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The molecular structure of a rapidly formed oligomeric adenosine tetraphosphate derivative from rat heart

Winston L. HUTCHINSON,* Peter G. MORRIS†‡ and John MOWBRAY*§

*Department of Biochemistry, University College London, Gower Street, London WC1E 6BT, U.K., and †M.R.C. Biomedical NMR Centre, National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K.

The inability to account for large systematic variations with time in soluble adenine nucleotides in perfused rat hearts [Bates, Perrett & Mowbray (1978) Biochem. J. **176**, 485–493; Mowbray, Bates & Perrett (1981) FEBS Lett. **131**, 55–59; Mowbray, Perrett & Bates (1984) Int. J. Biochem. **16**, 889–894] led us to show that the soluble nucleotides are in rapid equilibrium with some hitherto unrecognized trichloroacetic acid/methanol-precipitable highly phosphorylated heteropolymeric form [Mowbray, Hutchinson, Tibbs & Morris (1984) Biochem. J. **223**, 627–632]. Selective digestion coupled to chromatographic analysis together with m.s. and ^{31}P-n.m.r. spectrometry have now been used to show that the likely structure for a purified oligomer that is in specific-radioactivity equilibrium with tissue ATP is 3-phospho-[glyceroyl-γ-triphosphoroyl-5′-adenosine-3′-3-phospho]$_4$glyceroyl-γ-triphosphoroyl-5′-adenosine.

INTRODUCTION

Langendorff perfused rat hearts apparently under steady-state conditions have been observed to show large synchronous systematic variations with time in the contents of their purine nucleotides for the first 40 min or so of perfusion (Bates *et al.*, 1978; Mowbray *et al.*, 1981). Similar variations in ATP content of perfused hearts as measured by ^{31}P n.m.r. have been reported to follow a short period of experimental ischaemia (Bailey & Seymour, 1981). Most surprisingly, it proved impossible to account for the large net increases and decreases in the adenine nucleotide as interconversion with other known purine derivatives or macromolecules, and we had to suggest that there exist some hitherto unknown compound(s) capable of rapid exchange with the soluble nucleotides (Bates *et al.*, 1978). Selective radioactive labelling of heart purines showed that 10–15% of the radioactivity was rapidly incorporated into a trichloroacetic acid-insoluble species that was stable in acid but solubilized in alkali (Hutchinson *et al.*, 1981; Mowbray *et al.*, 1984), and a more-recent study from a Canadian laboratory has confirmed this observation (Fitt *et al.*, 1985). Although a very small fraction of this acid-insoluble radioactive material may be RNA (P. G. Heyworth & J. Mowbray, unpublished work), by far the bulk of it is present in a oligomeric tetraphosphate derivative of adenosine with a short organic acid (Mowbray *et al.*, 1984). Here we present evidence that the molecular structure of this material is a phosphate-linked linear co-polymer of adenosine and glyceric acid.

EXPERIMENTAL

Materials

The plates used for t.l.c. were 20 cm × 20 cm pre-coated plastic sheets of either PEI-(polyethylenimine-)cellulose F (0.1 mm layer thickness) or of silica gel 60 (0.2 mm layer thickness) obtained from BDH Chemicals, Poole, Dorset, U.K. Snake-venom diesterase was bought from the Boehringer Corporation, Lewes, Sussex, U.K., and S_1 nuclease from Sigma Chemical Co., Poole, Dorset, U.K. All other materials were as previously described (Bates *et al.*, 1978; Mowbray *et al.*, 1984) and were of the highest purity commercially available.

Methods

Radioactive labelling of heart nucleotides by Langendorff perfusion with [8-^{14}C]adenosine and the recovery of the trichloroacetic acid/methanol-insoluble radioactive material was carried out as described by Mowbray *et al.* (1984). The dried pellet (300 mg) of precipitated material was subjected to sonic oscillation (MSE 150 W ultrasonic disintegrater) at 23000 cycles/s with a 3 mm-diameter probe and an amplitude of 8 μm in 3 ml of 1% SDS/0.1 M-NaCl/1 mM-EDTA/10 mM-sodium acetate buffer, pH 4.5, in an ice bath for three bursts of 6 s with 10 s intervals between bursts. This mixture was phenol-extracted by the procedure of Stein *et al.* (1977) except that no preliminary digestion with deoxyribonuclease 1 was performed. Fast-atom-bombardment m.s. was carried out by M-Scan Ltd., Mass Spectrometry Consultants and Analysts, Sunninghill, Ascot, Berks., U.K. PEI-cellulose t.l.c. was initially performed with the alkaline solvent of Norman *et al.* (1974), but this led to breakdown of the extracted nucleotides, whereas more acidic conditions (Randerath & Randerath, 1967) often failed to separate some of the nucleotides (especially GTP from ATP and AMP from ADP) satisfactorily. By manipulation of the acid pH and the electrolyte concentration it proved possible to arrive at a solvent composition, 0.5 M-LiCl/4 M-sodium formate, pH 2, which allowed good separation of adenine nucleotides and guanine nucleotides. The low pH did not lead to any observable nucleotide or nucleoside decomposition over a running period of 5–6 h. The R_F values of a range of nucleotides and their degradation products are given in Table 1. By combining this solvent with the 3.3 M-

‡ Present address: Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.
§ To whom correspondence should be addressed.

Table 1. R_F values for nucleotides and deriatives on PEI-cellulose t.l.c.

The solvent systems were the newly devised 0.5 M-LiCl/4 M-sodium formate, pH 2 and the 3.3 M-ammonium borate, pH 7, solvent of Neuhard *et al.* (1965). Values are also given for oligomeric derivative and its phosphodiester breakdown product.

	R_F	
	$(NH_4)_3BO_3$	$LiCl/HCO_2Na$
ATP	0.26	0.30
ADP	0.40	0.65
AMP	0.50	0.76
GTP	0.13	0.13
IMP	0.64	0.70
Adenosine	0.40	0.73
Adenine	0.28	0.69
Guanosine	0.47	0.63
Hypoxanthine	0.50	0.64
Xanthine	0.25	0.43
Oligomer	0	0
Phosphodiester	0.04	0.68

ammonium borate, pH 7.0, solvent of Neuhard *et al.* (1965) (see also Table 1) in a two-dimensional procedure, a good separation of all the nucleotides and nucleosides listed in Table 1 was achieved, irrespective of the order in which the solvents were used. Where the LiCl/formate was the first solvent the salt can be removed by immersion in methanol for 5 min (Randerath & Randerath, 1967) before running in the second dimension, although this did lead to some loss of sample from the plates.

The silver nitrate stain for reducing sugars was as described by Trevelyan *et al.* (1950), and the modified procedure for organic acids that results in the formation of the silver salt of the anion appearing as a white spot on a darkened background masking any reduced Ag^+ ion was that of Macek (1963).

RESULTS AND DISCUSSION

Purification of the rapidly labelled oligomeric trichloroacetic acid/methanol-insoluble nucleotide derivative

After phenol extraction of the rapidly-^{14}C-labelled species precipitated with denatured heart protein and polynucleotide (see the Experimental section) the products from the aqueous phase of the extraction (contains about 2% protein) were recovered by freeze-drying and subjected to DEAE-cellulose chromatography in the presence of 7 M-urea at pH 4.5. Fig. 1 shows a typical elution profile in which the major labelled fraction is eluted at 0.3 M-NaCl with a specific radioactivity of about 80000 d.p.m./μmol of adenosine, which corresponds to the specific radioactivity of ATP in the trichloroacetic acid-soluble extract (Mowbray *et al.*, 1984). More-extensive sonication of the dried pellet before phenol extraction led to increased quantities of earlier-eluted radioactivity. Less or no sonication produced some radioactive material (specific radioactivity about 24000–30000 d.p.m./μmol of adenosine) in the 2 M-NaCl wash. It is thus not clear whether the material eluted at 0.3 M-NaCl was originally in a more-polymerized

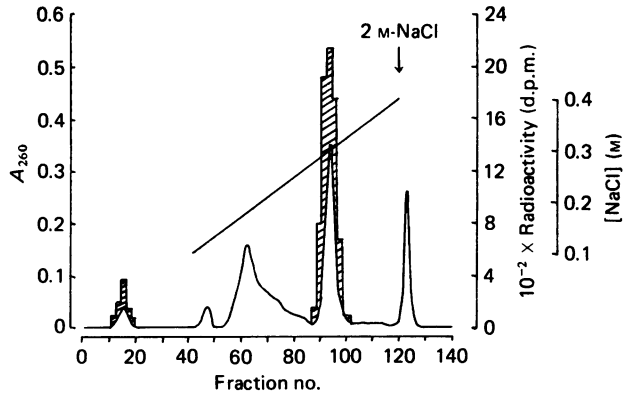


Fig. 1. Anion-exchange chromatography of the oligomeric species

Freeze-dried phenol extract of one heart was dissolved in about 0.4 ml of 1 mM-EDTA/7 M-urea/10 mM-sodium acetate buffer, pH 4.5, and applied to an 18 cm × 1 cm column of DEAE-cellulose and washed on with 30 ml of the buffer. The column was developed with a linear gradient of 0.1–0.4 M-NaCl in the same buffer at 25 ml/h, and 1.2 ml fractions were collected. At 0.4 M-NaCl the salt gradient was replaced by 2 M-NaCl, and a further 31 fractions were collected. Radioactivity was assayed in 50 μl samples.

form or merely attached to the higher-M_r species of RNA that are eluted at 2 M-NaCl. This material eluted at about 0.3 M-NaCl was relatively stable in acid conditions with 7 M-urea present, and was recovered from the combined eluate fractions by precipitation on the addition of 2 vol. of 96% (v/v) ethanol at −18 °C. This species had a phosphate/purine ratio of 4:1 (see Mowbray *et al.*, 1984). Its u.v.-absorption spectrum (not shown) is similar to that of other adenine nucleotides (such as ATP, A3′p5′A, A3′pppp5′A) except that it is slightly shifted to longer wavelengths ($\gamma_{max.}$ = 262 nm).

Identification of the components in the purified labelled material

The labile nature of the purified polyphosphorylated species had caused us to examine its principal breakdown product and to show that it is a phosphodiester of a purine nucleoside and a short carboxylic acid (Mowbray *et al.*, 1984). The precise identity of these components remained to be established.

The purine. A sample (approx. 100 nmol) of the ethanol-precipitated material was washed twice in ethanol, dried in a stream of N_2 and dissolved in 0.5 ml of water. To this was added 1 ml of 1 M-HCl, and the solution was placed in a boiling-water bath for 1 h. The hydrolysate was then freeze-dried and redissolved in 200 μl of sterile water. A volume containing between 5 and 10 nmol was applied to PEI-cellulose t.l.c. plates along with a range of standards in a sterile N_2 environment and developed either in an ammonium borate buffer, pH 7, or in an LiCl/sodium formate buffer, pH2, or in a two-dimensional separation using each buffer in turn (see the Experimental section). In each case all the recovered radioactivity (80–90% of that applied) co-chromatographed with adenine.

The sugar. The incubation procedure used for the liberation of adenine proved to be inadequate for sugar phosphate hydrolysis until the HCl was increased to 1.3 M. Otherwise the conditions were identical and the hydrolysate was freeze-dried and redissolved in water. Between 30 and 40 nmol of sugar was applied to silica-gel plates along with various hexoses, pentoses, sugar phosphates and deoxy sugars. The plates were developed in a butanol/ethanol water (10:1:2, by vol.) system (Spiro, 1960). Staining with silver nitrate (see the Experimental section) revealed two components of which the more strongly staining one (R_F 0.36) corresponded to the position of D-ribose. The faintly stained component (R_F 0.06) appeared only after prolonged heating of the plate and appeared to correspond in position to D-glyceric acid.

The carboxylic acid. Further samples from the sugar hydrolysis incubations were applied as above to silica-gel plates and developed in the butanol/ethanol/water system and also in an ethyl acetate/formic acid/water (3:1:1, by vol.) system (Buch *et al.*, 1952), which allows much further migration of organic acids. Both types of chromatogram were sprayed with Bromophenol Blue (0.05% in ethanol), which detects acid anions as yellow spots on a blue background, and with a modified silver nitrate strain (Macek, 1963; see the Experimental section). With the neutral butanol/ethanol/water system both stains strongly identified an acidic component (R_F 0.06) and the identically placed D-glyceric acid standard. In the acidic system both unknown (R_F 0.50) and the exactly corresponding D-glyceric acid stained weakly with silver nitrate and not at all with Bromophenol Blue.

The ammonium molybdate/ascorbic acid staining procedure of Hanes & Isherwood (1948) showed that none of these components contained residual phosphate.

Thus the evidence so far (see also Mowbray *et al.*, 1984) suggests that the trichloroacetic acid-insoluble derivative contains phosphate, adenosine and glyceric acid in the proportions 4:1:1. The approximate M_r of the material eluted from the ion-exchange column at 0.3 M-NaCl had been estimated at around 2800 (Mowbray *et al.*, 1984), and since no positive indications of other components had been obtained this suggests a possible oligomeric structure containing about four units.

Arrangement of phosphate groups in the compound

Study of the low-M_r breakdown product of the putative oligomeric species by ^{31}P n.m.r. had shown this to be a phosphodiester (Mowbray *et al.*, 1984). Information about the environments of the other phosphate groups associated with each molecule of adenosine and the relative proportions of these were also sought by ^{31}P-n.m.r. spectroscopy. Fig. 2 shows spectra obtained at 4 °C of a sample obtained by combining the three peak fractions of radioactive material eluted from a DEAE-cellulose column (e.g. see Fig. 1) at about 0.3 M-NaCl. These studies were conducted close to the sensitivity limit of the Bruker WM200 spectrometer employed, so fully relaxed spectra could not be obtained. On the basis of a knowledge of the T_1 value of ATP and other phosphorylated metabolites, the conditions under which Fig. 2(*a*) was obtained (60° pulses repeated at 4 s intervals) should give a reasonable approximation to the fully relaxed ideal. On the basis of the shifts of known phosphorylated metabolites (see Gadian, 1982), the resonances in Fig. 2(*a*) are consistent with a phosphodiester, confirming previous observations, and to pyrophosphate groups linked as in the α-, β- and γ-phosphate groups of ATP.

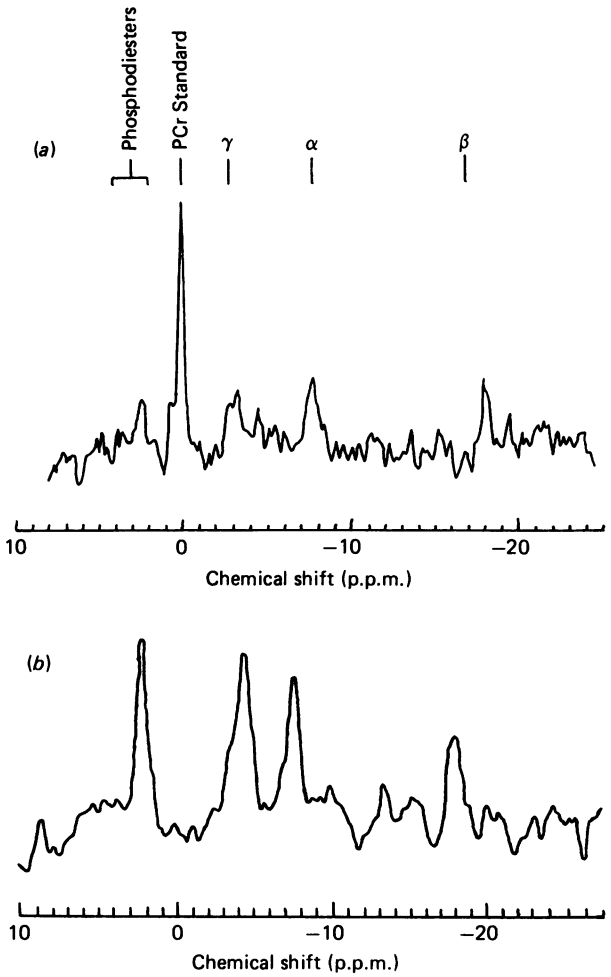


Fig. 2. N.m.r. spectrometry of the oligomeric species

The Figure shows ^{31}P-n.m.r. spectra at 81MHz of the oligomeric species in 0.3 M-NaCl/1 mM-EDTA/7 M-urea/10 mM-sodium acetate buffer, pH 4.5. Spectrophotometric estimation gave the apparent adenosine concentrations of the samples as 64 μM (*a*) and 73 μM (*b*), and the capillary-contained phosphocreatine (PCr) reference was present at an equivalent concentration of 90 μM. (*a*) The pulse interval was 4 s and the pulse angle 60°. The sweep width was 8 kHz and the total number of scans 12000. (*b*) The pulse interval was 2 s and the pulse angle 36 °. The sweep width was 4 kHz and the number scans 180000.

Approx. 15% more material was available from the n.m.r. study that resulted in the spectrum shown in Fig. 2(*b*). This was conducted under conditions where partial saturation of some peaks may occur, but where minor peaks show up maximally, and demonstrates that there are only four distinct environments for the phosphorus nuclei in the sample. Referring the areas under each peak (Fig. 2*a*) to the phosphocreatine standard gives a total phosphate concentration of 210 μM and the relative proportions of each species to be phosphodiester/α-phosphate/β-phosphate/γ-phosphate 1:1:1:1. These results are supported by our finding that after mild alkaline hydrolysis of the trichloroacetic acid precipitate

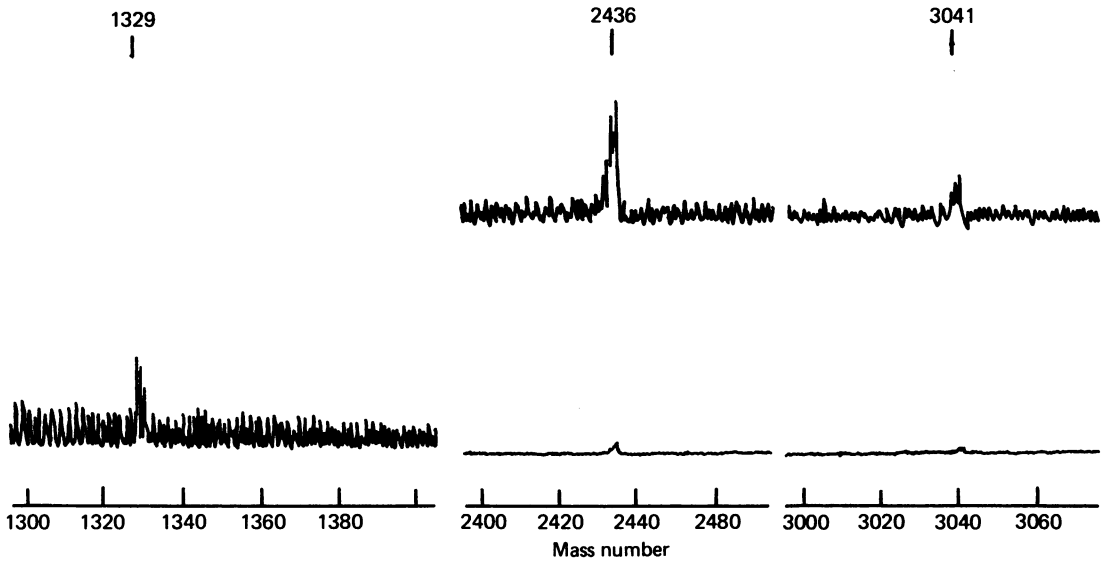


Fig. 3. Fast-atom-bombardment m.s. of the oligomeric species

The Figure shows three sections of the high-field positive-ion spectrum of a sample of the purified oligomer precipitated at −18 °C from column effluent buffer with 2 vol. of ethanol, dried in a stream of N_2, taken up in 100 μl of water and immediately frozen. The mass marker was calibrated against CsI. The upper detector trace is 10 times the sensitivity of the lower one. The approximate proportion of the material detected showing a mass of 1329 was 75–80%, of 2436 was 10% and of 3041 was 5%. Also seen were fragments containing about 1–2% of the material at mass numbers of 2871, 2961, ~ 3227, ~ 3325 and ~ 3378.

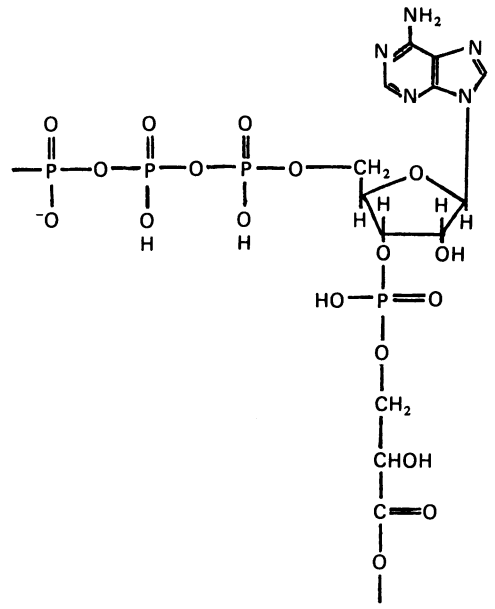


Fig. 4. Repeating subunit of oligomeric species

most of the adenine nucleotide in the hydrolysate is present as ATP when assayed enzymically (Lamprecht & Trautschold, 1974).

M.s. of the putative oligomeric derivative

Although no evidence for components other than adenosine, glycerate and phosphate had been found, an attempt was made to examine the composition of the compound by fast-atom-bombardment m.s. The presence of salt in our sample suppressed ionization and no negative ions were seen. However, a number of peaks were found in the high-field positive-ion spectrum, and these have allowed assessment of the unit M_r and hence comparison with model structures deduced on the basis that the compound is a phosphodiester of ATP and glycerate.

Material from DEAE-cellulose chromatography was precipitated at −18 °C by the addition of 2 vol. of 96% (v/v) ethanol, washed twice in ethanol, dried in a stream of N_2, dissolved in 100 μl of sterile water and snap-frozen before being transported to M-Scan Ltd. packed in solid CO_2. Fig. 3 shows three sections of the positive-ion spectrum. Most of the detected material gave a mass number of 1329 with other substantial peaks at 2436 and 3041: five minor fragments were detected with masses ranging from 2871 to 3378 (see the legend to Fig. 3). Since these values are accurate to within 1–2 mass units, it should be possible to match them all by breaking selected bonds in any proposed oligomeric structure and conversely to eliminate untenable proposals. On the basis that the compound is an oligomer of the unit shown in Fig. 4, it proved possible to suggest molecular structures for the fragments produced by the fast atom bombardment. For example, abbreviating the unit to

A
|
ppp5′R3′pGri

(unit M_r 656), then a dimer

A A
| |
HOppp5′R3′p3Gri1ppp5′R3′pGri

would have a mass of 1329 and a modified trimer of the form

A
|
HO-ppp[5′R3′p3Gri1ppp]$_3$-5′R3′p-OH

would have a mass of 2436. The largest unit, 3378, would correspond to a pentamer

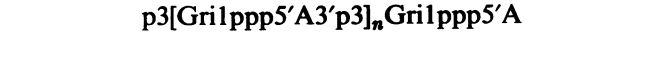
where A represents adenine, R represents ribose and Gri represents glyceric acid.

Ribose phosphate links

The presumption that the triphosphate is linked via the 5'-hydroxy group of the ribose is supported by the finding that alkaline hydrolysis of the oligomer produces enzymically assayable ATP. To discriminate between phosphodiesters at the 2'-hydroxy and 3'-hydroxy groups of ribose 100 nmol of the freeze-dried phosphodiester derived from the oligomer (Mowbray *et al.*, 1984) was dissolved in 60 μl of 83 mM-$MgCl_2$/67μM-Tris/HCl buffer, pH 7.5. To this was added 40 μl of snake-venom diesterase, and the solution was incubated at 37 °C for 30 min before being examined by t.l.c. on both PEI-cellulose and silica gel as described above. The ^{14}C-labelled digestion product co-chromatographed with 5'-AMP, as did samples of ATP digested under the same conditions. Snake-venom diesterases are known to liberate 5'-AMP from ATP and from 3',5'-polynucleotide phosphodiesters (Brownlee, 1972) and to work relatively poorly against 2',5'-phosphodiesters (Kerr & Brown, 1978). Digestion of the phosphodiester with S_1 nuclease in 50 mM-NaCl/1 mM-$ZnSO_4$/5% (v/v) glycerol/30 mM-sodium acetate buffer, pH 4.6, was without effect, confirming the presence of no free ribose 3'-phosphate ester.

Conclusions

At least 10% (Mowbray *et al.*, 1984) and perhaps as much as 30% (Bates *et al.*, 1978) of heart adenine nucleotide is present in an oligomeric form in combination with phosphoglycerate. This compound rapidly exchanges with soluble nucleotides. For comparison total heart RNA contains less than 20% of the adenosine phosphate found in soluble nucleotides. The structure of this oligomeric form appears to be



where the value of n is about 4 for the compound purified as described above.

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