate that unadenylylated RNA ligase may be coprecipitating with the manganese pyrophosphate. We are currently examining this phenomenon.

The addition of dimethylsulfoxide in concentrations up to 20% (v/v) inhibited the addition of pdGp to dA(pdA)_4 in both the presence and absence of the regeneration system. This effect is opposite to that reported for the single nucleotide addition reaction with ribonucleotide substrates (15,16) and the single-strand deoxyoligomer joining reaction (McCoy and Gumport, manuscript in preparation).

Because polyamines are known to interact with oligodeoxyribonucleotides (17), we examined the effect of spermine, spermidine, and putrescine on the addition of pdTp to dA(pdA)_4 in the presence of the ATP regeneration system (Table 2). Spermine at 2.0 mM or 5.0 mM greatly enhanced both the rate and yield resulting in greater than 90% product formation in 180 hr. Spermidine and putrescine exhibited similar but lesser effects. Since spermine, the most highly charged polyamine tested, gave the greatest stimulation, polymin P (a polyethylenimine) was also tested but showed little effect. We determined that the stimulatory effect of spermine was not simply replacement of the metal cation in the reaction. Substituting spermine for Mn^{++} in the presence of Mg^{++} (to allow ATP regeneration), greatly depressed the rate and yield.

Because of its ability to bind single strand oligodeoxyribonucleotides (18), RNase A was also tested (Table 2). Under these conditions, the addition of 3.7 mg/ml RNase A represented a one-to-one molar ratio of RNase A to dA(pdA)_4 acceptor. Both the rate and yield of the reaction were stimulated with a 90% yield in 180 hr. RNase A to dA(pdA)_4 ratios of 1:2 and 1:5 (1.75 and 0.72 mg/ml RNase A) did not affect initial velocities but did cause modest improvements in yields. The addition of both 2.0 mM spermine and 3.7 mg/ml RNase A to the pdTp and dA(pdA)_4 reaction resulted in an increase in the initial velocity over that obtained by either component alone. A yield of 90% product was obtained in 144 hr. Ethanol, the detergent Triton X-100, glass beads, and T4 or E. coli DNA ligase were tested, but they had little effect on the reaction (data not shown).
Table 2. Effect of various components on the reaction of pdTp and dA(pdA)₄

<table>
<thead>
<tr>
<th>Added Component</th>
<th>initial velocity (pmol/hr)</th>
<th>product yield at 180 hr (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>26</td>
<td>70</td>
</tr>
<tr>
<td>spermine (0.10 mM)</td>
<td>32</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>(0.25 mM)</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>(2.0 mM)</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>(5.0 mM)</td>
<td>39</td>
</tr>
<tr>
<td>spermidine (2.0 mM)</td>
<td>39</td>
<td>85</td>
</tr>
<tr>
<td>putrescine (2.0 mM)</td>
<td>30</td>
<td>73</td>
</tr>
<tr>
<td>polymine (0.01% v/v)</td>
<td>27</td>
<td>65</td>
</tr>
<tr>
<td>spermine (2.0 mM) +</td>
<td>7</td>
<td>30</td>
</tr>
<tr>
<td>10 mM Mg²⁺ minus Mn²⁺</td>
<td>24</td>
<td>75</td>
</tr>
<tr>
<td>RNase A (0.72 mg/ml)</td>
<td>28</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>(1.75 mg/ml)</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>(3.7 mg/ml)</td>
<td>90</td>
</tr>
<tr>
<td>spermine (2.0 mM) +</td>
<td>56</td>
<td>90</td>
</tr>
<tr>
<td>RNase A (3.7 mg/ml)</td>
<td></td>
<td></td>
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</tbody>
</table>

Reactions were performed as described in Materials and Methods. Reaction mixtures contained 2.0 mM pdTp, 0.25 mM [³²P]dA(pdA)₄, 0.10 mM ATP, 50 mM HEPES, pH 7.9, 30 µM RNA ligase, 10 mM MnCl₂, 20 mM DTT, 10 µg/ml BSA, and an ATP regeneration system of 1.0 mM phosphocreatine, 175 U/ml creatine kinase, and 170 U/ml myokinase.

The results in Table 2 show that spermine and RNase A can be used to enhance the rates and yields of the reaction. In addition, the reactions of 1) pdTp and dG(pdA)₂pdU, 2) pdGp and dT(pdT)₂pdG, 3) pdTp and dT(pdT)₇pdG, and 4) pdG(pdA)₂pdUpdTₗ and dT(pdT)₇pdG have also been stimulated by the addition of spermine (unpublished observations). Table 1.C shows that the addition of 2 mM spermine raises the velocity of the reaction with all the donors in the presence of the ATP regeneration system (compare 1.B and 1.C). Figure 3 compares the use of these optimum conditions for the addition of the best single deoxyribonucleoside bisphosphate donor, pdCp, and one of the poorest, pdTp. After incubation for 24 hr, the reaction of dA₅ with pdCp results in 95% of the dA₅ acceptor being converted to the product dA(pdA)₄pdCp. No nuclease activity is evident as demonstrated by the lack of radioactivity comigrating with pdA. The reaction of dA₅ with the poorer donor pdTp results in only 40% product after 24 hr, but a conversion of 92% of the dA₅ to product.
Figure 3. Paper chromatography of the single nucleotide addition reaction. Reactions were performed as described in Table 1.C. and reaction aliquots resolved by chromatography on Whatman No. 1 paper in solvent II. A. Reaction of pdCp with labeled dA(pdA)₄ after 24 hr incubation. B. Reaction of pdTp with labeled dA(pdA)₄ after 24 hr incubation. C. As in B, but after 177 hr incubation.

is observed after 177 hr. Again, no nuclease activity is detected. These results demonstrate that the conditions reported here allow for the efficient addition of single deoxyribonucleoside bisphosphates to oligodeoxyribonucleotides with no detectable nuclease degradation of either substrates or products.

Summary. In order to use RNA ligase for the synthesis of oligodeoxyribonucleotides of reasonable length it is necessary to have high yields at each individual nucleotide addition step. The conditions reported here allow the addition of any common deoxyribonucleoside bisphosphate to the oligodeoxyribonucleotide dA(pdA)₄ with yields greater than 85%. Because previous work (6) has shown no dramatic base specificity of DNA acceptors in the single nucleotide addition reaction of RNA ligase, we believe that these conditions can probably be extended to any oligodeoxyribonucleotide acceptor. We have begun
using these conditions for syntheses with acceptors of various base compositions (dG and dU terminated) and have observed results similar to those reported here using concentrations of 1 mM acceptor, 8 mM donor, and 0.4 mM ATP. The single nucleotide addition reaction conditions reported here enhance the usefulness of RNA ligase as a reagent for the stepwise enzymatic synthesis of oligodeoxyribonucleotides.

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