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CYTOTOXICITY OF DICHLOROMETHANE DIPHOSPHONATE AND OF 1-HYDROXYETHANE-1,1-DIPHOSPHONATE IN THE AMOEBAE OF THE SLIME MOULD *DICTYOSTELIUM DISCOIDEUM*

A ^{31}P NMR STUDY

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Abstract—Two pyrophosphate analogues, dichloromethane diphosphonate (Cl_2MDP), and 1-hydroxyethane-1,1-diphosphonate (EHDP), at concentrations of 0.5–1 mM, efficiently inhibited the growth of amoebae of the slime mould *Dictyostelium discoideum*. Cell viability decreased markedly upon incubation with the diphosphonates. The mechanism of toxicity was investigated by *in vivo* ^{31}P NMR spectroscopy and the formation of analogues of ATP [adenosine 5′-(β,γ-dichloromethane triphosphate) and adenosine 5′-(β,γ-1-hydroxyethane triphosphate)] was demonstrated. These two compounds were identified from their ^{31}P NMR spectra in perchloric acid extracts prepared from amoebae poisoned with Cl_2MDP or EHDP and may have been synthesized by reversible pyrophosphate exchange catalysed by cytosolic aminoacyl-tRNA synthetases.

Inorganic pyrophosphate is a cellular metabolite involved in many biochemical reactions such as the synthesis of proteins, nucleic acids and lipids as well as the generation of cAMP. Among the structural analogues of pyrophosphate, geminal diphosphonates with their characteristic P-C-P group are resistant to metabolic hydrolysis in eukaryotic cells because of their P-C bonds. These molecules have an important medical value in indications such as malignancy-associated hypercalcemia and other metabolic bone diseases [1, 2]. The cytotoxicity of diphosphonates has been investigated in various cell types [3–8]. In the macrophage lineage diphosphonate toxicity appeared while cells were acquiring endocytosis competence and it was proposed that toxicity could be linked to a high endocytic activity [9]. Endocytosis is extremely active in *Dictyostelium discoideum* amoebae and it is the primary feeding mechanism of this organism [10]. We report here that dichloromethane diphosphonate (Cl_2MDP‡) and 1-hydroxyethane-1,1-diphosphonate (EHDP) are cytotoxic towards *Dictyostelium*. By *in vivo* and *in vitro* ^{31}P NMR, we have demonstrated that Cl_2MDP and EHDP lead to the synthesis of nucleoside triphosphate analogues.

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‡ Abbreviations: AppC(CH_3)(OH)p, adenosine 5′-(β,γ-1-hydroxyethane triphosphate); AppCCl_2p, adenosine 5′-(β,γ-dichloromethane triphosphate); Cl_2MDP, dichloromethane diphosphonate; EHDP, 1-hydroxyethane-1,1-diphosphonate; IP_6, inositol hexakisphosphate; MDP, methylene diphosphonate; PCA, perchloric acid.

MATERIALS AND METHODS

Culture conditions. D. discoideum, strain AX2 (ATCC 24397) was grown at 22° in axenic medium [11] containing maltose (18 g/L) and dihydrostreptomycin (0.25 g/L).

Perchloric acid (PCA) extracts. PCA extracts of amoeba suspensions were performed as described previously [12].

^{31}P *NMR spectroscopy. In vivo* ^{31}P NMR spectra of whole cells (3–6 × 10^8/mL) were recorded at 22° on a Bruker AMX 400 WB spectrometer at 162 MHz, in 25 mm diameter tubes in a final volume of 20 mL. Acquisition conditions used 60° (40 μsec) pulses at 1.2 sec intervals with two levels of decoupling. A power level of 1 W was applied during the 0.2 sec acquisition time and a level of 0.5 W during the interpulse delay (1 sec). A WALTZ-16 pulse sequence was used for proton-decoupling. ^{31}P NMR spectra of PCA extracts were acquired in 10 mm diameter tubes on a Bruker WM 250 spectrometer at 101.25 MHz with spinning of the tube at 12 Hz. Acquisition conditions used 60° (10 μsec) pulses at 4 sec intervals with two levels of decoupling. A power level of 0.5 W was applied during the 0.48 sec acquisition time and a level of 0.2 W during the interpulse delay (3.52 sec). As stated above, a WALTZ-16 pulse sequence was used. All chemical shifts are given relative to 85% orthophosphoric acid at 0 ppm. The ^{31}P NMR spectra of diphosphonate

metabolites in PCA extracts were analysed as an AMX or an ABX system [13]. Chemical shifts and coupling constants obtained from the *in vitro* ^{31}P NMR spectra of PCA extracts of cells incubated with Cl_2MDP and EHDP were used for stimulation by an iterative calculation program (PANIC) on the Bruker Aspect 3000 computer.

Free Mg^{2+} measurement in Dictyostelium *amoebae.* Free Mg^{2+} was calculated using the separation of αP- and βP-NTP resonances in the *in vivo* ^{31}P NMR spectra of amoebal suspensions. A value of 50 μM was taken for the dissociation constant of Mg-ATP [14].

RESULTS

Effects of Cl_2MDP and EHDP on Dictyostelium *growth and cellular viability*

The growth of *Dictyostelium* amoebae in axenic medium was measured in the presence of various concentrations of Cl_2MDP and EHDP in the range of 1–4 mM (Fig. 1). Both Cl_2MDP and EHDP inhibited *Dictyostelium* growth. At a concentration of 1 mM, Cl_2MDP or EHDP allowed only two doublings of the cell population. At 4 mM Cl_2MDP or EHDP, growth was completely prevented and cells lysed slowly with a half-time of about 20 hr. The IC_{50} values of the two diphosphonates were close to 0.7 mM. The inhibitory effects of Cl_2MDP and EHDP on growth were not reversible and the two diphosphonates decreased cell viability in a concentration-dependent manner (Table 1). More than 90% of cells died after incubation for 5 hr with either 7.5 mM EHDP or Cl_2MDP.

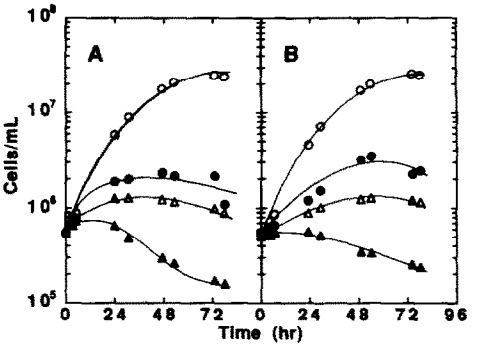


Fig. 1. Effect of Cl_2MDP and EHDP on the growth of *Dictyostelium* amoebae in axenic medium. (A) Cl_2MDP; (B) EHDP. The following concentrations were used: (●) 1 mM, (△) 2 mM, (▲) 4 mM and (○) control without diphosphonate.

^{31}P *NMR characteristics of Cl_2MDP and EHDP*

The variation in chemical shifts (δ) of EHDP and Cl_2MDP was measured in the pH range 4–9, both in the absence of Mg^{2+} and in the presence of excess Mg^{2+}. Apparent pK and limiting δ on the acidic and alkaline sides are indicated in Table 2. Under both conditions, δ (EHDP) was only slightly dependent on pH and the amplitude of variation reached only +0.4 ppm. In contrast, δ (Cl_2MDP) increased markedly with increasing pH and the amplitude of variation reached +1.8 ppm in the absence of Mg^{2+} and +1.0 ppm in the presence of excess Mg^{2+}. Apparent pK values of 5.9 and 7.1 were found in the absence of Mg^{2+} for Cl_2MDP and EHDP, respectively. They corresponded closely to the reported acidic dissociation constants pK_3 = 5.7–6.1 for Cl_2MDP and 6.9–7.3 for EHDP [15–17].

Table 1. Viability of *Dictyostelium* amoebae incubated with Cl_2MDP or EHDP

Concentration (mM)	EHDP	Cl_2MDP
	Viable cells (%)	
0	96 ± 4	
1	72 ± 6	71 ± 12
3	52 ± 18	47 ± 14
4	47 ± 9	31 ± 9
5	21 ± 9	18 ± 3
7.5	7 ± 2	8 ± 2

Dictyostelium amoeba (6×10^5 cells/mL) were incubated for 5 hr in axenic medium in the presence of Cl_2MDP or EHDP at the indicated concentrations. The percentage of viable cells was determined by comparing the number of cells obtained by counting with the Coulter counter to the number of plaques appearing after plating in association with the bacteria *Klebsiella aerogenes*.

Values are means ± SD obtained in four independent experiments.

Table 2. ^{31}P NMR characteristics of Cl_2MDP and EHDP

	pK	A	B
Cl_2MDP	5.87 ± 0.04	8.46 ± 0.03	10.28 ± 0.08
Cl_2MDP-Mg^{2+}	4.99 ± 0.11	8.12 ± 0.06	9.33 ± 0.07
EHDP	7.07 ± 0.14	19.12 ± 0.03	19.48 ± 0.03
EHDP-Mg^{2+}	5.7	18.4	18.8

^{31}P NMR parameters were determined with 5 mM Cl_2MDP or EHDP in 40 mM KCl, 4 mM NaCl, 2.5 mM P_i, 0.2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, containing 6.2 mM $MgCl_2$ for the Mg^{2+} condition and adjusted to required pH values between 4 and 9 with NaOH or HCl. Titration data were fitted to an equation of the form $pH = pK + \log[(\delta - A)/(B - \delta)]$; where δ represents the chemical shift at a given pH and A and B the limiting chemical shifts on the acidic and basic sides, respectively. In the case of EHDP-Mg^{2+}, data points were fitted only in the pH range 4–7.

Values are means ± SD obtained in three independent experiments, except for EHDP-Mg^{2+} (one experiment).

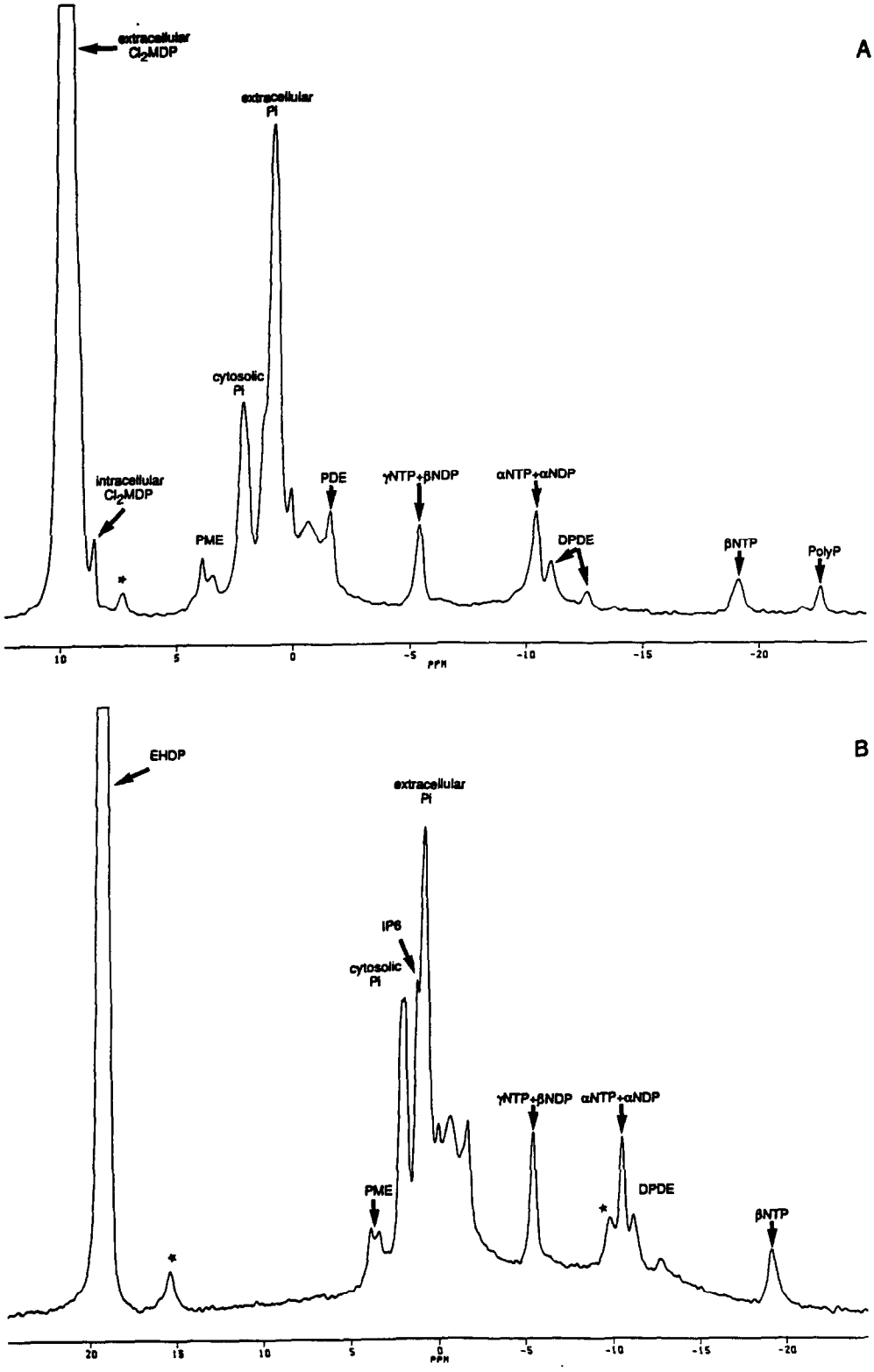


Fig. 2. *In vivo* ^{31}P NMR spectra of *Dictyostelium* amoebae incubated with Cl_2MDP or EHDP. Cells (5×10^8/mL) in 40 mM 2-(*N*-morpholino)-ethanesulfonic acid-Na, pH 5.8, were incubated with 7.5 mM Cl_2MDP (panel A) or 7.5 mM EHDP (panel B) and spectra were recorded on an Bruker AMX 400 WB spectrometer. Free induction decay corresponding to the period 150–255 min was added and transformed with Gaussian multiplication (line broadening = −10 Hz and Gaussian broadening = 0.02). Star symbols (★) correspond to new resonances distinct from parent diphosphonates and arising during incubation.

^{31}P *NMR analysis of* Dictyostelium *amoebae poisoning with diphosphonates*

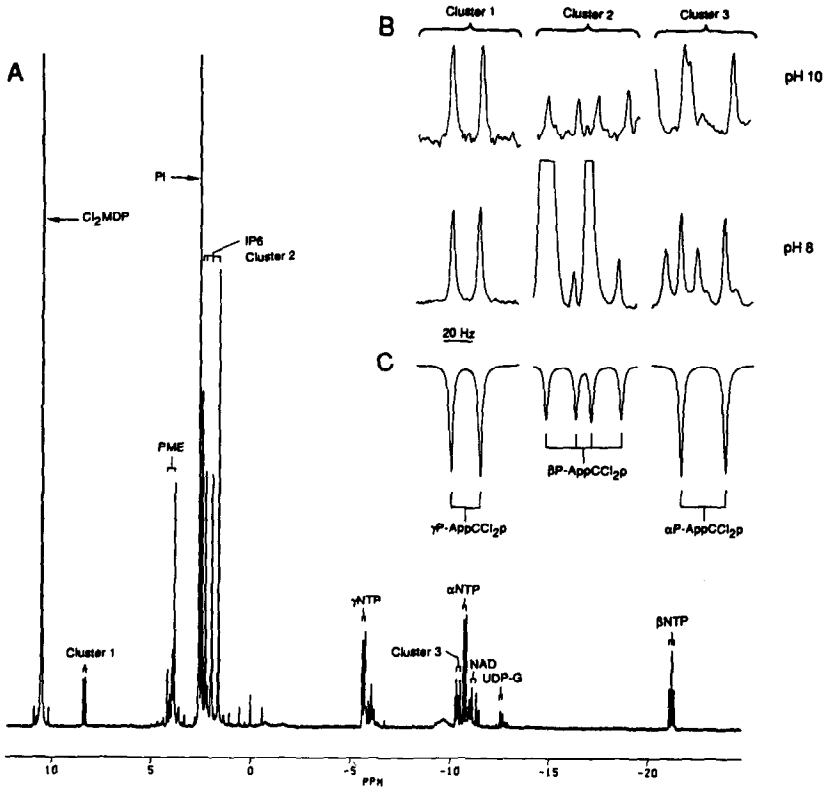
First, *in vivo* ^{31}P NMR spectroscopic analysis was performed to analyse the mechanism of diphosphonate toxicity. The ^{31}P NMR spectrum shown in Fig. 2A corresponds to a suspension of *Dictyostelium* amoebae incubated in the presence of 7.5 mM Cl_2MDP. The resonance arising from extracellular Cl_2MDP itself appears as a huge peak at +9.3 ppm, well separated from the resonances of naturally occurring intracellular phosphorylated metabolites in *Dictyostelium* amoebae, mainly nucleotides, inositol hexakisphosphate (IP_6), phosphomonoesters and P_i [12]. In addition, two smaller resonances were detected: one was located at +8.5 ppm as a shoulder on the extracellular Cl_2MDP peak and the other at +7.3 ppm. Both resonances appeared progressively during incubation and they were demonstrated to be intracellular either on the basis of washing experiments or by addition of a non-permeant paramagnetic broadening agent such as Mn^{2+} (not shown). From the *in vitro* pH titration data (Table 2), the resonance at +8.5 ppm could represent Cl_2MDP in an acidic intracellular compartment [18] and this aspect is currently under investigation. In contrast, the peak at +7.3 ppm resonated quite outside the experimental titration curves for Cl_2MDP or Cl_2MDP-Mg. Thus, it arose from a compound chemically different from Cl_2MDP. Very similar results were obtained upon incubation of *Dictyostelium* amoebae with 7.5 mM EHDP. As illustrated in the *in vivo* ^{31}P NMR spectrum shown in Fig. 2B, the extracellular EHDP line was found at +19.2 ppm and two new, smaller resonances were detected at +15.5 and −9.9 ppm. As discussed above in the case of Cl_2MDP, these two resonances must arise from metabolite(s) chemically different from EHDP.

Identification of new phosphorylated compounds synthesized during Dictyostelium *poisoning with* Cl_2MDP *and EHDP*

To identify potential diphosphonate metabolites, a ^{31}P NMR spectroscopic analysis was performed on PCA extracts obtained from amoebae incubated with Cl_2MDP or EHDP and washed to eliminate the extracellular diphosphonate. The ^{31}P NMR spectrum of a PCA extract of cells incubated for 4 hr with 7.5 mM Cl_2MDP is shown in Fig. 3A. Besides the natural phosphorylated metabolites and the peak at 10.4 ppm corresponding to Cl_2MDP itself, new resonances showed up in three clusters around +8.4, +2.5 and −10 ppm. The isolated doublet (J = 18.5 Hz) around +8.4 ppm (cluster 1) in the spectrum of the PCA extract corresponded to the peak at +7.2 ppm in the *in vivo* spectrum. In the PCA extract at pH 8.3, two peaks were apparent around +2.5 ppm (cluster 2) in a region crowded with the resonances of P_i and IP_6. The pH of the extract was raised to pH 10 to shift all the resonances of IP_6 and P_i around +2.85 ppm. Two more lines that were previously masked were evident at this pH and the cluster appeared as a set of four resonances of equal intensities. Other new resonances around −10 ppm (cluster 3; Fig. 3A) consisted of a doublet (J = 30.3 Hz). The large peak at 10.4 ppm in the extract arose from intracellular Cl_2MDP. The amount of Cl_2MDP was determined from the intensity of the resonance in a fully relaxed spectrum (repetition time 20 sec) and on the basis of a mean cellular volume of 520 μm^3 [19]. It corresponded to an average intracellular concentration of 3.1 ± 0.8 mM (mean ± SD; N = 6). On the basis of earlier work on the toxicity of methylene diphosphonate (MDP) in *Dictyostelium* [20] and on ^{31}P NMR characteristics of β,γ-methylene ATP analogues [21], all new resonances discussed above could be accounted for by the formation of a single metabolite: adenosine 5′-(β,γ-dichloromethane triphosphate) ($AppCCl_2p$). The simulation of a ^{31}P NMR spectrum of this compound as an AMX system with γP (A), βP (M) and αP (X) is shown in Fig. 3C. The simulated ^{31}P NMR spectrum is in full agreement with the observed resonances in the PCA extract and thus confirms the postulated AMX system. Clusters 1, 2 and 3 correspond to the γP, βP and αP atoms of the $AppCCl_2p$, respectively.

A PCA extract of cells incubated for 4 hr in the presence of 7.5 mM EHDP and analysed by ^{31}P NMR showed the natural phosphorylated cellular metabolites and EHDP itself, together with five new resonances around +15 ppm (clusters 4 and 5, Fig. 3A′) and two resonances around −10 ppm (cluster 6, Fig. 3A′). The large peak at +19.3 ppm in the extract arose from intracellular EHDP. The amount of EHDP was determined from the intensity of this resonance in a fully relaxed spectrum (repetition time 20 sec), proton decoupled only during acquisition time (0.48 sec) to avoid NOE effect. It corresponded to an intracellular average concentration of 4.0 ± 0.5 mM (mean ± SD; N = 3).

Fig. 3. Proton decoupled ^{31}P NMR spectra of PCA extracts of *Dictyostelium* amoebae incubated for 4 hr with 7.5 mM Cl_2MDP or EHDP. (A and A′) Free induction decay of 20 hr (Cl_2MDP) (A) or 18 hr (EHDP) (A′) of accumulation was transformed by Fourier transformation. Clusters 1–3 and 4–6 were resonances of the two new phosphorylated compounds (spectra recorded at 22° for Cl_2MDP and 4° for EHDP), respectively. (B and B′) Expansion of ^{31}P NMR spectral regions of the PCA corresponding to clusters 1–3 (B) and 4–6 (B′). Clusters of part B are represented at pH 8 and pH 10 as indicated. The experiment at pH 10 permits resolution of all of the resonances of the βP of $AppCCl_2p$ that are masked by the IP_6 lines at pH 8. The γP (two resonance lines) of $AppCCl_2p$ resonated in cluster 1 at +8.4 ppm, the βP (four resonance lines) resonated in cluster 2 at +2.2 ppm and the αP (two resonance lines) resonated in cluster 3 at −10.4 ppm. In B′, the γP (two resonance lines) and the βP (three resonance lines because the two central ones are superimposed) of $AppC(CH_3)(OH)p$ resonated in cluster 4 and 5 at +15.4 ppm, whereas the αP (two resonance lines) resonated in cluster 6 at −9.9 ppm. (C and C′) PANIC-simulated spectrum of $AppCCl_2p$ (C) and of $AppC(CH_3)(OH)p$ (C′) drawn to the same scale but in an inverted position.


A
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C
Cluster 1
Cluster 2
Cluster 3
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Cl_2MDP
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γNTP
αNTP
βNTP
Cluster 3
NAD
UDP-G
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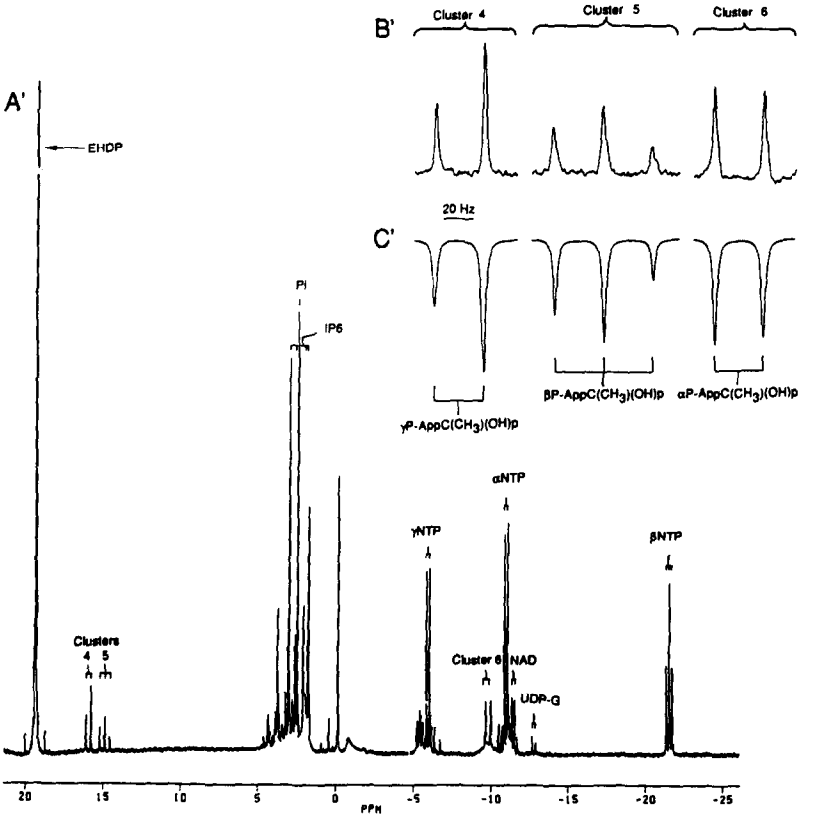
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αP-AppC(CH3)(OH)p
γP-AppC(CH3)(OH)p
20
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-20
-25
PPM

Table 3. Iterated chemical shift (δ) and coupling constant (J) values of $AppCCl_2p$ and $AppC(CH_3)(OH)p$

	$AppCCl_2p$	$AppCCl_2p$*	$AppC(CH_3)(OH)p$
Chemical shifts (ppm)			
$\delta_{P\gamma}$	8.3	8.55	15.8
$\delta_{P\beta}$	2.2	2.45	14.8
$\delta_{P\alpha}$	−10.4	−10.16	−9.8
Coupling constants (Hz)			
$J_{P\alpha,P\beta}$	30.3	30.5	33.2
$J_{P\beta,P\gamma}$	18.5	17.7	34.4

* Taken from [21].

Following similar reasoning to that discussed above for Cl_2MDP, the new resonances are likely to arise from the presence of a single metabolite that could be tentatively identified as adenosine 5′-(β,γ-1-hydroxyethane triphosphate) ($AppC(CH_3)(OH)p$). A simulated NMR spectrum was calculated as an ABX system [13] with γP (A), βP (B) and αP (X) by using the chemical shifts and coupling constants measured in the ^{31}P NMR spectrum of the PCA extract. As shown in Fig. 3B′ and C′, full agreement was obtained between experimental and simulated data. Cluster 4 corresponds to the γP and βP and cluster 5 to the αP of the $AppC(CH_3)(OH)p$. Table 3 summarizes the iterated chemical shifts (δ) and coupling constants (J) of $AppCCl_2p$ and $AppC(CH_3)(OH)p$.

Evolution of phosphorylated metabolites and calculation of free Mg^{2+} concentration during poisoning by Cl_2MDP and EHDP

The formation of the metabolites $AppCCl_2p$ and $AppC(CH_3)(OH)p$ synthesized during incubation with Cl_2MDP or EHDP, respectively, was followed as a function of time. Their intracellular concentrations increased progressively with time. After 3 hr of incubation with diphosphonates they reached a plateau value of 0.20 mM for $AppCCl_2p$ and 0.30 mM for $AppC(CH_3)(OH)p$.

Cl_2MDP and EHDP showed a strong chelating capacity towards divalent cations [15–17]. The entry of Cl_2MDP and EHDP into the cytosol could lead to a decrease in cytosolic free divalent cation concentration and thus perturb cell metabolism. To investigate this point, the free magnesium concentration in *Dictyostelium* cytosol ($[Mg^{2+}]_{free}$) was calculated using *in vivo* ^{31}P NMR spectra of amoeba suspensions during diphosphonate poisoning [22]. Before any addition of diphosphonate, $[Mg^{2+}]_{free}$ was 0.53 ± 0.14 mM (mean ± SD; N = 9). After 3 hr of incubation with 7.5 mM Cl_2MDP or EHDP, $[Mg^{2+}]_{free}$ remained unchanged: 0.50 ± 0.20 mM (mean ± SD; N = 6) or 0.55 ± 0.11 mM (mean ± SD; N = 3), respectively.

DISCUSSION

The two geminal diphosphonates, Cl_2MDP and EHDP, inhibited the growth of *Dictyostelium* amoebae. Their IC_{50} (0.7 mM) was three times lower than those of MDP [20]. Cl_2MDP or EHDP was internalized by the amoebae but the intracellular concentration remained lower than the concentration of diphosphonate in the incubation medium. *Dictyostelium* plasma membrane is devoid of carriers for nutrients such as sugars, amino acids or P_i [10]. Similarly to these compounds, and as shown previously for methyl phosphonate [22] or MDP [20], it is likely that Cl_2MDP and EHDP are internalized by fluid-phase pinocytosis. The mechanisms of toxicity of Cl_2MDP and EHDP in *Dictyostelium* amoebae were investigated by ^{31}P NMR spectroscopy. Progressive synthesis of new intracellular phosphonate derivatives $AppCCl_2p$ and $AppC(CH_3)(OH)p$ was demonstrated during poisoning of cells with Cl_2MDP or EHDP. Their intracellular concentration reached 0.2–0.3 mM. These analogues of ATP may have been synthesized by interaction of diphosphonates with the reversible aminoacyl intermediate of cytosolic aminoacyl-tRNA synthetases [20] and it is possible that they could act as toxic compounds by inhibiting cellular enzymes [23].

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