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The discovery of a rapidly metabolized polymeric tetraphosphate derivative of adenosine in perfused rat heart

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The predicted presence in perfused rat hearts of a rapidly metabolized but hitherto unrecognized form of adenosine phosphate has been confirmed by specific radioactive labelling. The properties of the purified compound suggest that it is a heteropolymer of a small organic acid, phosphate and purine nucleoside in the proportions 1:4:1.

Perfused rat hearts extracted under conditions where proper precautions were taken against adenylate kinase or creatine kinase remaining active (Seraydarian *et al.*, 1962; Lowry *et al.*, 1964; Williamson & Corkey, 1969) have been found to show large systematic variations with time in their purine nucleotide contents (Bates *et al.*, 1978). Statistical analysis has shown that the ATP/ADP ratio, as well as the contents of ADP, GTP, cyclic GMP, cyclic AMP and the sum of the contents of ATP, ADP and AMP, oscillate for 40–80 min after perfusion begins (Mowbray *et al.*, 1981). Most surprisingly, it proved impossible to account for large net increases and decreases in total adenine nucleotide content as breakdown and resynthesis of known macromolecules, interconversion with other nucleotides or nucleosides, or degradation to free bases: accordingly we were obliged to propose that some as yet unidentified form of nucleotide must exist which was capable of rapid interconversion with the soluble nucleotides (Bates *et al.*, 1978). Here we report that specific radiolabelling of the soluble nucleotides has led to the isolation and purification of a labile polymeric form of adenine nucleotide which is distinct from nucleic acid and which, in heart, contains around half the quantity of adenine present in tissue RNA (see Bates *et al.*, 1978).

Experimental

Methods

Langendorff perfusion was performed as previously described (Mowbray & Ottaway, 1973*a*; Bates *et al.*, 1978), except that the medium additionally contained 0.01% (w/v) streptomycin sulphate. Hearts were freeze-clamped at liquid-N_2 temperature and extracted in trichloroacetic acid/methanol at −10°C (Mowbray & Ottaway, 1973*b*). The nucleotides, nucleosides and bases were separated by high-performance liquid chromatography (Perrett, 1976) and assayed for ^{14}C by liquid-scintillation counting (Mowbray *et al.*, 1981). The heart nucleotides were radioactively labelled by single wash-through perfusion with medium containing 0.25 μM-[8-^{14}C]adenosine (59 Ci/mol) for 10 min, followed by a further 10 min washout with adenosine-free medium. The trichloroacetic acid/methanol-insoluble radioactivity was determined by washing the precipitate twice with 2 ml of trichloroacetic acid/methanol at −10°C, once with 10 ml of acetone and three times with 10 ml of diethyl ether before drying under a stream of N_2 gas. Weighed amounts (approx. 10 mg) were dissolved overnight in 200 μl of water + 1 ml of NCS solvent, and the ^{14}C was measured after the addition of 4 ml of PCS scintillant. The trichloroacetic acid-insoluble radioactive derivatives from precipitated protein were separated under sterile conditions from the heart precipitate, which had been washed and dried as for scintillation counting. The dried pellet was extracted with phenol, by the procedure of Stein *et al.* (1977), except that no preliminary digestion with deoxyribonuclease 1 was performed. The products from the aqueous phase (< 2% protein) were recovered by freeze-drying. The A_{260} of column effluents was monitored with a Cecil 2012 u.v. monitor (path length 1 cm, 74 μl cell). T.l.c. was carried out as described previously (Hutchinson *et al.*, 1983).

Materials

[8-^{14}C]Adenosine (59Ci/mol), NCS solvent and PCS scintillant were brought from Amersham International, Amersham, Bucks, U.K. DEAE-cellulose was from Whatman, Maidstone, Kent, U.K., streptomycin sulphate from Glaxo Laboratories, Greenford, Middx., U.K., and Sephadex gels were from Pharmacia Fine Chemicals AB, Uppsala, Sweden. All other materials were as previously described (Bates *et al.*, 1978) and were of the highest purity commercially available.

Results and discussion

The discovery of a rapidly labelled trichloroacetic acid-insoluble nucleotide

The nucleotides in heart can be radiolabelled by perfusion with [^{14}C]adenosine (Jacob & Berne, 1960; Liu & Feinberg, 1971; Hutchinson *et al.*, 1981). At concentrations $\leqslant 0.3\,\mu$M, adenosine was found to have no effect on the coronary flow rate or the mechanical performance of the hearts, and tissue radioactivity was shown to increase linearly with time of perfusion with [8-^{14}C]adenosine (Hutchinson *et al.*, 1981). Continued perfusion in the absence of label washes out 96% of the unincorporated precursor in 5min and >99% in 10min. Table 1 shows that, after a labelling period with adenosine containing ^{14}C tracer only in the purine ring, over 95% of the radioactivity in the soluble extract was confined to the adenine nucleotides for up to 50min of subsequent perfusion. The only other compounds containing measurable radioactivity were GTP and IMP.

Radioactivity was also incorporated into the trichloroacetic acid/methanol-insoluble precipitate from these hearts in a form that could not be solubilized by repeated extraction with trichloroacetic acid. The rapidity with which this insoluble material is ^{14}C-labelled (Fig. 1) implies an active exchange with the soluble nucleotides and hence an equilibration of the specific radioactivity between the insoluble and soluble pools. On the basis of the observation that the trichloroacetic acid-insoluble species had apparently reached specific-radioactivity equilibration with soluble nucleotide (see below) and that the content of soluble nucleotide in these hearts was about $4\,\mu$mol/g wet wt. (Fig. 1 legend), a likely estimate for the quantity of this insoluble substance is $0.4\,\mu$mol/g wet wt., given that about 10% of the radioactivity is trichloroacetic acid-insoluble (Fig. 1). The estimated quantity of this insoluble material (and its radioactivity) is rapidly doubled by ischaemia induced by halting perfusion (Hutchinson *et al.*, 1981; W. L. Hutchinson, unpublished work) and is generally found to be increased in groups of hearts in which the average sum of the soluble nucleotides is lowered.

Table 1. *Distribution of soluble radioactivity with time*
After 10min initial washout perfusion and 10min perfusion in the presence of $0.25\,\mu$M-[8-^{14}C]-adenosine, hearts were freeze-clamped, and the nucleotides separated by high-performance liquid chromatography and assayed for ^{14}C. The distributions shown are from the means of three hearts at each time. The radioactivity in 'other' compounds was virtually all in GTP and IMP.

Time after withdrawal of ^{14}C precursor (min)	Percentage of trichloroacetic acid/methanol-soluble radioactivity co-chromatographing with nucleotides			
	ATP	ADP	AMP	Other
10	79.2	14.7	3.8	2.3
20	80.2	11.6	4.0	4.2
30	78.0	15.6	2.7	3.7
50	74.5	18.1	3.8	3.6

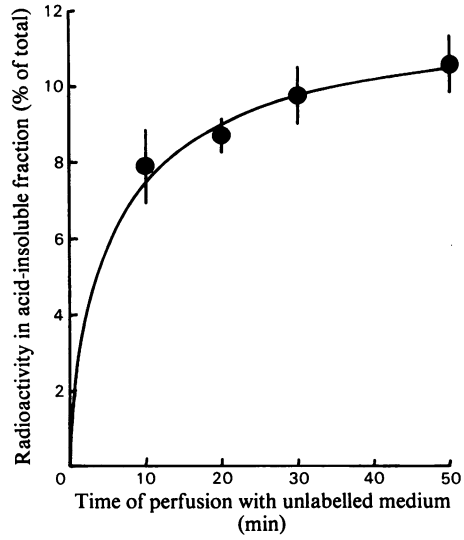

Fig. 1. *Incorporation of [8-^{14}C]purine derivatives into the trichloroacetic acid/methanol-insoluble fraction*
The labelling procedure was as described under 'Methods', and washout with unlabelled medium continued for the time shown. After freeze-clamping and homogenization of the hearts in trichloroacetic acid/methanol, the resulting precipitate was centrifuged down, washed and dried. Weighed amounts (approx. 10mg) were dissolved overnight in NCS solvent, and the samples were assayed for radioactivity. The points are the mean values $\pm$S.E.M. for three hearts at each time. The mean soluble adenine nucleotide content was $4.1 \pm 0.7\,\mu$mol/g wet wt. (S.D.; $n = 12$).

Properties and purification of the trichloroacetic acid/methanol-insoluble purine derivatives

The precipitated radioactivity, although unaffected by repeated acid extraction, was rapidly solubilized by dilute alkali in the cold, a property shared by *O*-phospho-serine or -threonine diesters (Perlmann, 1955) and perhaps by relatively short oligonucleotides or by co-precipitated P^1,P^4-bis(adenosyl) tetraphosphates found as purine-storage compounds in lower orders (Clegg *et al.*, 1967; Warner & McClean, 1968; Goh & Le John, 1977). The alkali-solubilized radioactivity migrates as three distinct spots near to, but less far than, ATP, ADP and AMP respectively on silica-gel t.l.c. (results not shown). A fourth radioactive spot co-migrated with authentic adenosine. Selective extraction of detergent-solubilized precipitate with guanidine hydrochloride (Strohman *et al.*, 1977) or phenol (Stein *et al.*, 1977), techniques designed to separate chromatin protein from polynucleotide, resulted in about 70% of the ^{14}C partitioning with the nucleic acid-rich fraction: re-extraction can remove virtually all the radioactivity from the protein fraction.

When the polynucleotide (aqueous) fraction separated by phenol extraction of the denatured heart precipitate was subjected to gel filtration on Sephadex G-200, two major peaks containing both ^{14}C and purine were found (Fig. 2): one peak is excluded from the gel and thus presumably has M_r 10^5 or more, and the second penetrates approximately to the same extent as mononucleotides.

Rechomatography of the high-M_r species always yielded a proportion of the radioactivity in the low-M_r position. While we were attempting to purify the high-M_r species, it became clear that it breaks down readily to yield low-M_r compounds (see below). T.l.c. and high-voltage electrophoresis of the mononucleotide-like compounds showed that some were more polar at pH8 even than ATP. Hence, on the premise that the high-M_r derivatives would be more polar than the contaminating RNA, an attempt was made to purify them by DEAE-cellulose ion-exchange chromatography. Fig. 3(*a*) shows that under certain conditions this was possible. The finding that the specific radioactivity of this ^{14}C-labelled species was around 75000d.p.m./μmol, which coincides with estimates of the specific radioactivity of the ATP at the time the heart was sampled, suggests that the material is relatively free of other compounds. The apparent M_r was estimated to be about 3000 by Sephadex G-50 chromatography (see Fig. 4 inset) compared with poly(A) standards generously supplied by Dr. P. W. Piper of this Department. Larger radioactive species were also found, which required a much higher NaCl concentration for elution. The purine content of the compound was estimated spectrophotometrically ($\varepsilon_{259} = 15.4 \times 10^3 M^{-1} \cdot cm^{-1}$; Morell & Bock,

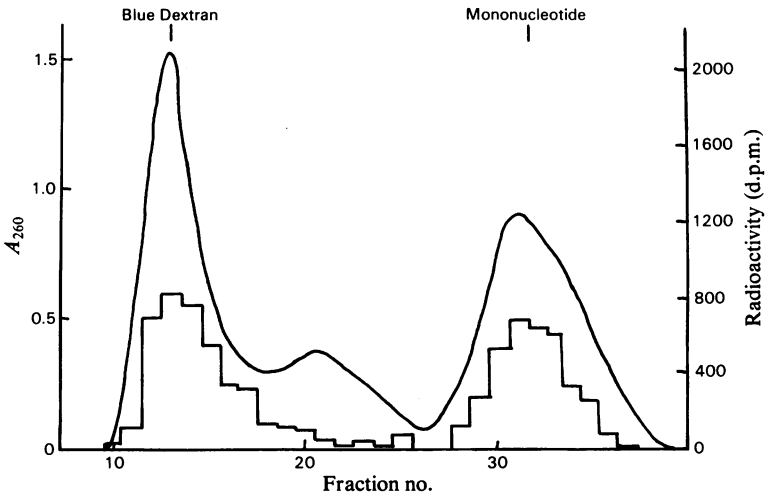

Fig. 2. *Sephadex G-200 chromatography of the trichloroacetic acid/methanol-insoluble radioactivity*
Two hearts (^{14}C-labelled) were homogenized, extracted and the trichloroacetic acid/methanol-insoluble fractions thoroughly washed and dried. All further operations were carried out under sterile conditions. After phenol extraction and freeze-drying (see under 'Methods'), the residue was taken up in 500μl of 8M-urea/0.02% NaN_3/0.5M-sodium acetate buffer, pH4.5. A 300μl portion was applied to a column (18cm × 1cm) of Sephadex G-200 equilibrated with the same buffer and developed at 3.3ml/h. The effluent was monitored at 260nm and 500μl fractions were collected; 100μl samples were assayed for radioactivity.

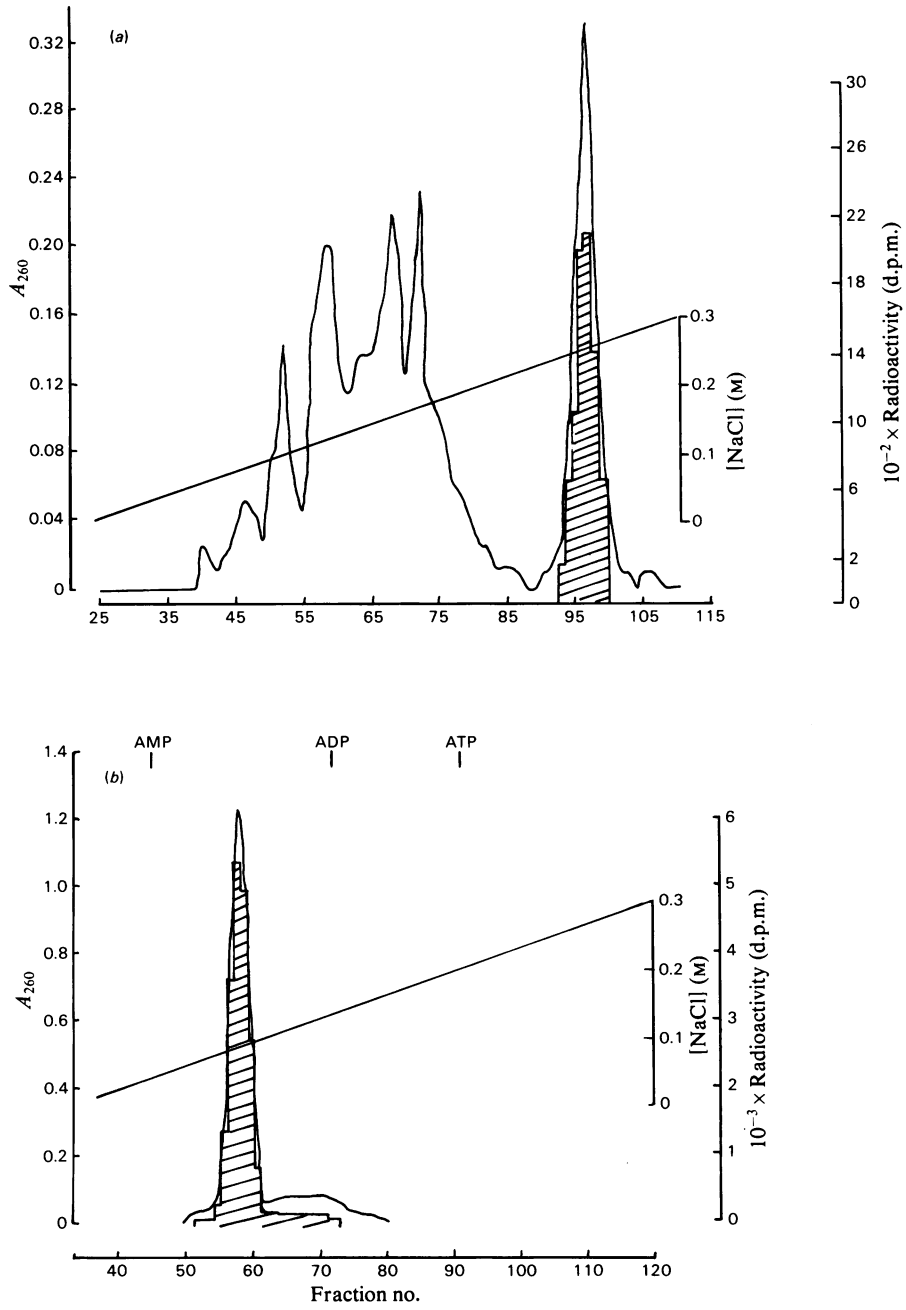

Fig. 3. *Anion-exchange chromatography of the trichloroacetic acid/methanol-insoluble derivatives.*
(*a*) Freeze-dried phenol extract of two hearts was dissolved in 0.3–0.5ml of 1mM-EDTA/7M-urea/10mM-sodium acetate buffer, pH4.5, and applied to a column (18cm × 1cm) of DEAE-cellulose and washed on with 30ml of the EDTA/acetate/urea buffer. The column was developed with a 0–0.3M linear gradient of NaCl in the same buffer at 25ml/h, and 1.2ml fractions were collected. Radioactivity was assayed on 50μl samples. (*b*) An oligomeric sample prepared as in (*a*) was stored for some days before being concentrated by freeze-drying and applied in water to a sterile column (50cm × 3cm) of Sephadex G-10 and eluted with water at 12ml/h. Fractions containing nucleotide were freeze-dried before being taken up in 0.3ml of 1mM-EDTA/7M-urea/10mM-sodium acetate buffer, pH4.5, and chromatographed as in (*a*).

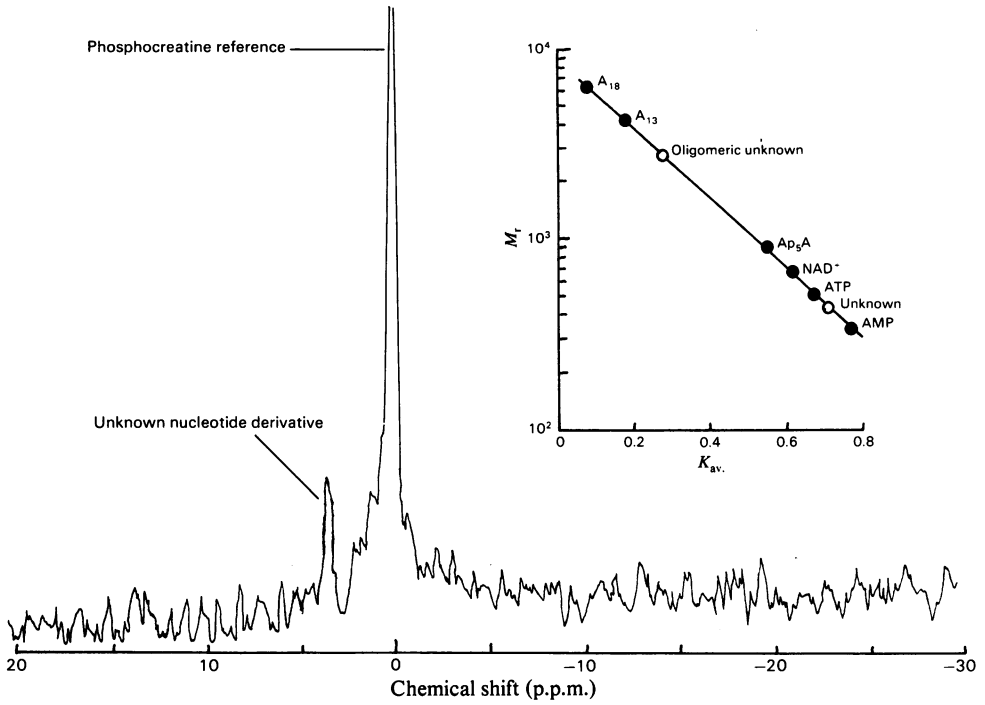

Fig. 4. *Nuclear-magnetic-resonance spectrometry and M_r estimation of the mononucleotide-like end product of the breakdown of oligomeric derivatives*

^{31}P-n.m.r. spectrum at 81 MHz of a 142 μM solution of the mononucleotide-like material shown in Fig. 3(*b*). The pulse interval was 4s and the pulse angle 50°. The sweep width was 10kHz and the total number of scans 14000. The temperature was 4°C. Inset: samples were applied to a sterile column (18cm × 1cm) of Sephadex G-50 in 7M-urea/100mM-sodium acetate buffer, pH 5.5, and developed in the same buffer. Ap_5A represents P^1, P^5-bis(adenosyl) pentaphosphate; A_{18} and A_{13} refer to ^{32}P-labelled poly(A) containing those numbers of units, supplied by Dr. P. W. Piper of this Department. $K_{av.} = (V_e - V_0)/(V_t - V_0)$, where V_e = elution volume, V_0 = void volume and V_t = total packed volume.

1954), and the phosphorus content was assayed by using molybdate after ashing (Ames, 1966). Somewhat surprisingly, the phosphate:purine molar ratio was 4.2:1, compared with values of 2.6:1, 1.4:1 and 0.87:1 for ATP, ADP and AMP standards respectively, and this has been confirmed with several separate preparations. Unfortunately, after desalting this species has proved to be very labile, with a half-life of around 2 weeks at −20°C. However, the mononucleotide-like breakdown product, easily purified (Fig. 3*b*) from these preparations, can give some clues to the structure of the oligomeric species. As Fig. 3(*b*) shows, it is more polar than AMP. Its phosphate:purine ratio is about 1:1, so it presumably contains some other anionic group. Direct estimation (Lloyd, 1966) for $-SO_3^-$ proved negative and, since on t.l.c. the compound migrates well with formic acid/LiCl at pH2 but poorly with borate at pH7 (results not shown), this may be carboxylate. Most interestingly, a ^{31}P-n.m.r. study of this compound (Fig. 4) shows that the phosphate environment is similar to that in a phosphodiester, and this, coupled with an apparent M_r (Fig. 4, inset) of around 110 greater than AMP and the extra anionic character, suggests that this stable breakdown product of the higher-M_r species may contain a short carboxylic acid linked via a phosphate group to purine nucleoside. Alkaline treatment of the labelled oligomeric compound yields a radioactive breakdown product which co-migrates with adenosine on t.l.c. (see above). Since the solvent system used separates adenosine, inosine and hypoxanthine (W. L. Hutchinson, unpublished work), this tentatively suggests that the base in the derivative may be adenine.

^{31}P-n.m.r studies of intact tissues raise the possibility that the adenosine phosphodiester may indeed be a normal metabolic intermediate. Unassigned resonances have been observed in the phosphodiester region of the spectra with rat kidney and heart (Seeley *et al.*, 1977) as well as with

skeletal muscles from rabbit (Seeley *et al.*, 1976), rat (Seeley *et al.*, 1977), frog and toad (Dawson *et al.*, 1977; Burt *et al.*, 1976) and dystrophic chicken (Burt *et al.*, 1976). These are consistent with the much earlier reports of unidentified phosphate esters on paper chromatography of muscle extracts from frog, tortoise and man (Caldwell, 1953; Caldwell & Prankerd, 1954). In trichloroacetic acid-soluble extracts of [8-^{14}C]adenosine-labelled hearts we have observed an unidentified radiolabelled spot which co-migrates with the adenosine phosphodiester on t.l.c. (Hutchinson *et al.*, 1983).

Conclusion

These findings, together with our earlier observations (Bates *et al.*, 1978; Mowbray *et al.*, 1981; Hutchinson *et al.*, 1981), suggest that the soluble adenine nucleotides in heart rapidly interconvert with a highly phosphorylated heteropolymeric form which also involves some other small molecule. The true function of this sequestered nucleotide is at present conjectural, although its increased synthesis during ischaemia and release on reperfusion suggest that it may be part of a homoeostatic mechanism for the ATP/ADP ratio.

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