# Studies on Polynucleotides. LIII. Syntheses of Trinucleotides Containing Adenylate and 2′-Deoxyadenylate Units and a Study of Their Effect on the Binding of Lysyl Soluble Ribonucleic Acid to Ribosomes*

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ABSTRACT: Chemical syntheses of the following five trinucleoside diphosphates containing adenylate and 2′-deoxyadenylate units are described: ApdApdA, dApdApA, ApdApA, ApApdA, dApApA. These oligonucleotides were tested for their ability to stimulate the binding of $^{14}$C-lysyl soluble ribonucleic acid (s-RNA) to ribosomes.

Four of the oligonucleotides stimulated the binding, the stimulation being in the range of 50–70% of that given by the control ApApA. dApdApA, which was inactive at the standard (0.01–0.02 M) $Mg^{2+}$ ion concentrations used in the binding experiments, also stimulated the binding of lysyl s-RNA at higher divalent ion concentration.

Denatured deoxyribonucleic acid (DNA) has been shown to bind to ribosomes (Takanami and Okamoto, 1963). However, in studies of the stimulation of the binding of aminoacyl soluble ribonucleic acid (s-RNA) to ribosomes by oligonucleotides, deoxyribooligonucleotides were found to be inactive (Nirenberg and Leder, 1964). Thus, *e.g.*, the ribotrinucleotide, ApApA, stimulated the binding of $^{14}$C-lysyl-s-RNA to ribosomes, whereas the deoxyribooligonucleotide, $d(pA)_8$, failed to show any effect. It was of interest to determine whether mixed oligonucleotides containing both ribo- and deoxyribonucleotide units would mediate the complex formation between the aminoacyl-s-RNA and ribosomes. It seemed possible, *e.g.*, that the complex formation required only one or the other terminal unit of a trinucleotide to be a ribonucleotide, whereas the remaining two units could be deoxyribose counterparts. If this were so, then in the problem of the deciphering of the codon sequences for different amino acids by this technique (Nirenberg and Leder, 1964) specific oligo- and polynucleotides containing predominantly deoxyribonucleotide units would be adequate. This problem was expected to be somewhat simpler than the synthesis of ribopolynucleotides because the synthetic methodology in the deoxyribopolynucleotide field was further advanced than in the ribose series at the time the present work was undertaken.[1]

The present work describes the synthesis of five of the possible six trinucleoside diphosphate (I–V)[2] containing adenylate and 2′-deoxyadenylate units. Furthermore, tests of their ability to stimulate the binding of $^{14}$C-lysyl-s-RNA to ribosomes are reported.[3] Prior to this work, no oligonucleotide consisting of both ribo- and deoxyribonucleotide units has been described, and it is clear that oligonucleotides of this general type are of much wider interest for studies of the nucleic acids. Their availability, *e.g.*, would facilitate further studies

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[1] In the meantime the syntheses of all of the possible 64 ribotrinucleotides derived from the four major ribonucleotides by purely chemical methods have been satisfactorily accomplished (Lohrmann *et al.*, 1966).

[2] For the general system of diagrammatic representations of oligonucleotides see Khorana (1961). In the diagrammatic representations of protected nucleosides and nucleotides, the benzoyl group on the adenine ring is shown by adding Bz after the initial A and the protecting groups on the sugar hydroxyl groups are indicated by OAc (for acetyl group) and OBz (for benzoyl group). DMTr is abbreviation for the dimethoxytrityl group. The general system of abbreviations is that as currently used in the *Journal of Biological Chemistry*. In the specification of the mixed oligonucleotides, deoxyadenosine units are designated by adding the letter d immediately before the nucleoside initial A. For designating the protected oligonucleotides (*e.g.*, those in Table I), the system used is that which has been developed recently (Lohrmann *et al.*, 1965). Thus, the benzoyl groups on the adenine ring are designated by the superscript added to the nucleoside initial A. Protecting groups on the 2′ and 3′-hydroxyl groups are shown by adding the abbreviations OBz or OAc to the right of the nucleoside initial. The 5′-hydroxyl group is represented by adding OH- to the left of the nucleoside initial, or, if acylated, by adding AcO- or BzO- in this position.

[3] Since this work was completed, McCarthy and Holland (1965) have demonstrated the ability of denatured deoxyribonucleic acid (DNA) to stimulate the incorporation of amino acids into acid-insoluble material in a cell-free amino acid incorporating system. The presence of antibiotics such as streptomycin is required for this stimulation.

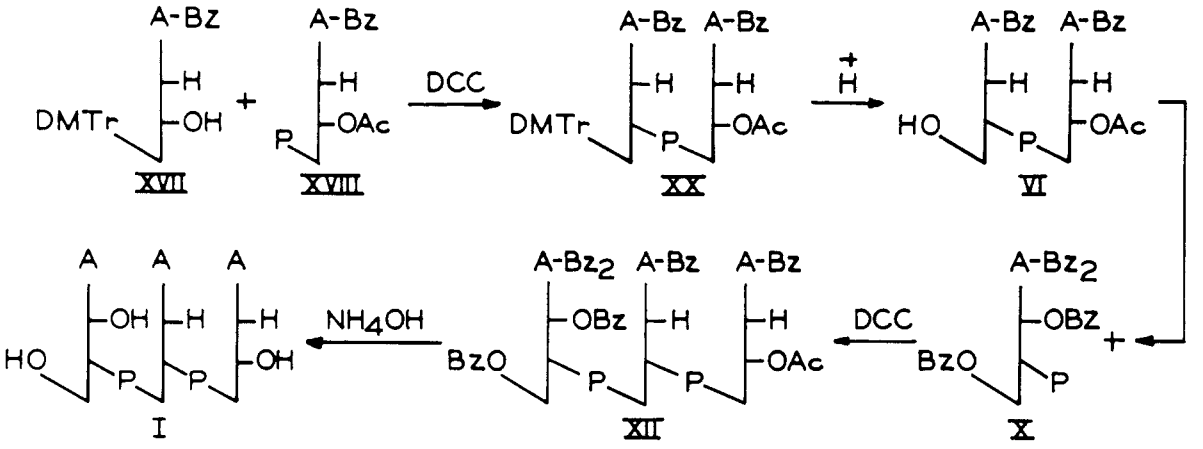


FIGURE 1: Steps in the synthesis of ApdApdA (I).

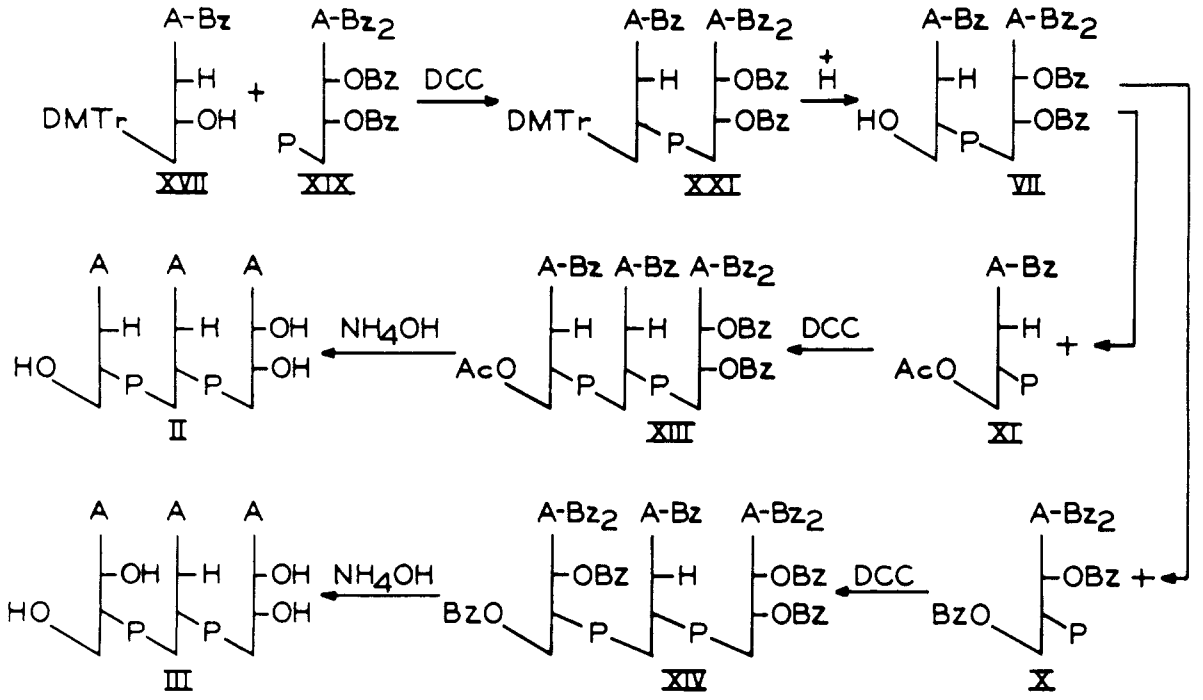


FIGURE 2: Steps in the synthesis of dApdApA (II) and of ApdApA (III).

of the specificities of a variety of nucleases and of other enzymes of nucleic acid metabolism.

*Synthesis of Trinucleoside Diphosphates.* The methods used in the stepwise synthesis of the five oligonucleotide (I–V) are illustrated in Figures 1–4.[2] It is seen that in all cases, the first steps involved the synthesis of suitably protected dinucleoside phosphates bearing only the 5′-hydroxyl end group free (VI–IX). Each one of the latter type of intermediate was condensed with suitably protected adenosine 3′-phosphate (X) or deoxyadenosine 3′-phosphate (XI) to give the trinucleoside diphosphates in the totally protected form (XII–XVI). The condensing agent used throughout was dicyclohexylcarbodiimide (DCC).

The protected dinucleoside phosphates themselves (VI–IX) were prepared by either of two routes depending on whether the left hand terminus carrying the free 5′-hydroxyl group was a ribonucleoside or a deoxyribonucleoside unit. Thus, the synthesis of the intermediates VI and VII involved the condensation of 5′-*O*-dimethoxytrityl-*N*-benzoyldeoxyadenosine (XVII) with protected deoxyadenosine 5′-phosphate (XVIII) and protected adenosine 5′-phosphate (XIX), respectively. The fully protected dinucleoside phosphates XX and XXI were thus obtained. On the other hand, the synthesis of the intermediates VIII and IX involved the condensation of 5′-*O*-dimethoxytrityl-*N*,*N*,$O^2$′-tribenzoyladenosine 3′-phosphate (XXII) with, re-

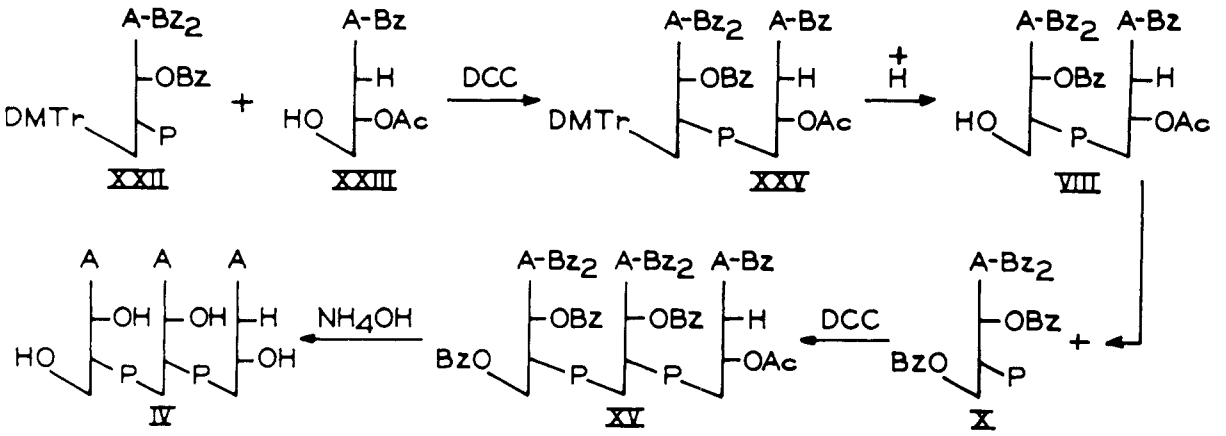


FIGURE 3: Steps in the synthesis of ApApdA (IV).

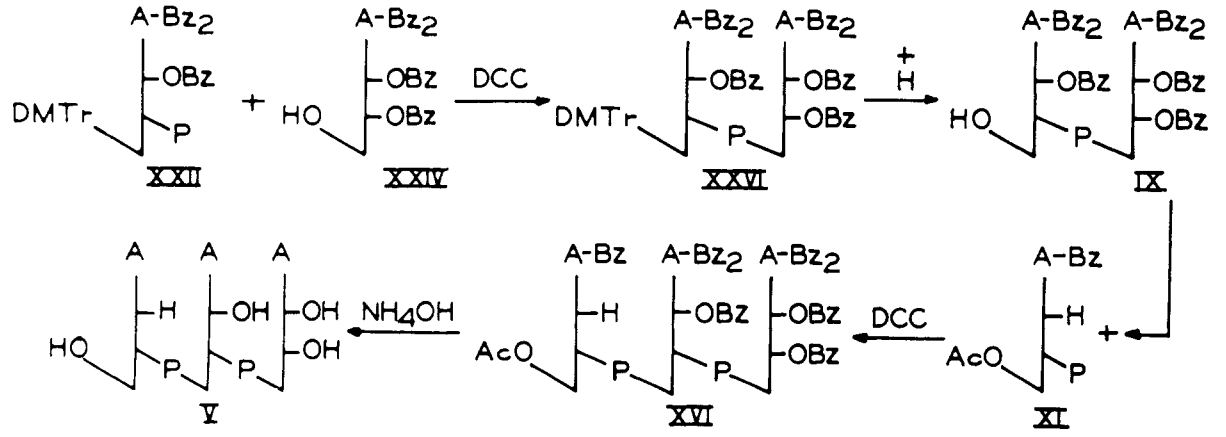


FIGURE 4: Steps in the synthesis of dApApA (V).

TABLE I: Experimental Conditions for the Synthesis of Trinucleoside Diphosphates.

| Oligonucleotide | Protected Dinucleoside Phosphate[a] | Amount Used ($\mu$moles) | Protected Mononucleotide[a] | M Equiv of Mono-nucleotide | Yield (%) |
|---|---|---|---|---|---|
| ApdApdA | HO-dA$^{Bz}$-p-dA$^{Bz}$-OAc (VI) | 8 | BzO-A$^{Bz_2}$-OBz-p | 7 | 45 |
| dApdApA | HO-dA$^{Bz}$-p-A$^{Bz_2}$-(OBz)$_2$ (VII) | 10 | Ac-O-dA$^{Bz}$-p | 2 | 21 |
| ApdApA | HO-dA$^{Bz}$-p-A$^{Bz_2}$-(OBz)$_2$ (VII) | 12 | BzO-A$^{Bz_2}$-OBz-p | 4 | 14 |
| ApApdA | HO-A$^{Bz_2}$-OBz-p-dA$^{Bz}$-OAc (VIII) | 10 | BzO-A$^{Bz_2}$-OBz-p | 6 | 41 |
| dApApA | HO-A$^{Bz_2}$-OBz-p-A$^{Bz_2}$-(OBz)$_2$ (IX) | 10 | AcO-dA$^{Bz}$-p | 3 | 35 |

[a] See footnote 2 in text.

spectively, *N*-benzoyl-3′-*O*-acetyldeoxyadenosine (XXIII) and *N*,*N*,*O*$^{2'}$,*O*$^{3'}$-tetrabenzoyladenosine (XXIV). The fully protected dinucleoside phosphates XXV and XXVI were thus obtained. The removal of the dimethoxytrityl group in the fully protected dinucleoside phosphates (XX, XXI, XXV, XXVI) was effected by brief treatment with 80% acetic acid. Except for the case of IX where both of the glycosyl bonds were completely stable to this treatment, considerable amount of depurination was observed, the presence of *N*-benzoyl group on the adenine ring increasing the sensitivity of the purine deoxyglycosyl bond. The resulting products (VI–IX) were all purified by chromatography on diethylaminoethyl- (DEAE-) cellulose columns.

Following condensations of the protected dinucleoside phosphates (VI–IX) with protected mononucleotides (X or XI) (Table I) the protecting groups were all removed by an ammoniacal treatment and the trinucleoside diphosphates were purified by preparative paper chromatography or, in two cases, by a combination of paper chromatography and paper electrophoresis. The yields of the purified products are recorded in Table I. All of the trinucleoside diphosphates (I–V) thus obtained were ascertained to be pure by extensive paper chromatography (four solvents) and by paper electrophoresis. The mobilities of the synthetic products are compiled in Table II. Further characterization was accomplished by the results of enzymic degradation. All the products were completely susceptible to the action of spleen and venom phosphodiesterases and the expected mononucleotides and nucleosides were liberated in the correct proportions. The results of these degradations are given in Tables III and IV.

TABLE II: Paper Chromatography of Trinucleoside Diphosphates Containing Adenylate and Deoxyadenylate Units.

| Compound | Solvent A ($R_F$ Relative to Ap) | Solvent B ($R_F$ Relative to Ap)[a] | Solvent C ($R_F$ Relative to Ap) | Solvent D ($R_F$ Relative to Ap) |
|---|---|---|---|---|
| pA | 0.57 | 0.17 | 0.87 | 0.89 |
| dApdA, ApdA | 2.5 | | 1.39 | |
| dApA, ApA | 1.74 | | 1.20 | 1.61 |
| ApApdA, ApdApdA | 1.0 | 0.92 | 1.00 | 0.73 |
| ApdApA, dApdApA, dApApA, ApApA | 0.57 | 0.17 | 0.87 | 0.58 |

[a] Chromatography was carried out for 36 hr.

TABLE III: Enzymic Degradation of Trinucleotides Using Venom Phosphodiesterase.[a]

| Compound | Quantity Degraded ($OD_{259}$ Units) | Products Formed | Ratio of Products |
|---|---|---|---|
| dApApA | 5.15 | dA,pA | dA:pA = 1:2.06 |
| ApdApA | 8.05 | A,dpA,pA | A:dpA:pA = 1:1.16:1.09 |
| ApApdA | 3.03 | A,pA,dpA | A:pA:dpA = 1:1.14:1.23 |
| ApdApdA | 6.21 | A,dpA | A:dpA = 1:2.09 |

[a] For details see text.

TABLE IV: Enzymic Degradation of Trinucleotides Using Spleen Phosphodiesterase.

| Compound | Quantity Degraded ($OD_{259}$ Units) | Found, Ratio of Nucleotide:Nucleoside[a,b] (Theoretical 2:1) |
|---|---|---|
| dApdApA | 4.2 | dAp:A = 2.36:1 |
| dApApA[c] | 3.9 | dAp + Ap:A = 2.14:1 |
| ApApdA | 6.9 | Ap:dA = 2.18:1 |
| ApApA | 2.7 | Ap:A = 2.28:1 |

[a] The solvent system used for separation (solvent A) did not separate Ap from dAp, although A and dA were clearly resolved. [b] A deaminase present in the enzyme preparation converted about 30% of A and dA into inosine and deoxyinosine, respectively. The inosine spot was eluted and its absorption at 249 mμ measured and this was allowed for in the calculation of the nucleoside:nucleotide ratio. [c] Of this product 4% was resistant to spleen diesterase, appearing as ApA.

*Results of Binding Experiments and Discussion.* Figure 5 shows the results of the experiments carried out to test the stimulation of the binding to ribosomes of $^{14}$C-lysyl-s-RNA. In these experiments the stimulation of binding was measured as a function of trinucleotide concentration using 0.02 M $Mg^{2+}$ ion concentration and 25°. Figures 6 and 7 show the kinetics of the binding of lysyl-s-RNA to ribosomes as a function of time at 25 and 0°. The results of Figure 5–7 show that the saturation levels and the rate of the complex formation are similar in all cases. Four of the mixed trinucleoside diphosphates caused stimulation of the binding, although to a lesser extent than ApApA. It is interesting that, of the two trinucleoside diphosphates, dApdApA and ApdApdA, which contain only one ribonucleoside unit, the former was inactive while the latter carrying a ribonucleoside unit at the 5′-hydroxyl terminus significantly stimulated the complex formation. Thus, the left hand terminus would appear to be more important in the process of complex formation. However, the presence of the ribonucleoside unit at this terminus was not essential for the activity since dApApA gave good (about 60% of that given by ApApA) response in the reaction. The second trinucleoside diphosphate, ApdApA, which again contained two ribonucleoside units, was also quite effective. An experiment was carried out to test the influence of the magnesium ion concentration on the stimulation of the binding and the results are

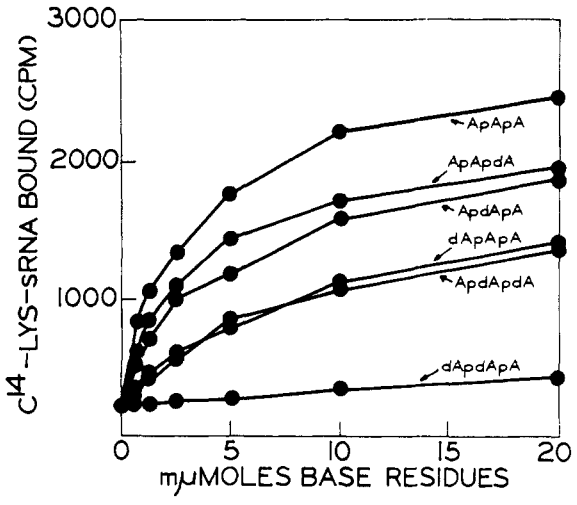


FIGURE 5: Stimulation by trinucleoside diphosphates of the binding of $^{14}$C-lysyl-s-RNA to ribosomes. Each reaction mixture contained 6740 cpm of unfractionated $^{14}$C-lysyl-s-RNA (0.7 OD unit at 260 mμ), ribosomes (1.0 OD unit), and the trinucleoside diphosphate in a total volume of 0.05 ml. The concentration of $Mg^{2+}$ ions was 0.02 M. The incubation was at 25° for 20 min.

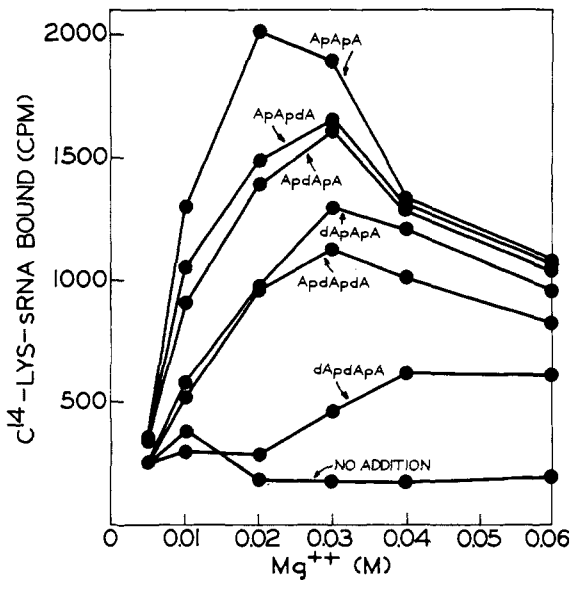


FIGURE 6: The effect of magnesium ion concentration on the stimulation of the binding of $^{14}$C-lysyl-s-RNA to ribosomes by trinucleotides The incubation mixtures contained, in a final volume of 0.05 ml, 20 mμmoles of base residues of trinucleotide, 6740 cpm of unfractionated $^{14}$C-lysyl-s-RNA, ribosomes (1.0 OD), and $MgCl_2$. The incubation was for 20 min at 25°.

shown in Figure 8. While in most of the present experiments, 0.02–0.03 M $Mg^{2+}$ ion concentration was optimal for the complex formation, with dApdApA significant stimulation was observed only at or above 0.04 M divalent ion concentration.

It would appear that while single-stranded deoxyribopolynucleotides have affinity for the ribosomes and similarly complex formation between complementary regions of s-RNA and deoxyribopolynucleotides would also occur, the formation of the ternary complex (messenger, aminoacyl-s-RNA, and ribosome) requires the attainment of a suitable three-dimensional conformation. The present results show that the nature of the sugar moiety (ribose *vs.* deoxyribose) in the nucleoside units plays a very significant role in the attainment of the required conformation. It is worthy of note that in the presence of antibiotics single-stranded deoxyribopolynucleotides bring about amino acid incorporation in the cell-free system (McCarthy and Holland, 1965), and it would be highly interesting to study the effects of the selected antibiotics on the formation of aminoacyl-s-RNA–ribosome complex in the presence of synthetic deoxyribopolynucleotides and of the mixed deoxyribo- and ribooligonucleotides described in the present paper.

## Experimental Section

*Materials and General Methods.* The preparation of $^{14}$C-lysyl-s-RNA and ribosomes has been described previously (Nishimura *et al.*, 1965). For assay of $^{14}$C-lysyl-s-RNA binding to ribosomes the assay procedure described by Nirenberg and Leder (1964) was used. The reaction mixture (0.05 ml) contained 0.1 M Tris–HCl pH 7.5, 0.05 M KCl, 1.0 $OD_{260}$ unit[4] of ribosomes, 0.005–0.06 M magnesium acetate, 6740 cpm $^{14}$C-lysyl-s-RNA (0.7 $OD_{260}$ unit). Incubation was carried out for 20 min at 25° unless otherwise stated.

Paper chromatography was performed by the descending technique using Whatman No. 1 paper. The solvent systems used were solvent A, isopropyl alcohol–concentrated ammonia–water (7:1:2); solvent B, isopropyl alcohol–concentrated ammonia–0.1 M boric acid (7:1:2); solvent C, 1-propanol–concentrated ammonia–water (55:10:35); solvent D, 1 M ammonium acetate (pH 7.5)–ethanol (3:7). Preparative paper electrophoresis was carried out using Whatman No. 1 paper and a buffer of 0.05 M ammonium acetate (pH 7.5), in an apparatus similar to that described by Markham and Smith (1952). Analytical paper electrophoresis was carried out using Whatman 3MM paper and 0.03 M phosphate buffer (pH 7.1) in a commercially available apparatus at 4000 v across the length of the standard Whatman 3MM paper sheet. Silica gel G was used for thin layer chromatography.

Degradations of oligonucleotides with spleen phosphodiesterase and venom phosphodiesterase were per-

[4] The abbreviation OD refers to the extinction of a nucleotidic or polynucleotidic solution at neutral pH in a 1-ml solution using a 1-cm light path quartz cell. The wavelength at which the extinction is measured is indicated by the subscript after the abbreviation OD.

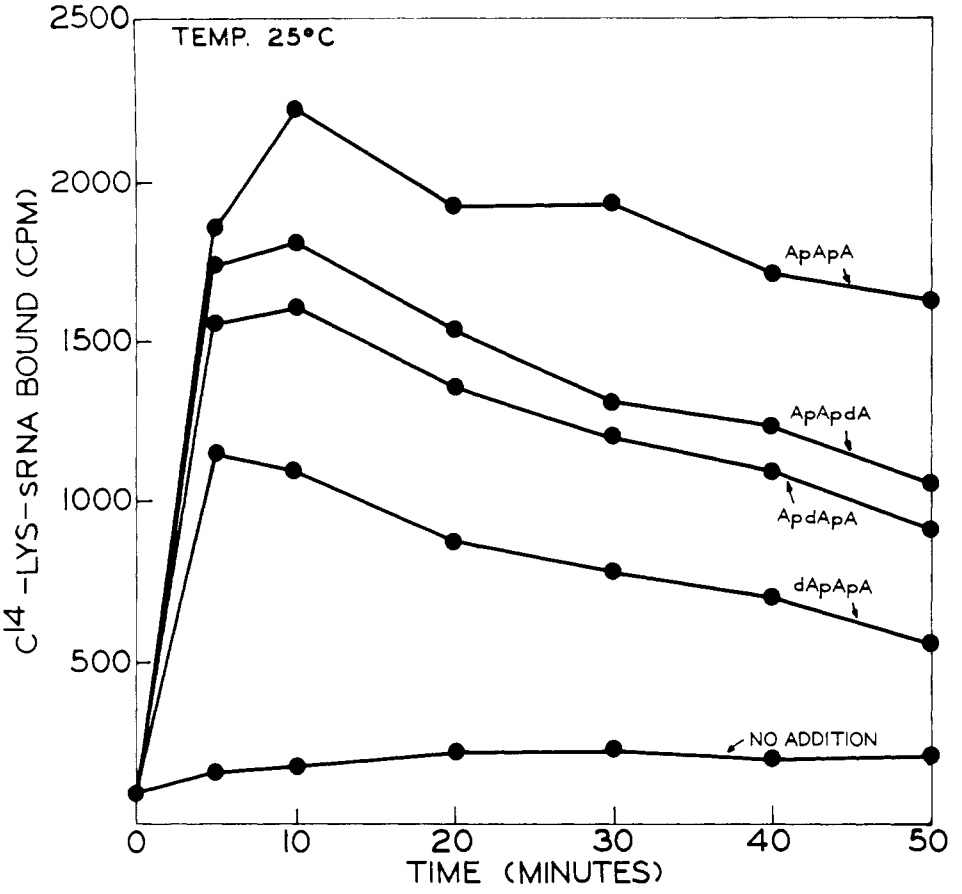


FIGURE 7: Kinetics of the trinucleotide-stimulated binding of $^{14}$C-lysyl-s-RNA to ribosomes at 25°. Each reaction mixture contained the trinucleotide (20 mμmoles of base residues), unfractionated $^{14}$C-lysyl-s-RNA (6740 cpm), ribosomes 1.0 OD, in a final volume of 0.05 ml. The magnesium ion concentration was 0.02 M.

formed as described previously (Khorana *et al.*, 1961). The products of enzymic degradation were separated by paper chromatography in solvent A, and the products were eluted with water and adjusted to pH 7.5 with 0.05 M phosphate buffer. The four possible products (dA, A, dpA, and pA) of venom phosphodiesterase action on the oligonucleotides were completely separated from each other in the solvent system. The nucleotides dAp and Ap formed by action of spleen phosphodiesterase on the oligonucleotides traveled as a single band in solvent A, which was again used for analysis of the products of enzyme degradation.

*Pyridinium 5'-O-Acetyl-N-benzoyldeoxyadenosine 3'-Phosphate (XI).* An aqueous solution of the triethylammonium salt of 5'-*O*-dimethoxytrityl-*N*-benzoyldeoxyadenosine 3'-phosphate (2300 $OD_{282}$ units)[4] (Schaller *et al.*, 1963) was lyophilized and the product was dissolved in 80% acetic acid (10 ml). After 8 min at room temperature the solvent was evaporated over a period of 4 min. Pyridine was added and the mixture was evaporated to a gum, dissolved in water (5 ml), and applied to the top of a DEAE-cellulose column (50 cm × 1 cm, bicarbonate form). The column was eluted with a linear salt gradient (2 l. of water in the mixing vessel and 2 l. of 0.3 M ammonium bicarbonate in the reservoir), fractions of 10 ml being collected in 20 min. Pure *N*-benzoyldeoxyadenosine 3'-phosphate (1850 $OD_{282}$ units)[4] was eluted as the sole product in fractions 67–81 at 0.06 M salt concentration. The product was lyophilized to remove ammonium bicarbonate and converted to the pyridinium salt by passage through a column of Dowex-50 pyridinium ion-exchange resin. The column washings were lyophilized, dissolved in anhydrous pyridine (5 ml), and treated with acetic anhydride (0.5 ml) for 8 hr. Water (20 ml) was then added to the mixture, and the product was evaporated to a gum and rendered anhydrous by repeated evaporation of added pyridine. The residual gum was dissolved in pyridine (1 ml) and precipitated in ether (40 ml) to afford 5'-*O*-acetyl-*N*-benzoyldeoxyadenosine 3'-phosphate (46 mg; $R_F$ in solvent D, 0.62). This was contaminated with a small amount (about 5%) of the corresponding pyrophosphate, $P^1$,$P^2$-di(5'-*O*-acetyl-*N*-benzoyldeoxyadenosine) 3'-pyrophosphate ($R_F$ 0.72 in solvent D).

*3'-O-Acetyl-N-benzoyldeoxyadenosine (XXIII).* 5'-*O*-dimethoxytrityl-*N*-benzoyldeoxyadenosine (DMTr-$dA^{Bz}$) (250 mg) (Schaller *et al.*, 1963) was treated with

acetic anhydride (2.0 ml) in anhydrous pyridine (5 ml) for 8 hr at room temperature, after which time ethanol (2 ml) was added to destroy excess acetic anhydride. The product was evaporated to a gum, dissolved in anhydrous pyridine, and precipitated from ether. Thin layer chromatography showed two trityl-containing products ($R_F$ 0.69 and 0.78 compared with an $R_F$ of 0.57 for DMTr-$A^{Bz}$, benzene, 20% alcohol being used to develop the plate) which were presumed to be 3′-*O*-acetylated products bearing one and two protecting groups on the adenine ring. As these could not be separated by silica gel column chromatography the product was treated with 80% acetic acid for 8 min. The acetic acid was then evaporated under reduced pressure over a period of 4 min and pyridine was immediately added to the residue. The pyridine was removed and the product was dissolved in chloroform and applied to a silica gel column (1 × 30 cm) which was eluted successively with chloroform (200 ml), chloroform–10% ethanol (200 ml), and chloroform–20% ethanol (200 ml), 10-ml fractions being collected every 10 min. The mixture of 3′-*O*-acetyl-*N*-benzoyldeoxyadenosine and, presumably, 3′-*O*-acetyl-*N*-acetyl-*N*-benzoyldeoxyadenosine was eluted from the column with the last solvent. The fractions were examined by thin layer chromatography and those containing the required product were pooled and evaporated. A pyridine solution of the residue was precipitated in petroleum ether to yield 105 mg (70%) of the protected nucleoside ($R_F$ 0.40 and 0.53 on thin layer chromatography using benzene–20% alcohol to develop the plate).

*Pyridinium N,N′,O²′,O³′-Tetrabenzoyladenosine 5′-Phosphate (XIX).* A mixture of adenosine 5′-phosphate (710 mg, 2 mmoles) and benzoyl chloride (5.6 g, 40 mmoles) in anhydrous pyridine (20 ml) was allowed to stand at room temperature for 2 hr, and the products were then partitioned between chloroform (50 ml) and water (50 ml). The chloroform layer was washed with water, evaporated, and rendered anhydrous by evaporation of pyridine. The residue was dissolved in anhydrous pyridine (10 ml) and precipitated in cyclohexane (400 ml). The gummy precipitate was redissolved in pyridine and added to an excess of cyclohexane to yield 1.7 g of a solid powder. This was dissolved in pyridine (20 ml) and acetic anhydride (5 ml) and left overnight. Aqueous pyridine (150 ml of 50%) was then added and the mixture was left at room temperature for 4 hr. The mixture was then evaporated and rendered anhydrous, and a solution of the product in anhydrous pyridine was precipitated in cyclohexane to afford 1.1 g of *N,N′,O²′,O³′*-tetrabenzoyladenosine 5′-phosphate. In solvent D, this product had $R_F$ 0.67, and on brief alkaline treatment was converted to *N*-benzoyladenosine 5′-phosphate which had an $R_F$ of 0.36 in the same solvent.

*Pyridinium 5′-O-Dimethoxytrityl-N,N′,O″-tribenzoyladenosine 3′-Phosphate (XXII).* Dry adenosine 3′-phosphate (356 mg, 1 mmole) in 40 ml of anhydrous pyridine was treated with dimethoxytrityl chloride (1.7 g, 5 mmoles) for 45 min at room temperature (Lapidot and Khorana, 1963). Ammonium hydroxide solution (10 ml of 1 M) was added and the mixture was

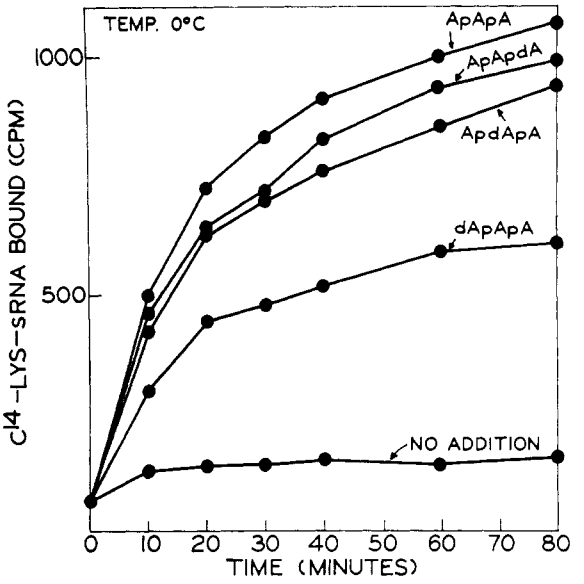


FIGURE 8: Kinetics of the trinucleotide stimulated binding of $^{14}$C-lysyl-s-RNA to ribosomes at 0°. Other details are as in Figure 3.

evaporated to a gum and dissolved in 25 ml of water. After ether extraction the aqueous layer was applied to the top of a DEAE-cellulose column (40 cm × 3 cm, bicarbonate form), and the column was eluted with a linear salt gradient (4 l. of water in the mixing vessel with 4 l. of 0.3 M triethylammonium bicarbonate in the reservoir); fractions of 20 ml were collected in 10 min. The unchanged mononucleotide was eluted at 0.1 M salt concentration, at which time 260 ml of ethanol and 520 ml of ethanol were added to the mixing vessel and reservoir, respectively. The product, 5′-*O*-dimethoxytrityladenosine 3′-phosphate (7800 $OD_{258}$ units), was eluted in fractions 200–300, and was pure by paper chromatography ($R_F$ in solvent A, 0.53). The pooled fractions were evaporated and rendered free of salt by repeated evaporation of added pyridine. Tetraethylammonium benzoate (5 mmoles) was added to the residue which was rendered anhydrous by evaporation of pyridine. Pyridine was removed by addition and evaporation of three 10-ml portions of benzene. Benzoic anhydride (3 g) was added to the residual gum and after thorough mixing the syrup was allowed to stand at room temperature for 2 days. Benzoyl chloride (5 ml) and pyridine (10 ml) were then added to ensure complete benzoylation of the nucleotide (Lohrmann *et al.*, 1966). After 2 hr the mixture was partitioned between chloroform and water. The chloroform layer was washed with water and evaporated and the residue was rendered anhydrous, dissolved in pyridine (10 ml), and precipitated in cyclohexane (200 ml). The precipitation step was repeated and the solid product thus obtained was then dissolved in pyridine (10 ml) and treated with acetic anhydride (4 ml) overnight. Water (10 ml) was added and the mixture was allowed to stand for 4 hr

at room temperature. The product was passed through a column of Dowex-50 pyridinium resin, the column washings were rendered anhydrous by addition and evaporation of pyridine, and the product, 5′-*O*-dimethoxytrityl-*N*,*N*′,*O*$^{2'}$-tribenzoyladenosine 3′-phosphate, was dissolved in pyridine and precipitated in ether. It was dissolved in purified dioxane containing a trace of pyridine and stored at −20°. On treatment with methanolic ammonia, it was converted to 5′-*O*-dimethoxytrityladenosine 3′-phosphate ($R_F$ in solvent A, 0.53).

*N-Benzoyldeoxyadenylyl-(3′→5′)-3′-O-acetyl-N-benzoyldeoxyadenosine (HO-dA$^{Bz}$-p-dA$^{Bz}$-OAc) (VI).* An anhydrous mixture of 5′-*O*-dimethoxytrityl-*N*-benzoyladenosine (50 mg), 3′-*O*-acetyl-*N*-benzoyldeoxyadenosine 5′-phosphate (50 mg), Dowex-50 pyridinium resin (100 mg), and dicyclohexylcarbodiimide (DCC)[5] (0.4 g) in pyridine (1 ml) was shaken at room temperature for 3 days. After addition of (50%) aqueous pyridine (10 ml) the solution was extracted with petroleum ether and filtered. It was evaporated to a gum and treated with 80% acetic acid for 8 min after which time the acetic acid was rapidly removed and the product was dissolved in ethanol–pyridine and applied to the top of a DEAE-cellulose column (50 cm × 3 cm, acetate form) and eluted at 0° with a linear salt gradient (2 l. of 95% ethanol in the mixing vessel and 2 l. of 0.1 M triethylammonium acetate in 95% ethanol in the reservoir); 15-ml fractions were collected every 10 min. The product was eluted from the column in fractions 171–187 and amounted to 450 $OD_{282}$ units. The fractions were pooled and evaporated in the presence of pyridine and an anhydrous pyridine solution of the product was precipitated in petroleum ether. The precipitate was dissolved in aqueous pyridine and passed through a column of Dowex-50 pyridinium resin. The column washings were lyophilized to yield 15 mg of the protected dinucleoside phosphate. When an aliquot was hydrolyzed with concentrated ammonia, dApdA was obtained as the sole product.

*Pyridinium N-Benzoyldeoxyadenylyl-(3′→5′)-N,N′,O*$^{2'}$*,O*$^{3'}$*-tetrabenzoyladenosine (HO-dA$^{Bz}$-pA$^{Bz_2}$-(OBz)$_2$) (VII).* 5′-*O*-dimethoxytrityl-*N*-benzoyldeoxyadenosine (0.125 mmole) and pyridinium *N*,*N*′,O$^{2'}$,O$^{3'}$-tetrabenzoyladenosine 5′-phosphate (360 mg, 0.5 mmole) were dissolved in anhydrous pyridine (2 ml) and anhydrous Dowex-50 pyridinium resin (200 mg) and DCC (0.5 g) was added. After shaking at room temperature for 3 days the reaction mixture was treated with 50% aqueous pyridine (20 ml) for 4 hr. The solution was extracted with petroleum ether, and the aqueous layer was evaporated to a gum, dissolved in dioxane containing a few drops of pyridine, and lyophilized. The powder was dissolved in 80% acetic acid (20 ml) for 8 min. The acetic acid was then evaporated over a period of 6 min and pyridine (0.5 ml) was immediately added to the residue. The product was dissolved in ethanol (20 ml) and applied to the top of a DEAE-cellulose column (3 × 50 cm acetate form in 95% ethanol) and the column was eluted at 0° with a linear salt gradient (3 l. of ethanol in the mixing vessel and 3 l. of 0.3 M triethylammonium acetate in 95% ethanol in the reservoir); 10-ml fractions were collected in 10 min. The product was eluted in fractions 55–75 and amounted to 960 $OD_{282}$ units; an aliquot was hydrolyzed with concentrated ammonia solution to a yield dApA as the sole product and this was degraded by venom phosphodiesterase to equal amounts of dA and pA. The product was concentrated in the presence of pyridine, converted to the pyridinium salt, and lyophilized three times from aqueous pyridine to remove pyridinium acetate. The yield of dry powder was 31 mg.

*Pyridinium N,N′,O*$^{2'}$*-Tribenzoyladenylyl-(3′→5′)-3′-O-acetyl-N-benzoyldeoxyadenosine (HO-A$^{Bz_2}$-OBz-p-dA$^{Bz}$-OAc) (VIII).* An anhydrous mixture of pyridinium 5′-*O*-dimethoxytrityl-*N*,*N*′,O$^{2'}$-tribenzoyladenosine 3′-phosphate (2000 $OD_{282}$ units), 3′-*O*-acetyl-*N*-benzoyldeoxyadenosine (100 mg), Dowex-50 pyridinium resin (200 mg), and DCC (0.6 g) in pyridine (2 ml) was shaken at room temperature for 3 days. Aqueous pyridine was then added and the mixture was extracted with petroleum ether and filtered to remove dicyclohexylurea and Dowex-50 resin. Solvents were removed and the residue was dissolved in 80% acetic acid (5 ml) for 8 min after which time the acetic acid was rapidly evaporated (4 min) under reduced pressure. The residue in ethanol–5% pyridine (10 ml) was applied to the top of a triethylaminoethyl- (TEAE-) cellulose column which was eluted at 0° with a linear salt gradient (2 l. of 95% ethanol in the mixing vessel, and 2 l. of 0.2 M triethylammonium acetate in 95% ethanol in the reservoir); fractions of 10 ml were collected every 10 min. The protected dinucleoside phosphate was eluted in fractions 125–141 and amounted to 1250 $OD_{282}$ units. The pooled fractions were evaporated, converted to the pyridinium salt, and lyophilized repeatedly. The product weighed 40 mg. Methanolic ammonia treatment afforded ApdA as the only product.

*Pyridinium N,N′,O*$^{2'}$*-Tribenzoyladenylyl-(3′→5′)-N,N′,O*$^{2'}$*,O*$^{3'}$*-tetrabenzoyladenosine, (HO-A$^{Bz_2}$-OBz-p-A$^{Bz_2}$-(OBz)$_2$) (IX).* Pyridinium 5′-*O*-dimethoxytrityl-*N*,*N*′,O$^{2'}$-tribenzoyladenosine 3′-phosphate (3000 $OD_{282}$ units) and *N*,*N*′,O$^{2'}$,O$^{3'}$-tetrabenzoyladenosine (100 mg) was rendered anhydrous and dissolved in anhydrous pyridine (2 ml). Dowex-50 pyridinium resin (about 200 mg) and DCC (0.5 g) were added and the reaction mixture was shaken at room temperature for 3 days. Aqueous pyridine was then added to the mixture and after extraction with petroleum ether the solution was filtered. After 6 hr the aqueous layer was diluted with water, frozen, and lyophilized. The resulting powder was dissolved in 80% acetic acid for 10 min, the acetic acid was then evaporated under reduced pressure, and the product was dissolved in ethanol–5% pyridine (20 ml) and applied to the top of a DEAE-cellulose column (50 cm × 3 cm acetate form in 95% ethanol). The column was eluted at 0° with a linear salt gradient (2 l. of 95% ethanol in the mixing vessel,

[5] Abbreviation used: DCC, dicyclohexylcarbodiimide.

2 l. of 0.2 M triethylammonium acetate in 95% ethanol in the reservoir); fractions of 12 ml were collected every 10 min. The protected dinucleoside phosphate was eluted in fractions 140–152 and amounted to 1920 $OD_{282}$ units. The pooled fractions were evaporated in presence of pyridine, converted to the pyridinium salt, and lyophilized three times from aqueous pyridine to remove pyridinium acetate. The powder obtained weighed 80 mg. It was converted by methanolic ammonia treatment to ApA which was degraded by purified snake venom diesterase into equivalent amounts of A and pA.

*General Procedure for the Synthesis of Trinucleoside Diphosphates.* The protected dinucleoside phosphate and protected nucleotide as the pyridinium salt were dissolved in anhydrous pyridine (10 ml) and the solution was evaporated to 1 ml. Anhydrous Dowex-50 pyridinium resin (about 150 mg) and DCC (60 mg) was added and the mixture was shaken at room temperature for 3 days, when 50% aqueous pyridine (2 ml) and petroleum ether (5 ml) were added. After separation of the aqueous phase, the resin and precipitated dicyclohexylurea were washed with aqueous pyridine (two 1-ml portions). The combined aqueous layers were allowed to stand at room temperature for 8 hr, and were then evaporated and treated with excess methanolic ammonia for 18 hr. The product was separated by preparative paper chromatography in solvent D. The slowest moving band corresponded to the trinucleoside diphosphate. After elution of the band from the paper, it was rechromatographed in solvent A to remove ammonium acetate. The purity of the product thus obtained was examined by paper electrophoresis at pH 7.1. In two cases this technique showed the presence of some Ap which was not removed in the initial chromatography. This contaminant was removed by paper electrophoresis using 0.05 M ammonium acetate pH 7.5 buffer. The product thus purified was freed from ammonium acetate by rechromatography in solvent A. The trinucleoside diphosphates which were pure on paper electrophoresis were also pure in solvents A–D. Their mobilities on paper chromatography are shown in Table II, and their enzymic characterization is shown in Tables III and IV.

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