ISOLATION AND CHARACTERIZATION OF DINUCLEOSIDE TETRA- AND TRI-PHOSPHATES FORMED IN THE PRESENCE OF LYSYL-sRNA SYNTHETASE

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In the preceding paper we presented evidence that new adenine nucleotides are formed from ATP in the presence of a purified lysyl-sRNA synthetase. The present communication describes isolation and characterization of these compounds as diadenosine polyphosphates. In extension of these studies we have found that the addition to this system of other nucleoside di- and triphosphates results in the synthesis of a family of mixed dinucleoside tri- and tetraphosphates.

Dinucleoside tri- and tetraphosphates were synthesized by incubating 1-10 μmoles of the appropriate nucleotides with E. coli lysyl-sRNA synthetase (Stern and Mehler, 1965), lysine, Mg^{++}, and Tris buffer at pH 7.8. A small amount of E. coli sRNA (mixed) was added in some cases. All reactions were followed by anion-exchange thin-layer chromatography (TLC) on PEI-cellulose (Randerath and Randerath, 1964, 1965, 1966).* Nucleotides were isolated by preparative TLC. After pressure dialysis, the dialyzable material was streaked onto one or two 20 cm wide PEI-cellulose plastic sheets (Randerath and Randerath, 1966) and chromatographed. A typical chromatogram is shown in Fig. 1.

*Poly(ethyleneimine) ("Polymin P") was obtained from BASF Colors and Chemicals, Inc., 845 Third Avenue, New York, N.Y. 10022.

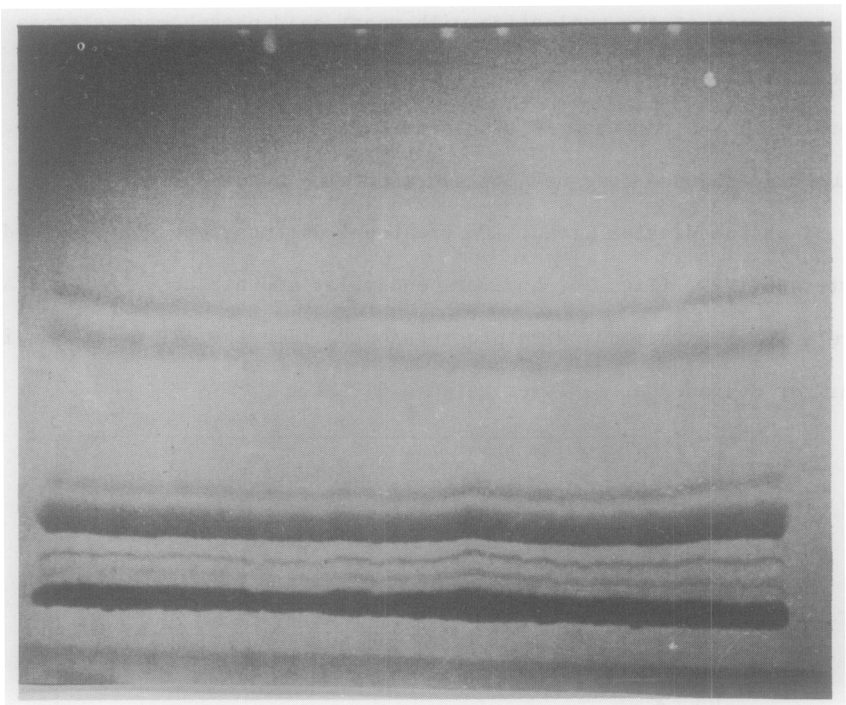

Fig. 1. Preparative thin-layer chromatogram of nucleotides from an incubation of ATP with lysyl-sRNA synthetase. The incubation was carried out for 4 hours and 20 minutes at 37°C in a total volume of 1.0 ml. The tube contained 0.04 M Tris-HCl at pH 7.8, 0.008 M $MgCl_2$, 0.002 M ATP, 0.0015 M l-lysine, 0.24 mg lysyl-sRNA synthetase, and 2.0 mg stripped E. coli sRNA (unfractionated). Chromatography was carried out with 1 M LiCl on a 20 cm wide PEI-cellulose sheet. The chromatogram was photographed in short-wave ultraviolet light. The compounds represent from top to bottom: 5'-AMP, AP_3A, ADP, AP_4A, ATP, an unknown compound in the sRNA used, and sRNA. It can be seen that AP_4A is the predominant low molecular weight nucleotide in the mixture.

One M LiCl was found to be a suitable solvent for most separations. After desalting with methanol, compounds were eluted from cut-outs with 3 N ammonia for 15 min in the cold. The eluates were filtered in the cold through a fritted-glass funnel. After lyophilization, the compounds were taken up in water, centrifuged, and the supernatant solutions were used for further experiments.

The main product obtained by incubating ATP with lysyl-sRNA synthetase was characterized in the following way. The ultraviolet spectrum of the compound at neutral pH closely resembles that of ATP.

On treatment of the nucleotide with yeast acid phosphomonoesterase (Schmidt et al., 1963), no change in its TLC characteristics was observed. The compound is stable to 0.25 N LiOH at 30°C for 24 hr. Thus the compound does not contain a free phosphomonoester or a 3',5'-phosphodiester group. On treatment with snake venom phosphodiesterase (VPD) (Fig. 2), however, equimolar amounts of 5'-AMP and ATP are initially formed. With progression of time the ATP so formed is further degraded at a slower rate to 5'-AMP.

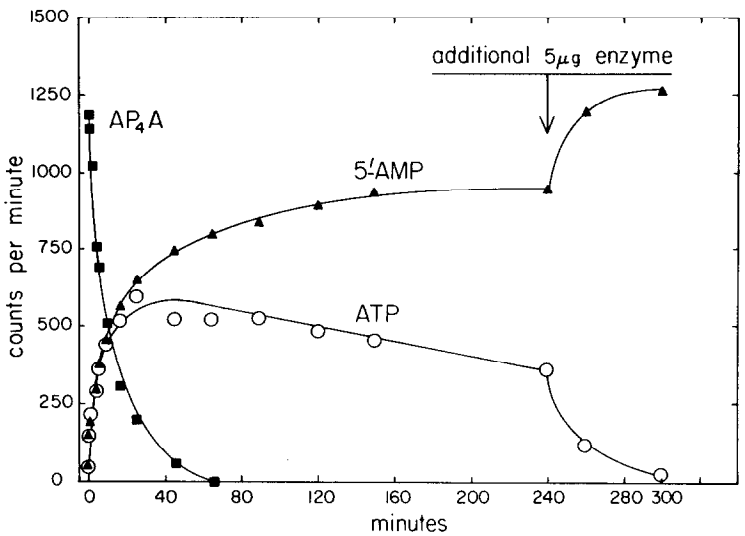

Fig. 2. Enzymatic degradation of $\alpha,\delta P^{32}$-AP_4A with snake venom phosphodiesterase. The incubation was carried out at 27°C in a total volume of 0.2 ml. The tube contained 0.05 M $MgCl_2$, 0.05 M Tris-HCl at pH 8.4, 0.0004 M AP_4A ($\alpha,\delta P^{32}$-AP_4A containing 330 cpm/mμmole), and 1.25 μg VPD (Crotalus adamanteus, Worthington). Aliquots of 10 μl were chromatographed with 1 M LiCl on PEI-cellulose sheets at various time intervals. At 240 minutes, a solution of 5 μg of VPD in 5 μl water was added to the residual incubation mixture in order to complete the reaction. The nucleotides, located by their quenching of ultraviolet light, were cut from the sheets and counted in an end-window gas-flow counter. The results are expressed as total counts in each spot isolated from 10 μl of incubation mixture. The 260 and 300 minute values are corrected for the volume change.

The VPD degradation of the compound was also followed spectrophotometrically at 259 mμ (Fig. 3). Upon addition of VPD, a rapid increase in optical density is observed, which corresponds to 16-17% hypochromicity of the compound.

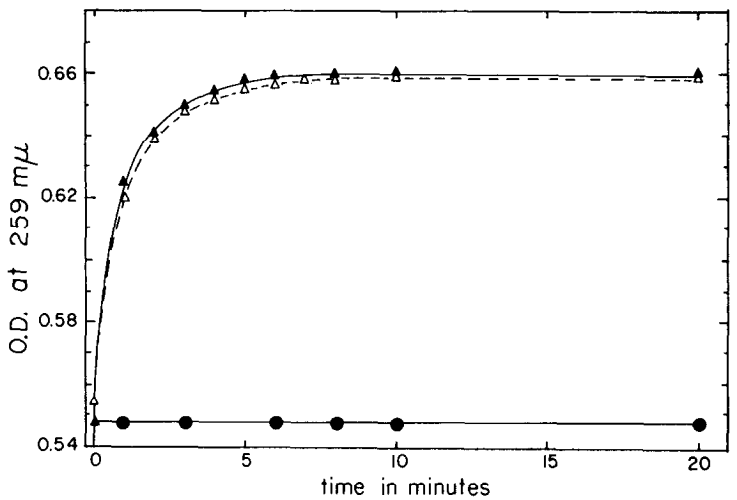

Fig. 3. Change in optical density during digestion of AP_4A with VPD. A 1 cm path divided cell containing a volume of 1.3 ml in each side was used. Both sides contained 0.05 M Tris-HCl at pH 7.8 and 0.01 M $MgCl_2$. In addition, one side contained 0.021 mM AP_4A and the other side 10 μg/ml of VPD. The A_{259} was measured against a buffer blank. The two sides were mixed and the optical density was measured at the time intervals indicated in the figure. The determinations were run in duplicate, with one control. The bottom straight line represents the control cell in which the compound was not mixed with the enzyme.

The phosphorus to adenine ratio was found to be 3.97:2.00. Adenine was determined spectrophotometrically and corrected for 17.6% hypochromicity (see also below).

The analytical data indicate that the compound contains one molecule each of 5'-AMP and ATP linked together by a pyrophosphate bond. This nucleotide, P^1,P^4-di(adenosine-5')tetraphosphate, (AP_4A) has been synthesized chemically by Moffatt et al. (1965, a-d), and our analytical results are in agreement with those reported by these authors. They are also comparable to those described by Finamore and Warner (1963) and by Adam and Moffatt (1966), the former in the identification of P^1,P^4-di(guanosine-5')tetraphosphate from brine shrimp eggs. AP_4A was first suggested by Smith and Khorana (1958) as an intermediate in the reaction of ADP with dicyclohexylcarbodiimide.

The hypochromicity of AP_4A was also calculated as follows: $\alpha,\delta\ P^{32}$-AP_4A was prepared enzymatically from αP^{32}-ATP. Aliquots of solutions of both compounds were chromatographed in quadruplicate on PEI-cellulose sheets with 1 M LiCl. Compounds were cut from the sheets and counted in an end-window gas-flow counter. Nucleotides and corresponding blank areas were subsequently eluted with 2 M LiCl-0.02 M Tris-HCl, pH 7.4. Extinctions (A_{260}) were measured at 260 mμ against the blank eluates. The ratios A_{260}/cpm were found to be 0.957×10^{-4} for ATP and 0.790×10^{-4} for AP_4A. From these data and the molar extinction coefficient of ATP ($\varepsilon_{ATP_{260}} = 15.4 \times 10^3$; Bock et al., 1956), the molar extinction coefficient, $\varepsilon_{AP_4A,\ 260}$, at neutral pH of AP_4A was calculated according to the equation

$$\varepsilon_{AP_4A,260} = \frac{2 \times \varepsilon_{ATP,260} \times A_{AP_4A,260} \times cpm_{ATP}}{A_{ATP,260} \times cpm_{AP_4A}} = 25.4 \times 10^3$$

Thus the hypochromicity on a "per adenosine residue basis" is 17.6%, which is in satisfactory agreement with the value obtained by VPD digestion of AP_4A.

In addition to AP_4A, another nucleotide is formed on incubation of ATP with lysyl-sRNA synthetase (cf. Table 1 of the preceding paper). Larger amounts of this compound may be obtained by incubating ADP and ATP with the enzyme. Treatment of this nucleotide with VPD results in a rapid initial cleavage to 5'-AMP and ADP. No ATP or other nucleotides could be detected by TLC. AMP and ADP are formed initially in equimolar amounts from an equivalent amount of compound (Table 1). ADP is subsequently hydrolyzed at a very slow rate to 5'-AMP. The reaction indicates that the nucleotide is P^1,P^3-di(adenosine-5')triphosphate (AP_3A), a compound that has been synthesized chemically and been shown by Reiss and Moffatt (1965a) to behave in an identical manner when treated with VPD.

If GTP or dGTP are similarly added to the incubation mixture in

addition to ATP, P^1-adenosine-5'P^4-guanosine-5'tetraphosphate (AP_4G) and P^1-adenosine-5'P^4-deoxyguanosine-5' tetraphosphate (AP_4dG) appear to be formed, respectively, plus AP_4A. These two "hybrid" nucleotides are hydrolyzed by VPD to give initially, in addition to 5'-AMP and ATP, 5'-GMP plus GTP and dGMP plus dGTP, respectively. No diphosphates are formed in these reactions. From the incubation mixture containing GTP another nucleotide has been isolated by preparative TLC which appears to be P^1-adenosine-5'P^3-guanosine-5' triphosphate (AP_3G). VPD digestion of this compound gave, in addition to 5'-AMP and ADP, 5'-GMP and GDP. Preliminary experiments with UTP and dTTP indicate formation of AP_4U and AP_4dT plus the corresponding "hybrid" dinucleoside triphosphates.

If dATP is used as the sole nucleotide in the reaction mixture, in the absence of ATP, it appears that P^1,P^4-di(deoxyadenosine-5')tetraphosphate (dAP_4dA) is formed rapidly and in high yield. This compound is degraded by VPD initially to dAMP and dATP as the only nucleotides. It is interesting in this connection that dATP can substitute for ATP in the aminoacylation reaction resulting in lysyl-sRNA formation (unpublished data).

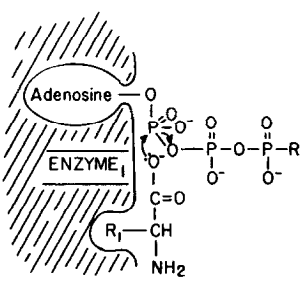

Fig. 4. Proposed mechanism for the formation of nucleoside diphosphates (R = nucleoside) and triphosphates (R = nucleoside monophosphate) from dinucleoside tri- and tetraphosphates in the presence of lysyl-sRNA synthetase.

The observation that in the presence of lysyl-sRNA synthetase mixed "hybrids" of the type AP_4N may be formed in which N is a nucleoside other than adenosine, opens up the possibility that the reversal of this reaction (Fig. 4) may generate ribo- and deoxyribonucleoside triphosphates as primers for other synthetic reactions within the cell. Key precursors for DNA and RNA synthesis may thus become available from dinucleoside tetraphosphates. The unusual situation that dATP can substitute for ATP in the lysine activation reaction and that dAP_4dA can be formed is also of special interest in this connection.

TABLE 1

Enzymatic Degradation of $\alpha,\gamma P^{32}$-AP_3A with Snake Venom Phosphodiesterase

Minutes	AP_3A degraded	AMP formed	ADP formed
2	0.95	0.96	1.00
4	1.48	1.54	1.60
12	2.67	2.64	2.91
30	3.50	3.55	3.65

The incubation was carried out at 30°C in a total volume of 0.2 ml. Each tube contained 0.05 M $MgCl_2$, 0.05 M Tris-HCl at pH 8.4, 0.0004 M AP_3A ($\alpha,\gamma P^{32}$-AP_3A containing 250 cpm/mμmole), and 1.25 μg of VPD. Aliquots of 10 μl were chromatographed on PEI-cellulose sheets at various time intervals. Compound areas were cut out and counted. The results are expressed as mμmoles of compounds in each spot isolated from 10 μl of incubation mixture.

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