The Phosphorylation of 5'-Oligoadenylic Acids by Adenylate Kinase and Adenosine Triphosphate

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1. Radioactivity was incorporated into 5'-O-phosphoryl-adenylyl-(3'-5')-adenosine (pApA) on incubation with adenylate kinase and [γ - 32 P]ATP. The corresponding triadenylate and tetra-adenylate reacted more slowly. 2. Only oligoadenylate with a terminal 5'-phosphate served as the substrate. 3. The product formed from pApA was shown to behave like 5'-O-pyrophosphoryl-adenylyl-(3'-5')-adenosine (ppApA) on hydrolysis with alkaline phosphatase, potassium hydroxide and hydrochloric acid. 4. The characteristics of the reaction indicated that it was catalysed by adenylate kinase, but the rate of phosphate transfer from ATP to pApA was about 0.01% of that in the typical reaction with AMP. 5. The reverse reaction between ADP and ppApA occurred readily, but no additional phosphorylation of ppApA (to give pppApA) could be demonstrated.

Nucleic acids are phosphorylated by 5'-hydroxyl polynucleotide kinase (Richardson, 1965; Novogrodsky & Hurwitz, 1966; Novogrodsky, Tal, Traub & Hurwitz, 1966), which attaches a single 5'-O-phosphoryl group to a molecule with a terminal 5'-hydroxyl group. The occurrence of oligonucleotides that may have terminal pyrophosphoryl groups (Finamore, 1964) suggests the existence of additional phosphorylation reactions for nucleic acids. Stahl & Ebel (1964) found that, contrary to the claims of earlier workers (e.g. Dounce & Kay, 1953), adenylate kinase did not phosphorylate RNA. However, it is now shown that in the presence of large amounts of adenylate kinase short-chain 5'-oligoriboadenylic acids such as pApA* incorporate 32 P from [γ - 32 P]ATP.

MATERIALS AND METHODS

Enzymes. Adenylate kinase (ATP-AMP phosphotransferase, EC 2.7.4.3) from rabbit muscle (360 units/mg.) and hexokinase (EC 2.7.1.1) (140 units/mg.) were purchased from Boehringer Corp. Ltd. (London, W. 5); chromatographically purified alkaline phosphatase (EC 3.1.3.1) from *Escherichia coli* (20 units/mg.) was purchased from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.); sheep kidney endonuclease was purified as described by

Kasai & Grunberg-Manago (1967); rat liver polyadenylate nucleotidyltransferase (EC 2.7.7.19) was prepared as described by Klemperer (1965).

Labelled nucleotides. [γ - 32 P]ATP (specific activity approx. 10^8 counts/min./ μ mole) was prepared by the method of Glynn & Chappell (1964). All 32 P in this material was located in the terminal phosphate. This was shown by incubating [32 P]ATP (2.5 μ moles) for 10 min. at 37° in 1.0 ml. (final volume) with 10 μ moles of glucose, 10 μ g. of hexokinase, 50 μ moles of tris-HCl buffer, pH 7.4, and 5 μ moles of MgCl₂. ADP in the reaction product was separated on a 2.5 cm.-wide strip of DEAE-cellulose paper (Whatman DE-81) by descending chromatography for 4 hr. in 4 M-formic acid-0.1 M-ammonium formate (Ives, Morse & Potter, 1963) and was found to be non-radioactive.

[32 P]ADP was prepared by incubating 6 μ moles of [32 P]ATP with 8 μ moles of AMP, 25 μ g. of adenylate kinase, 50 μ moles of sodium phosphate buffer, pH 7.5, and 12.5 μ moles of MgCl₂ in 2.5 ml. (final volume) for 15 min. at 37°. The reaction mixture was applied to a 20 cm. $\times$ 1 cm. column of DEAE-cellulose (Whatman DE-11, HCO₃⁻ form) and the components were fractionated with a linear gradient of NH₄HCO₃, pH 7.8 (375 ml. of 0.02 M-NH₄HCO₃ in the mixing chamber, 375 ml. of 0.25 M-NH₄HCO₃ in the reservoir). [32 P]ADP was eluted at approx. 0.12 M-NH₄HCO₃ and was desalted at 0° by two successive treatments with Dowex 50 (X8; H⁺ form; 200-400 mesh) and then concentrated on a rotary evaporator at 37°.

[8- 14 C]ATP was purchased from The Radiochemical Centre (Amersham, Bucks.).

Oligoadenylate. The 5'-oligoadenylic acids were prepared by digestion of polyadenylic acid with sheep kidney endonuclease followed by fractionation on a column of DEAE-cellulose (HCO₃⁻ form) as described by Kasai & Grunberg-Manago (1967), except that the peaks from the column were concentrated by adsorption on 2 cm. $\times$ 1 cm. DEAE-cellulose (HCO₃⁻ form) columns followed by elution in 2 M-NH₄HCO₃ (Tomlinson & Tener, 1963). The eluate was

* Abbreviations: A, adenosine; pA, adenosine 5'-phosphate; Ap, adenosine 3'-phosphate; ApA, adenylyl-(3'-5')-adenosine; pApA, 5'-O-phosphoryl-adenylyl-(3'-5')-adenosine; pApApA etc., the corresponding longer oligoadenylates; ppApA, 5'-O-pyrophosphoryl-adenylyl-(3'-5')-adenosine; ppAp, 5'-O-pyrophosphoryl-adenosine 3'-phosphate.

then desalted by passage over a 2 cm. $\times$ 30 cm. column of Sephadex G-10.

The products were characterized by the proportion of the total phosphate that was released by digestion with alkaline phosphatase. Oligonucleotide (approx. 0.2 μ mole) was incubated at 37° in 0.25 ml. (final volume) with 10 μ g. of alkaline phosphatase and 25 μ moles of tris-HCl buffer, pH 8.5. Samples for determination of free P_i and total phosphate (Ames & Dubin, 1960) were taken after 2 hr. and 4 hr. to ensure that the digestion was complete. The (total phosphate)/(terminal phosphate) ratios 2.05, 3.1 and 4.1 respectively were found for oligoadenylylates assumed from their position of elution from the DEAE-cellulose column to be pApA, pApApA and pApApApA. To confirm that these substances were 5'-oligonucleotides it was also demonstrated that they acted as acceptors for [14 C]AMP when incubated with [14 C]ATP and the rat liver polyadenylate nucleotidyltransferase, which attaches AMP to oligonucleotides with unsubstituted 3'(2')-hydroxyl groups (Klempere, 1965).

The reference substance ApA for electrophoresis was prepared as described by Klempere (1965).

Incubation for incorporation of 32 P into oligoadenylylates. The standard reaction mixtures (total volume 0.25 ml.) contained adenylate kinase (12.5 or 50 μ g. of protein, which had been dialysed against 0.02 M-sodium phosphate buffer, pH 7.5) together with 0.1 μ mole of oligoadenylylate, 0.3 μ mole of [γ - 32 P]ATP (specific activity 0.5 $\times 10^6$ –6.4 $\times 10^6$ counts/min./ μ mole), 5 μ moles of sodium phosphate buffer, pH 7.5, and 1.25 μ moles of $MgCl_2$. Samples (0.04 ml.) were taken at intervals during the incubation at 37° and applied together with non-radioactive carrier ATP to 2.5 cm.-wide strips of DEAE-cellulose paper. Descending chromatography was carried out for 4 hr. in 0.4 M-ammonium formate buffer, pH 6.5 (Falaschi, Adler & Khorana, 1963). Labelled ATP and ADP, together with AMP, moved ahead of the oligoadenylylates and their labelled derivatives (Fig. 1). The chromatograms were dried at 50° and then divided into 2 cm. bands for measurement of radioactivity. This allowed a clear distinction between 32 P in the product and ATP areas. The total radioactivity recorded in the areas corresponding to the product agreed within 5% on duplicate chromatograms. The values were corrected for the radioactivity found in corresponding areas with unincubated controls. These 'blank' values for areas corresponding to the product formed from pApA, pApApA and pApApApA respectively were 1.6%, 0.4% and 0.15% of the total radioactivity on the chromatogram.

In some experiments AMP (0.1 μ mole/0.25 ml.) was substituted for oligoadenylylate and the adenylate kinase concentration was decreased to 0.1 μ g./0.25 ml. After short periods of incubation the newly formed [32 P]ADP was separated from [32 P]ATP on DEAE-cellulose paper (see under 'Labelled nucleotides' above). From the percentage of total 32 P incorporated into ADP the rate of [32 P]ADP synthesis was approx. 400 μ moles/min./mg. of enzyme.

Determination of radioactivity. Paper areas (not larger than 2 cm. $\times$ 2.5 cm.) from chromatograms and electrophoresis strips were placed in vials together with 12 ml. of scintillation fluid [toluene containing 4 g. of 2,5-diphenyl-oxazole and 0.05 g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene/l.]. For determinations of specific activity samples of material were first dried on to 2 cm. $\times$ 2.5 cm. areas of Whatman no. 1 filter paper, which were then placed in

vials in the same way. Radioactivity was measured in a liquid-scintillation counter (background 100 counts/min.; efficiency 42%).

Radioautography. Chromatograms or electrophoresis strips were exposed to Kodirex X-ray film (Kodak Ltd., Kirkby, Liverpool). The film was later developed for 4 min. with Pentelux X-ray developer (May and Baker Ltd., Dagenham, Essex) and fixed with Amfix high-speed fixer (May and Baker Ltd.).

Preparation of [32 P]ppApA. The standard reaction mixture (containing 0.4 μ mole of pApA/ml.) was increased to 5 ml. (total volume) and the concentration of [32 P]ATP was increased to 2.4 μ moles/ml. After incubation for 8 hr. at 37° the mixture was applied to a 20 cm. $\times$ 1 cm. column of DEAE-cellulose (HCO_3^- form). A linear gradient of NH_4HCO_3 , pH 7.8 (750 ml. of 0.02 M- NH_4HCO_3 in the mixing chamber, 750 ml. of 0.5 M- NH_4HCO_3 in the reservoir), eluted [32 P]ADP, [32 P]ATP, non-radioactive pApA and [32 P]ppApA respectively at approx. 0.12 M-, 0.14 M-, 0.19 M- and 0.21 M- NH_4HCO_3 . The contents of the tubes corresponding to the ppApA peak (approx. 1.3 μ moles) were combined. This material was concentrated and desalted as described above for the oligoadenylylates.

Characterization of [32 P]ppApA. Samples (approx. 0.2 μ mole, 1.5 $\times 10^6$ counts/min./ μ mole) were hydrolysed with phosphatase (as described for pApA etc.), 0.3 N-KOH (16 hr. at 37°, followed by neutralization with $HClO_4$ at 0° and removal of $KClO_4$ by centrifugation) or 1.0 N-HCl (7 min. at 90°, followed by neutralization with NaOH). Samples of these digests were taken for determination of free P_i and of total phosphate, or were diluted in 0.02 N-HCl for measurement of E_{260} (taking 14.25 as the average ϵ_{mM} value for an equimolar mixture of A and Ap; Beaven, Holliday & Johnson, 1955). Samples were also applied to Whatman no. 1 paper adjacent to marker compounds (A, Ap, ADP and ATP). The paper was subjected to electrophoresis at pH 3.5 at 50 v/cm. for 1 hr. with sodium citrate buffer (0.05 M, except after HCl digestion, when 0.5 M was used) between polyethylene sheets on a water-cooled metal block. The positions of the adenine derivatives were located by their absorption under u.v. light, and the distribution of radioactivity was measured as described above.

Incubations with [32 P]ppApA. [32 P]ppApA (0.3 $\times 10^6$ counts/min./ μ mole) was incubated under standard conditions with either non-radioactive ADP or [32 P]ADP (1.5 $\times 10^6$ counts/min./ μ mole). Samples (0.04 ml.) of the mixture were fractionated as described above by chromatography on DEAE-cellulose paper (0.4 M-ammonium formate buffer, pH 6.5) or by paper electrophoresis in 0.05 M-sodium citrate buffer, pH 3.5. To ensure separation of ATP and ADP from oligoadenylylate the appropriate regions of the electrophoresis paper were eluted with water and the eluates were applied to DEAE-cellulose paper for chromatography as described above.

RESULTS

Incorporation of 32 P into oligoadenylylate. On incubation of [32 P]ATP and oligoadenylylate with adenylate kinase there was a progressive synthesis of a radioactive product that ran immediately behind the oligoadenylylate on DEAE-cellulose paper chromatograms (Fig. 1). Other experiments

Fig. 1. Radioautogram of reaction products from $[^{32}\text{P}]\text{ATP}$ and oligoadenylate. Reaction mixtures (0.25 ml.) containing 50 μg . of adenylate kinase, 0.1 μmole of oligoadenylate, 0.3 μmole of $[^{32}\text{P}]\text{ATP}$ (6.4×10^6 counts/min./ μmole), 1.25 μmoles of MgCl_2 and 5 μmoles of sodium phosphate buffer, pH 7.5, were incubated at 37° and then subjected to chromatography in 0.4 M-ammonium formate buffer, pH 6.5, on DEAE-cellulose paper strips. The chromatograms were dried and exposed to X-ray film. The positions of the strips (broken line for origin) and of the u.v.-absorbing oligoadenylate areas were outlined on the film. ATP and ADP (and AMP) run together in the area that coincides with the region of maximum radioactivity. Darkening of the film obscures the outline of this area. In (a) reaction mixtures containing pApA were incubated for the time (min.) shown. A blank incubated without pApA for 60 min. is included. The exposure time was 20 hr. In (b) reaction mixtures containing pApApA ([pA]₃) or pApApApA ([pA]₄) were incubated for 240 min. The exposure time was 40 hr. The labelled P_i (ahead of ATP + ADP) shows up more clearly because of longer exposure and printing times.

showed that the initial rate of the reaction was proportional to the amount of enzyme added and that the optimum conditions were provided by 5 mM-magnesium chloride and 0.02 M-sodium phosphate buffer, pH 7.5 (rates at pH 6.5 and 8.5 were 90% of the rate at pH 7.5). When tris-hydrochloric acid buffer was substituted for phosphate buffer the reaction rate was approximately halved. Fig. 1 also shows that small amounts of $[^{32}\text{P}]\text{P}_i$ (running ahead of ATP + ADP) were released

Fig. 2. Incorporation of ^{32}P into pApA. The reaction mixtures were as described in Fig. 1(a). In one case (○) a further 50 μg . of adenylate kinase was added at 120 min. Another tube (---○) received an addition of 0.5 μmole of ADP/0.25 ml. at 240 min. and was incubated for a further 240 min.

during the incubation. This release represented a hydrolysis of ATP at the rate of about 0.5%/4 hr., and other experiments showed that it occurred independently of the presence of enzyme or of oligoadenylate.

Fig. 2 shows that the amount of ^{32}P incorporated into pApA was initially proportional to the time of incubation (3.6% of the total radioactivity/15 min.), but later approached a constant level (20%) that was not altered by adding more adenylate kinase or after longer incubation. This level represented an equilibrium position, since it decreased when extra ADP was added. Further, in other experiments, the final percentage of ^{32}P incorporated depended on the relative proportions of ATP and pApA. Thus 0.3 μmole of ATP plus 0.05 μmole of pApA/0.25 ml. gave a final 13%, whereas 0.15 μmole of ATP plus 0.1 μmole of pApA/0.25 ml. gave a final 34% of ^{32}P in the product. Starting with 0.3 μmole of $[^{32}\text{P}]\text{ATP}$ /0.25 ml. (Fig. 2) the incorporation of 3.6% of the total ^{32}P /15 min. and 20% at equilibrium corresponds roughly (see the Discussion section) to the synthesis of reaction product equivalent to 11 μmoles /15 min. and 50 μmoles at equilibrium.

Characteristics of the reaction. Table 1 shows that in the absence of oligonucleotide or enzyme no radioactive product was formed similar to that found in the standard incubation mixture. The enzyme was only slowly inactivated by heating at 100° in 0.1 N-hydrochloric acid. In the presence of 0.1 μmole of ADP/0.25 ml. the reaction rate was lowered by 70%. This may have been due to a direct effect or to AMP (which might compete with pApA) produced from ADP by the action of adenylate kinase. In the same way AMP was also an inhibitor.

Table 1. *Characteristics of the reaction between ATP and oligoadenylate*

The standard incubation mixture (0.25 ml.) contained 12.5 or 50 μ g. of adenylate kinase, 0.1 μ mole of oligonucleotide (as shown), 0.3 μ mole of [32 P]ATP (5.3×10^6 counts/min./ μ mole), 5 μ moles of sodium phosphate buffer, pH 7.5, and 1.25 μ moles of $MgCl_2$. These conditions were varied as shown by pretreating the enzyme with 0.1 N-HCl at 100° for 5 min. (followed by neutralization with NaOH), by adding 0.1 μ mole of ADP or AMP or 0.3 μ mole of GTP, CTP or UTP/0.25 ml., or by substituting [14 C]ATP (0.2 $\times 10^6$ counts/min./ μ mole) for [32 P]ATP. The amount of radioactivity incorporated into product was determined (a) after incubation for 1 hr. at 37° with 12.5 μ g. of adenylate kinase and (b) after 4 hr. with 50 μ g. of adenylate kinase.

Oligonucleotide	Incubation conditions	Radioactivity in product (%)	
		(a)	(b)
pApA	Standard	3.30	—
None	Standard	< 0.05	—
pApA	Enzyme omitted	< 0.05	—
pApA	HCl-treated enzyme	2.10	—
pApA	ADP added	1.00	—
pApA	AMP added	1.25	—
pApA	GTP added	3.20	—
pApA	CTP added	3.25	—
pApA	UTP added	3.35	—
pApA	[14 C]ATP substituted	< 0.05	—
ApA	Standard	< 0.05	—
ApAp	Standard	< 0.05	—
pApApA	Standard	0.15	3.20
pApApApA	Standard	< 0.05	0.75

Table 2. *Electrophoretic mobilities at pH 3.5*

Substances were subjected to paper electrophoresis at 50v/cm. for 60 min. with 0.05 M-sodium citrate buffer, pH 3.5, between polyethylene sheets on a water-cooled metal block. R_{ATP} is the electrophoretic mobility relative to that of ATP, both measured as distances from the origin. A negative value for R_{ATP} indicates that the compound moves towards the cathode.

Substance	R_{ATP}	Substance	R_{ATP}
A	-0.31	ATP	1.00
P _i	1.40	ApA	0.13
pA	0.21	pApA	0.46
Ap	0.26	ppApA	0.82
ADP	0.87	ppAp	1.08

The addition of non-radioactive GTP, CTP or UTP in amount equal to that of [32 P]ATP was without effect, indicating that the reaction specifically required ATP. No radioactive product was formed with [14 C]ATP and pApA, nor with [32 P]ATP and ApA or ApAp, suggesting that in the standard conditions 32 P was attached to the 5'-phosphate of pApA.

Table 1 also shows that the longer oligoadenylate chains were labelled more slowly: pApApA and pApApApA respectively incorporated 32 P at about 5% and 1% of the rate found in the initial linear phase of the reaction with pApA.

Characterization of [32 P]ppApA. The reaction

product formed from pApA was hydrolysed with alkaline phosphatase, potassium hydroxide and hydrochloric acid, and the hydrolysis products were characterized by the distribution of phosphate and by electrophoresis (see Table 2 for electrophoretic mobilities). The expected reactions in the presence of phosphatase and potassium hydroxide are shown by eqns. (1) and (2) respectively:

Table 3 shows that after phosphatase treatment the (total phosphate)/(P_i released) ratio was close to the expected value of 1.5. Phosphatase released all of the 32 P as P_i and gave rise to a u.v.-absorbing compound that was characterized as ApA on the basis of electrophoretic mobility and because on subsequent hydrolysis with potassium hydroxide it yielded Ap plus A.

Digestion of ppApA with potassium hydroxide produced A and a radioactive compound presumed to be ppAp (together with a small proportion of radioactive P_i). On subsequent hydrolysis with phosphatase the presumed ppAp yielded A plus [32 P]P_i. The compound moved just ahead of ATP on electrophoresis at pH 3.5, and this greater mobility of ppAp relative to ATP may be compared with the greater mobility of Ap relative to pA (Table 2), which is presumably a consequence of the lower pK of the amino group in Ap (Smith, 1955).

Table 3. *Characteristics of hydrolysis products of [³²P]ppApA*

Samples of [³²P]ppApA (approx. 0.2 μ mole, 1.5×10^6 counts/min./ μ mole) were hydrolysed by the procedure indicated and the hydrolysis products were characterized as described in the Materials and Methods section. Total P refers to total phosphate, which was determined as P_i after wet-ashing. Electrophoresis was in 0.05M-sodium citrate buffer, pH 3.5, except after hydrolysis with HCl, when 0.5M-citrate was used and in this case the only u.v.-absorbing areas visible were due to the reference compounds. Radioactivity (% of total) refers to total radioactivity on electrophoresis paper.

Hydrolysis procedure	Distribution of phosphate (molar ratios)	Ultraviolet-absorbing areas	Electrophoresis		
			Location of radioactivity		
			Substance	Counts/min.	% of total
Phosphatase	Total P/P _i = 1.47	ApA	P _i	4900	99
Phosphatase, then KOH	—	Ap + A	—	—	—
KOH	Total P/(A + Ap) = 1.58	ppAp + A	{ ppAp	10800	91
			{ P _i	950	8
KOH, then phosphatase	Total P/P _i = 1.05	A	{ P _i	2300	98
HCl	P _i /total P = 0.47	—	{ P _i area	4500	91
			{ ppAp area	400	8

The rapid release of at least 90% of the ³²P as [³²P]P_i by 1.0N-hydrochloric acid at 90° suggests that the ³²P in the product was attached to the 5'-phosphate of the original pApA. As expected, acid hydrolysis also gave rise to some ppAp. Other experiments showed that 80–90% of the terminal phosphate of ADP was released under similar conditions. However, the amount of P_i released from ppApA (47% of the total phosphate) was greater than the maximum expected (33%) for the terminal phosphate alone. This is accounted for by the fact that under similar conditions about 20% of the terminal phosphate of pApA was released.

Incubations with ppApA. In contrast with the relatively slow synthesis of ppApA the reverse reaction was more rapid. Thus when non-radioactive ADP (0.25 μ mole/0.25ml.) was incubated with [³²P]ppApA (0.1 μ mole/0.25ml., 0.3×10^6 counts/min./ μ mole) under the standard conditions (50 μ g. of adenylate kinase/0.25ml.) the reaction was complete within 5min., with 44% of the total radioactivity in ADP plus ATP and 56% remaining in ppApA.

On the other hand, no synthesis of pppApA could be demonstrated. Thus when [³²P]ADP (0.1 μ mole/0.25ml.; 1.5×10^6 counts/min./ μ mole) was incubated with [³²P]ppApA (0.125 μ mole/0.25ml.; 0.3×10^6 counts/min./ μ mole) for 4 or 8hr. under the standard conditions (50 μ g. of adenylate kinase/0.25ml.) electrophoresis of the reaction product showed the presence of labelled ppApA, ADP and ATP (R_{ATP} 0.82, 0.87 and 1.0 respectively), but no increase in radioactivity in the vicinity of these areas. Moreover, the region between and including the ADP and ATP areas (where pppApA might be expected to run) was eluted and the eluate was

subjected to chromatography on DEAE-cellulose paper, but the radioactivity in the region behind that of ADP plus ATP was the same as in the unincubated control (1–2% of the total radioactivity). Therefore no significant amounts of pppApA were formed under the conditions that allow the synthesis of ppApA.

DISCUSSION

The reaction of pApA with ATP was analogous to the adenylate kinase-catalysed phosphorylation of AMP by ATP. Thus the reaction product formed from pApA contained one additional equivalent of phosphate that could be released by alkaline phosphatase. Digestion with potassium hydroxide excluded groups other than the 5'-phosphate of pApA as the attachment site for this additional phosphate. This supports the finding (Table 1) that only compounds with a terminal 5'-phosphate served as substrates. The ready release of [³²P]P_i by acid hydrolysis confirmed that the product of the reaction was ppApA.

It seems likely that the phosphorylation of pApA was an atypical reaction of adenylate kinase. Lieberman (1956) has shown that this enzyme will also catalyse a phosphorylation of pyrophosphate by ADP at 0.1% of the rate of the typical adenylate kinase reaction. ADP, which is a competitive inhibitor of the typical adenylate kinase-catalysed reaction between ATP and AMP (see Noda, 1962), also inhibited the reaction with pApA, and the activity of the enzyme was fairly resistant to boiling in 0.1N-hydrochloric acid, as is also true for adenylate kinase (Colowick & Kalckar, 1943). However, the reverse reaction between ppApA and

ADP was much more rapid than the forward reaction between pApA and ATP. This may reflect a greater inhibition of the latter by its products (e.g. ADP). In the same way the failure to synthesize detectable amounts of pppApA from ppApA and ADP may possibly have been due to inhibition by the reactants themselves or by ATP and AMP derived from ADP.

Assuming that eqn. (3) describes the reaction with pApA, then the ADP formed would regenerate ATP via the typical adenylate kinase-catalysed reaction (eqn. 4):

At the same time ^{32}P would equilibrate via both reactions between ATP, ADP and ppApA. For this reason the amount of ppApA formed is probably underestimated by taking the value (% of ^{32}P in ppApA) $\times$ (initial amount of ATP). If this complication is neglected then the initial rate (in Fig. 2, with 50 μg . of enzyme/0.25 ml.) becomes 11 μmoles of ppApA formed/15 min./50 μg . of enzyme, or about 15 $\mu\text{moles}/\text{min.}/\text{mg.}$ of enzyme. Since under comparable conditions approx. 400 μmoles of AMP were phosphorylated/min./mg. of enzyme, the rate of reaction with pApA was less than 0.01% of the rate of the typical adenylate kinase reaction. Moreover, the longer-chain homologues were phosphorylated even more slowly, which makes it rather unlikely that these reactions have any significance *in vivo*.

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