Incorporation of bisphosphonates into adenine nucleotides by amoebae of the cellular slime mould *Dictyostelium discoideum*

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Bisphosphonates are a class of synthetic pyrophosphate analogues. Some are known to be potent inhibitors of osteoclast-mediated bone resorption *in vivo*, but their mechanisms of action are unclear. The order of potency of bisphosphonates as inhibitors of bone resorption closely matches the order of potency as inhibitors of growth of amoebae of the slime mould *Dictyostelium discoideum*, indicating that bisphosphonates may have a mechanism of action that is similar in both osteoclasts and *Dictyostelium*. Methylenebisphosphonate and several halogenated derivatives, which have low potency as antiresorptive agents and as growth inhibitors of *Dictyostelium*, are metabolized intracellularly by *Dictyostelium* amoebae into methylene-containing adenine nucleotides. We have used a combination of n.m.r. and f.p.l.c. analysis to determine whether incorporation into nucleotides is a feature of other bisphosphonates, especially those that are potent antiresorptive agents. Only bisphosphonates with short side chains or of low potency are incorporated into adenine nucleotides, whereas those with long side chains or of high potency are not metabolized. Bisphosphonate metabolism in cell-free extracts of *Dictyostelium* was accompanied by inhibition of aminoacylation of tRNA by several aminoacyl-tRNA synthetases. These enzymes were barely affected by the bisphosphonates that were not metabolized. The results indicate that some bisphosphonates are not metabolically inert analogues of pyrophosphate and appear to be metabolized by aminoacyl-tRNA synthetases. The cellular effects of some bisphosphonates may be the result of their incorporation into adenine nucleotides or inhibition of aminoacyl-tRNA synthetases, although the potent bisphosphonates appear to act by a different mechanism.

INTRODUCTION

Geminal bisphosphonates are analogues of pyrophosphate in which the labile phosphoanhydride bond (H_2O_3P-O-PO_3H_2) of pyrophosphate is replaced by a stable methylene group (H_2O_3P-CR^1R^2-PO_3H_2) to which two groups (R^1 and R^2) are attached. Since the discovery that bisphosphonates with simple side chains, such as dichloromethylenebisphosphonate (Cl_2MBP) and hydroxyethylidenebisphosphonate (HEBP), are inhibitors of bone resorption *in vitro* and *in vivo* (Fleisch et al., 1969; Russell et al., 1970), a large number of bisphosphonates with different side-chain structures have been synthesized (Fleisch, 1988). Many, especially those with a side chain containing a basic nitrogen group in an alkyl chain or heterocyclic ring, have been found to be even more potent inhibitors of bone resorption (Schenk et al., 1986; Sietsema et al., 1989). As a result, the bisphosphonates have become an important class of therapeutic drugs for the treatment of debilitating metabolic bone disorders including Paget's disease of bone (Meunier and Revault, 1991), hypercalcaemia of malignancy and bone metastases (Adami et al., 1987; Ralston et al., 1989) and postmenopausal osteoporosis (Papapoulos et al., 1992).

The mechanisms by which bisphosphonates inhibit bone resorption remain unclear but it is generally believed that they affect bone-resorbing osteoclasts either by a direct cytotoxic effect or by inhibiting processes vital for bone resorption (Flanagan and Chambers, 1989; Carano et al., 1990; Sato et al., 1991), or through effects on other cells such as osteoclast precursors or even osteoblasts (Hughes et al., 1991; Boonekamp et al., 1986; Sahni et al., 1993). Bisphosphonates have been found to be inhibitors of a number of enzymes, e.g. lysosomal enzymes (Felix et al., 1976) and adenylate cyclase (Pilczyk et al., 1972), and metabolic pathways, e.g. glycolysis (Fast et al., 1978) and sterol biosynthesis (Amin et al., 1992), but it is unclear whether these effects are important *in vivo* for inhibition of bone resorption. As bisphosphonates are analogues of pyrophosphate, they could also act by taking part in metabolic reactions in place of pyrophosphate, although it has usually been concluded that bisphosphonates are metabolically inert (Fleisch, 1981). However, methylenebisphosphonate (MBP) inhibits growth of amoebae of the cellular slime mould *Dictyostelium discoideum* and is incorporated intracellularly into the β,γ-methylene analogue of ATP ($AppCH_2p$) and also into an analogue of 5′,5‴-diadenosyl-P^1,P^4-tetraphosphate (Ap_4A) ($AppCH_2ppA$) (Klein et al., 1988). The nucleotide analogues were identified in intact cells and cell extracts by ^{31}P n.m.r. It has since been shown, by using a combination of n.m.r. and anion-exchange f.p.l.c., that simple halogenated bisphosphonates, including Cl_2MBP, are also met-

Abbreviations used: 3-PHEBP, 2-(3-pyridinyl)-1-hydroxyethylidene-1,1-bisphosphonic acid; ABuBP, 4-aminobutylidene-1,1-bisphosphonic acid; AHBuBP, 4-amino-1-hydroxybutylidene-1,1-bisphosphonic acid; AHPrBP, 3-amino-1-hydroxypropylidene-1,1-bisphosphonic acid; Ap_4A, 5′,5‴-diadenosyl-P^1,P^4-tetraphosphate; $AppCH_2p$, adenosine 5′-[β,γ-methylene]triphosphate; $AppCH_2ppA$, diadenosine 5′5‴-P^1,P^4-[P^2,P^3-methylene]-tetraphosphate; Cl_2MBP, dichloromethylene-1,1-bisphosphonic acid; CPTMBP, chloro-4-phenylthiomethylene-1,1-bisphosphonic acid; EBP, ethylidene-1,1-bisphosphonic acid; F_2MBP, difluoromethylenebisphosphonic acid; FMBP, monofluoromethylenebisphosphonic acid; HEBP, 1-hydroxyethylidene-1,1-bisphosphonic acid; HMBP, 1-hydroxymethylene-1,1-bisphosphonic acid; MBP, methylene-1,1-bisphosphonic acid; PCCP, ethane-1,2-bisphosphonic acid; PCPCP, bis-(phosphonomethyl)phosphinic acid; PMMP, phosphonomethylenemethylphosphinic acid; VBP, 1-ethenylidene-1,1-bisphosphonic acid.

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Table 1 Structure of bisphosphonate side chains (R^1 and R^2, attached to the geminal carbon atom) and potency as inhibitors of *Dictyostelium* growth of bisphosphonates examined for incorporation into either AppCp or AppCppA nucleotides by intact *Dictyostelium* amoebae or cell-free extracts of *Dictyostelium*

In addition, PMMP (IC_{50} > 4 mM) and PCPCP (IC_{50} > 2 mM) were metabolized to AppCp-type nucleotides. By contrast with our observations, HEBP has also been reported by Pelorgeas et al. (1992) to be incorporated into an AppCp nucleotide by *Dictyostelium*. IC_{50} values are taken from Rogers et al. (1994).

Abbreviation (or generic name)	R^1	R^2	IC_{50} (μM)	Metabolized to AppCp nucleotide	Metabolized to AppCppA nucleotide
MBP	H	H	2200	+	+
F_2MBP	F	F	2700	+	+
Cl_2MBP (clodronate)	Cl	Cl	480	+	−
HMBP	OH	H	600	+	+
EBP	CH_3	H	1100	+	−
VBP	CH_2=		1000	+	−
HEBP (etidronate)	CH_3	OH	380	−	−
CPTMBP (tiludronate)	Chloro-4 phenylthio	H	3100	+	−
AHPrBP (pamidronate)	$[CH_2]_2NH_2$	OH	167	−	−
ABuBP	$[CH_2]_3NH_2$	H	44	−	−
AHBuBP (alendronate)	$[CH_2]_3NH_2$	OH	32	−	−
3-PHEBP (risedronate)	CH_2-pyridyl	OH	14	−	−

abolized to nucleotide analogues by *Dictyostelium* amoebae (Rogers et al., 1992a). Pelorgeas et al. (1992) have reported that another bisphosphonate in clinical use (HEBP), as well as Cl_2MBP, is also incorporated into an ATP analogue by *Dictyostelium*.

It is probable that bisphosphonates are incorporated into analogues of ATP by *Dictyostelium* by the back reactions catalysed by aminoacyl-tRNA synthetases, as MBP has been found to be incorporated into $AppCH_2p$ by the back reaction of purified lysyl-tRNA synthetase from *Escherichia coli* (Zamecnik and Stephenson, 1969).

(i) E + amino acid + ATP $\rightleftharpoons$ E–amino acid–AMP + PP_i

(ii) E–amino acid–AMP + tRNA $\rightarrow$ E + aminoacyl-tRNA + AMP

(iii) E + amino acid + $AppCH_2p$ $\leftarrow$ E–amino acid–AMP + MBP

(iv) E + amino acid + AppppA $\leftarrow$ E–amino acid–AMP + ATP

(v) E + amino acid + $AppCH_2ppA$ $\leftarrow$ E–amino acid–AMP + $AppCH_2p$

It appears that the aminoacyl-tRNA synthetases can catalyse a series of reactions. First, they catalyse activation of amino acids in reactions with ATP to give enzyme-bound aminoacyladenylates and pyrophosphate (reaction i). Each aminoacyladenylate then usually reacts with the appropriate tRNA in a second enzyme-catalysed reaction (ii) giving aminoacyl-tRNA and AMP. However, the reaction leading to formation of aminoacyladenylate is freely reversible in the absence of pyrophosphatase and the back reaction is not specific for pyrophosphate but may use MBP to give $AppCH_2p$ (reaction iii), ATP to give AppppA (reaction iv) or $AppCH_2p$ to give $AppCH_2ppA$ (reaction v). Formation of aminoacyl-tRNA can therefore be inhibited by compounds such as MBP as they compete with tRNA for reaction with aminoacyladenylate.

We have found that a wide range of bisphosphonates have growth-inhibitory and cytotoxic effects on *Dictyostelium* amoebae (Rogers, 1993; Rogers et al., 1994). Furthermore, the order of potency of these compounds as inhibitors of growth of *Dictyostelium* closely matches their order of potency as inhibitors of bone resorption *in vivo* (Rogers et al., 1994). We have therefore been investigating the mechanisms by which bisphosphonates affect *Dictyostelium* growth, as these may be similar to the mechanisms by which they affect osteoclasts and inhibit bone resorption. Although it has already been shown that MBP and a few halogenated bisphosphonates (which are of low antiresorptive potency) can be metabolized by *Dictyostelium* to nucleotide analogues (Rogers et al., 1992a; Pelorgeas et al., 1992), we have now examined whether *Dictyostelium* is capable of metabolizing other bisphosphonates (especially the most potent antiresorptive ones with larger side chains) to determine whether their effects on cell proliferation and viability, and hence inhibition of bone resorption, may be the result of their incorporation into nucleotide analogues. We have also investigated whether bisphosphonate metabolism in cell-free extracts of *Dictyostelium* is associated with inhibition of tRNA aminoacylation by any of the aminoacyl-tRNA synthetases, as this would support the view that these enzymes are responsible for bisphosphonate metabolism in *Dictyostelium*.

EXPERIMENTAL

Chemicals

MBP was from Sigma. 3-Amino-1-hydroxypropylidene-1,1-bisphosphonic acid (AHPrBP) and 4-amino-1-hydroxybutylidene-1,1-bisphosphonic acid (AHBuBP) were from Gentili S.p.A., Pisa, Italy. Chloro-4-phenylthiomethylene-1,1-bisphosphonic acid (CPTMBP) was obtained from Sanofi Recherche, Montpellier, France. Difluoromethylenebisphosphonic acid (F_2MBP), 1-ethenylidene-1,1-bisphosphonic acid (VBP), ethane-1,2-bisphosphonic acid (PCCP) and bis-(phosphonomethyl)phosphinic acid (PCPCP) were synthesized as described previously (Rogers et al., 1994). All other bisphosphonates were from Procter and Gamble Pharmaceuticals, Cincinnati, OH, U.S.A. The bisphosphonate structures are shown in Table 1. All other chemicals were from Sigma. ^{3}H-labelled amino acids were purchased from NEN. [^{35}S]Cysteine and [^{14}C]asparagine were from Amersham International.

Growth of *D. discoideum*

Amoebae of the strain Ax-2 were grown axenically in HL5-glucose medium as previously described (Watts and Ashworth, 1970). Cultures were inoculated at an initial density of 10^4 cells/ml.

Identification of metabolites in amoebae incubated with bisphosphonates

To examine which bisphosphonates are metabolized by *Dictyostelium* amoebae, perchloric acid extracts were prepared from amoebae that had been incubated in 5 mM bisphosphonate for 2 h, as previously described by Rogers et al. (1992a).

^{31}P n.m.r. spectra were collected at 20 °C on a Bruker AMX-500 spectrometer as described previously (Rogers et al., 1992a). Signals in the n.m.r. spectra were identified by comparison with spectra of authentic compounds wherever possible. For f.p.l.c. analysis, 50 μl samples of extracts prepared from *Dictyostelium* cells were loaded on to a 1 ml Mono Q anion-exchange column (Pharmacia), and eluted in a gradient of NH_4HCO_3 (120–360 mM NH_4HCO_3 over 17 min followed by 360–120 mM NH_4HCO_3 over 3 min; 1.5 ml/min). Eluted nucleotides were detected by their A_{254}, and were identified by comparison with the retention times of standards and by spiking extracts with authentic compounds wherever possible. By using f.p.l.c., adenine nucleotides at concentrations of approx. 1 μM or more could be detected in the perchloric acid extracts.

Identification of metabolites in cell-free extracts incubated with bisphosphonates

The ability of cell-free extracts of amoebae to metabolize bisphosphonates was examined by f.p.l.c. analysis of these extracts after incubation with bisphosphonates. Identification of peaks representing bisphosphonate metabolites in the f.p.l.c. profiles was aided by removing some of the major nucleotides, especially ADP and ATP, after the incubation. This was achieved by adding apyrase, as this enzyme sequentially cleaves the 5′-phosphate groups from ATP and ADP to form AMP. AppCp nucleotides containing a β,γ-methylene group are resistant to the action of apyrase. Thus new nucleotide peaks eluted after AMP during f.p.l.c. analysis represented bisphosphonate metabolites.

Cell extracts were prepared from approx. 10^9 amoebae that had been washed twice in 20 mM Mes/NaOH buffer, pH 6.3, at 4 °C. The cell pellet was made up to 10 ml with 50 mM Tris/HCl buffer containing 1 mM EDTA, 0.5 mM dithiothreitol, 15 mM KCl, 10 mM $MgCl_2$ and 50% (v/v) glycerol, final pH 7.4, at 4 °C, and sonicated five times (15 s each) at 4 °C with an MSE Soniprep sonicator at maximum power. Cell membranes and debris were removed by centrifugation at 10000 *g* for 15 min, and the supernatant was centrifuged again at 100000 *g* for 2 h. The supernatant (5 ml) was removed and dialysed at 4 °C for at least 12 h against 500 ml of 10 mM Tris/HCl buffer containing 10 mM $MgCl_2$, 15 mM KCl, 1 mM EDTA, 0.5 mM dithiothreitol and 50% (v/v) glycerol, final pH 7.4. The final dialysed cell extract was stored at −20 °C.

Cell extract (20 μl) was mixed thoroughly with 20 μl of 2.65 mM bisphosphonate in 20 mM Tris/HCl, pH 7.4, and 40 μl of a solution containing 133 mM Tris/HCl, 40 mM $MgCl_2$, 132 mM KCl, 13 mM ATP, 1 mM CTP, 2.5 mM dithiothreitol and 0.3 mM EDTA in 50% glycerol, pH 7.4. The final concentrations of the components in the mixture were 500 μM bisphosphonate, 5 mM ATP, 0.35 mM CTP, 15 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, 0.10 mM EDTA and 52 mM Tris. These conditions have previously been used to measure aminoacyl-tRNA synthetase activity (Mathews et al., 1984). Each mixture was incubated at 22 °C for 2 h and after addition of 10 units of apyrase for a further 1 h at 22 °C. After the addition of 10 μl of 70% (w/v) perchloric acid to precipitate proteins, the mixtures were neutralized and treated as described previously (Rogers et al., 1992a). Samples (25 μl) were then analysed by ion-exchange f.p.l.c. as described above.

Assay of tRNA aminoacylation by a cell-free extract of *Dictyostelium* amoebae

The assay of tRNA aminoacylation was adapted from the method of Mathews et al. (1984). Solution A contained 133 mM Tris/HCl, 40 mM $MgCl_2$, 132 mM KCl, 13 mM ATP, 1 mM CTP, 2.5 mM dithiothreitol and 0.3 mM EDTA in 50% glycerol, pH 7.4. Tubes containing 100 μg of bovine liver tRNA (in 10 μl of 10 mM Tris/HCl, pH 7.4), 10 μl of 2.65 mM bisphosphonate in 10 mM Tris/HCl, pH 7.4, 3 μl of one ^{3}H-labelled L-amino acid (or L-[^{35}S]cysteine or L-[^{14}C]asparagine) (approx. specific radioactivity 2400 GBq/mmol for ^{3}H-labelled amino acids, 8.4 GBq/mmol for [^{14}C]asparagine, 5.5 GBq/mmol for [^{35}S]cysteine), and 20 μl of solution A were preincubated at 25 °C for 5 min. Dialysed cell-free extract (10 μl) was then added and the samples were mixed thoroughly before incubation at 25 °C. The final concentrations of assay components were approx. 1 μM radiolabelled amino acid, 500 μM bisphosphonate, 5 mM ATP, 0.35 mM CTP, 15 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, 0.10 mM EDTA and 52 mM Tris.

After 15 min, the contents of each tube were transferred to 21 mm-diameter Whatman 3MM filter circles. The filters were allowed to absorb the solution (approx. 1 min) and were then dropped into 150 ml of ice-cold 10% (w/v) trichloroacetic acid to precipitate the aminoacyl-tRNA complex on to the filter. After 30 min the filters were transferred to 150 ml of fresh 10% (w/v) trichloroacetic acid for a further 30 min, followed by two similar washes in 150 ml of ice-cold 5% (w/v) trichloroacetic acid. Finally, the filters were left for 10 min in 50 ml of ice-cold ethanol and dried under an i.r. lamp for 30 min. Radioactivity on filters suspended in 5 ml of Scintran O scintillation fluid (BDH) was determined in a Philips liquid-scintillation counter. Control incubations were set up as follows: control 1, tubes contained 10 μl of 10 mM Tris/HCl, pH 7.4, instead of 500 μM bisphosphonate to allow assay of enzyme activity in the absence of bisphosphonate; control 2, tubes lacked ATP, tRNA (replaced with 10 μl of 10 mM Tris/HCl) and bisphosphonate (replaced with 10 μl of 10 mM Tris/HCl) to allow measurement of the background radioactivity resulting from binding of material other than aminoacyl-tRNA to filters.

The activity of each enzyme in the presence of bisphosphonate was expressed as a percentage of the activity in the absence of bisphosphonate, i.e.

$$\frac{\text{c.p.m.}_{\text{(with bisphosphonate)}} - \text{c.p.m.}_{\text{(control 2)}}}{\text{c.p.m.}_{\text{(control 1)}} - \text{c.p.m.}_{\text{(control 2)}}} \times 100$$

RESULTS

Identification of bisphosphonate metabolites by n.m.r. spectroscopy

^{31}P n.m.r. analysis of cell extracts has previously been used to demonstrate incorporation of bisphosphonates into nucleotides (Klein et al., 1988; Rogers et al., 1992a; Pelorgeas et al., 1992) because most of the signals due to bisphosphonates and their metabolites are easily identified. They generally occur downfield in the spectrum, away from the signals due to naturally occurring phosphates (mostly nucleoside di- and tri-phosphates, P_i, inositol hexaphosphate, phosphomono- and phosphodi-esters).

An extract made from amoebae that had been incubated in ethylidene-1,1-bisphosphonic acid (EBP) exhibited, in addition to the bisphosphonate signal at +22 p.p.m., peaks at +19 p.p.m. (a doublet) and +17.5 p.p.m. (a singlet), together with a cluster of peaks at −10 p.p.m. (Figure 1a) which were absent from the control. By comparison with the spectrum of AppCp in a cell extract from amoebae incubated in MBP (Klein et al.,

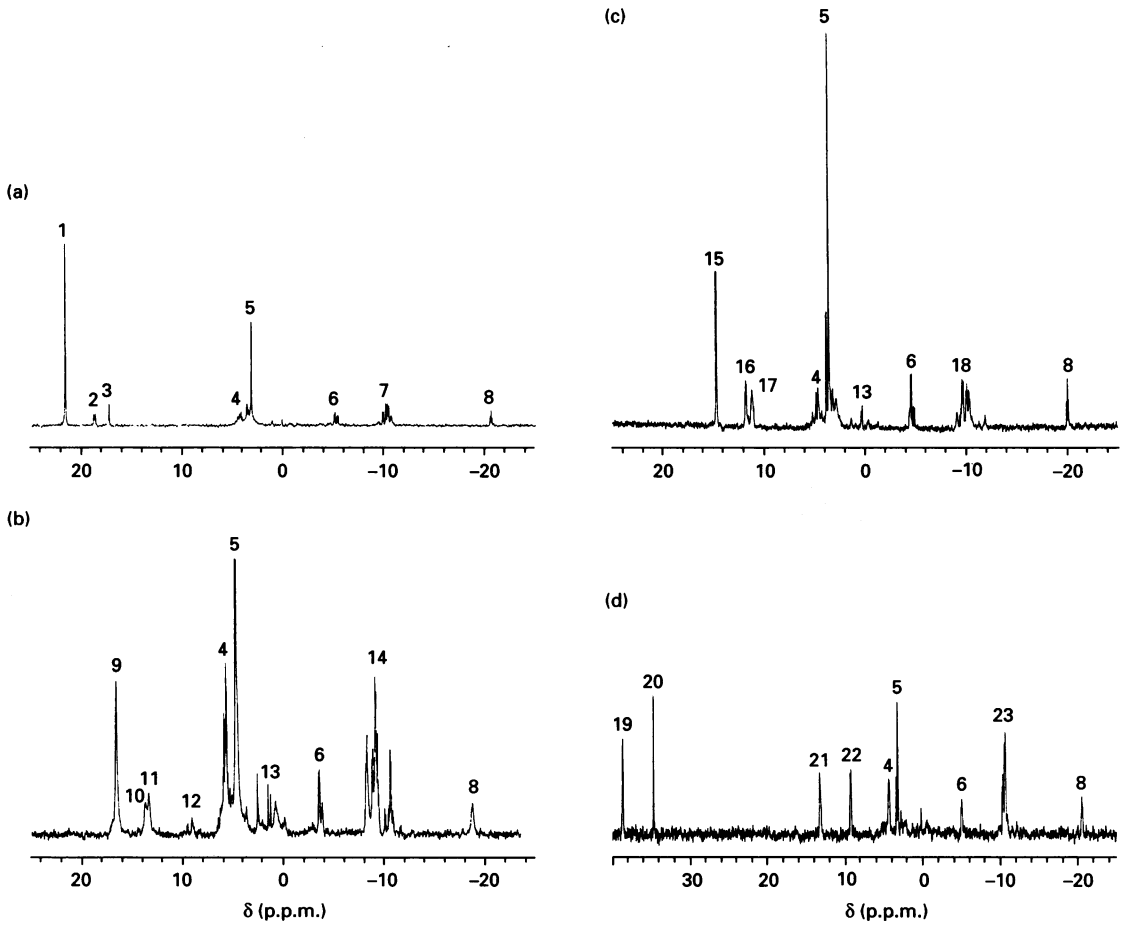

Figure 1 ^{31}P n.m.r. spectra of perchloric acid extracts from *Dictyostelium* amoebae incubated with bisphosphonates

Extracts were prepared from approx. 10^{10} intact amoebae that had been incubated for 2 h with 5 mM EBP (**a**), HMBP (**b**), CPTMBP (**c**) or PMMP (**d**). After incubation, protein was precipitated by the addition of perchloric acid and the supernatants were neutralized with a saturated solution of $KHCO_3$. Assignments of the major phosphorus-containing compounds are: (**a**) 1, EBP; 2, β-phosphonate of AppCH(CH_3)p; 3, γ-phosphonate of AppCH(CH_3)p; 4, phosphomonoesters; 5, P_i; 6, β-phosphate of nucleoside diphosphates and γ-phosphate of nucleoside triphosphates; 7, α-phosphate of AppCH(CH_3)p and nucleoside triphosphates; 8, β-phosphate of nucleoside triphosphates; (**b**) 9, HMBP; 10, β-phosphonate of AppCH(OH)p; 11, γ-phosphonate of AppCH(OH)p; 12, β-phosphonate of AppCH(OH)ppA; 13, phosphodiesters; 14, α-phosphate of AppCH(OH)p and nucleoside triphosphates; (**c**) 15, CPTMBP; 16, γ-phosphonate of AppCH(SC_6H_5Cl)p; 17, β-phosphonate of AppCH(SC_6H_5Cl)p; 18, α-phosphate of AppCH(SC_6H_5Cl)p and nucleoside triphosphates; (**d**) 19, phosphinate group of PMMP; 20, γ-phosphinate of AppCH$_2$p(CH_3); 21, phosphonate group of PMMP; 22, β-phosphonate of AppCH$_2$p(CH_3); 23, α-phosphate of AppCH$_2$p(CH_3) and nucleoside triphosphates.

1988; Rogers et al., 1992a), the resonances at +19 p.p.m., +17.5 p.p.m. and −10 p.p.m. observed in the cell extract after incubation of amoebae in EBP would appear to represent the β- and γ-phosphonates and α-phosphate respectively of an analogue of ATP in which the P_β-O-P_γ group has been replaced by P_β-CH(CH_3)-P_γ, i.e. EBP was metabolized to AppCH(CH_3)p.

Similarly, an extract prepared from amoebae that had been incubated in 1-hydroxymethylene-1,1-bisphosphonic acid (HMBP) (Figure 1b) contained the bisphosphonate peak at +16 p.p.m., two poorly resolved peaks at +12 p.p.m. and a cluster of peaks around −10 p.p.m. These probably arise from the β- and γ-phosphonates (at +12 p.p.m.) and α-phosphate (at −10 p.p.m.) of an analogue of ATP in which the P_β-O-P_γ group has been replaced by P_β-CH(OH)-P_γ, i.e. the HMBP metabolite was AppCH(OH)p. Smaller peaks at about +8 p.p.m. may be attributable to the β-phosphonate of AppCH(OH)ppA.

No AppCp or AppCppA metabolites of HEBP (which differs from HMBP by the presence of a methyl group on the geminal bisphosphonate carbon atom) or potent growth-inhibitory bisphosphonates with large side chains, such as AHPrBP, AHBuBP, 4-aminobutylidene-1,1-bisphosphonic acid (ABuBP) or 2-(3-pyridinyl)-1-hydroxyethylidene-1,1-bisphosphonic acid (3-PHEBP), could be detected either after a 2 h incubation of amoebae in 5 mM bisphosphonate or by growing cells for several days in low sublethal concentrations of bisphosphonate, although the intact bisphosphonates could be identified in the cell extracts. However, CPTMBP (a far less potent non-hydroxylated geminal bisphosphonate with a large chloro-4-phenylthiomethylene side chain) did appear to be incorporated into an AppCH(SC_6H_4Cl)p analogue of ATP. The ^{31}P n.m.r. spectrum of an extract of amoebae that had been incubated in 5 mM CPTMBP for 2 h contained two new clusters of peaks at +11 p.p.m. and +12 p.p.m., and the bisphosphonate peak at +14 p.p.m. (Figure 1c). Two-dimensional ^{31}P–^{31}P correlated spectroscopy indicated that the cluster at +11 p.p.m. was coupled to a cluster at −10 p.p.m. and to the cluster at +12 p.p.m., whereas the +12 p.p.m. cluster was coupled only to the +11 p.p.m. cluster. This agrees with the expected coupling in an AppCp-type nucleotide of the β-phosphonate (+11 p.p.m.) to both the α-phosphate (−10 p.p.m.) and the γ-phosphonate (+12 p.p.m.), and the

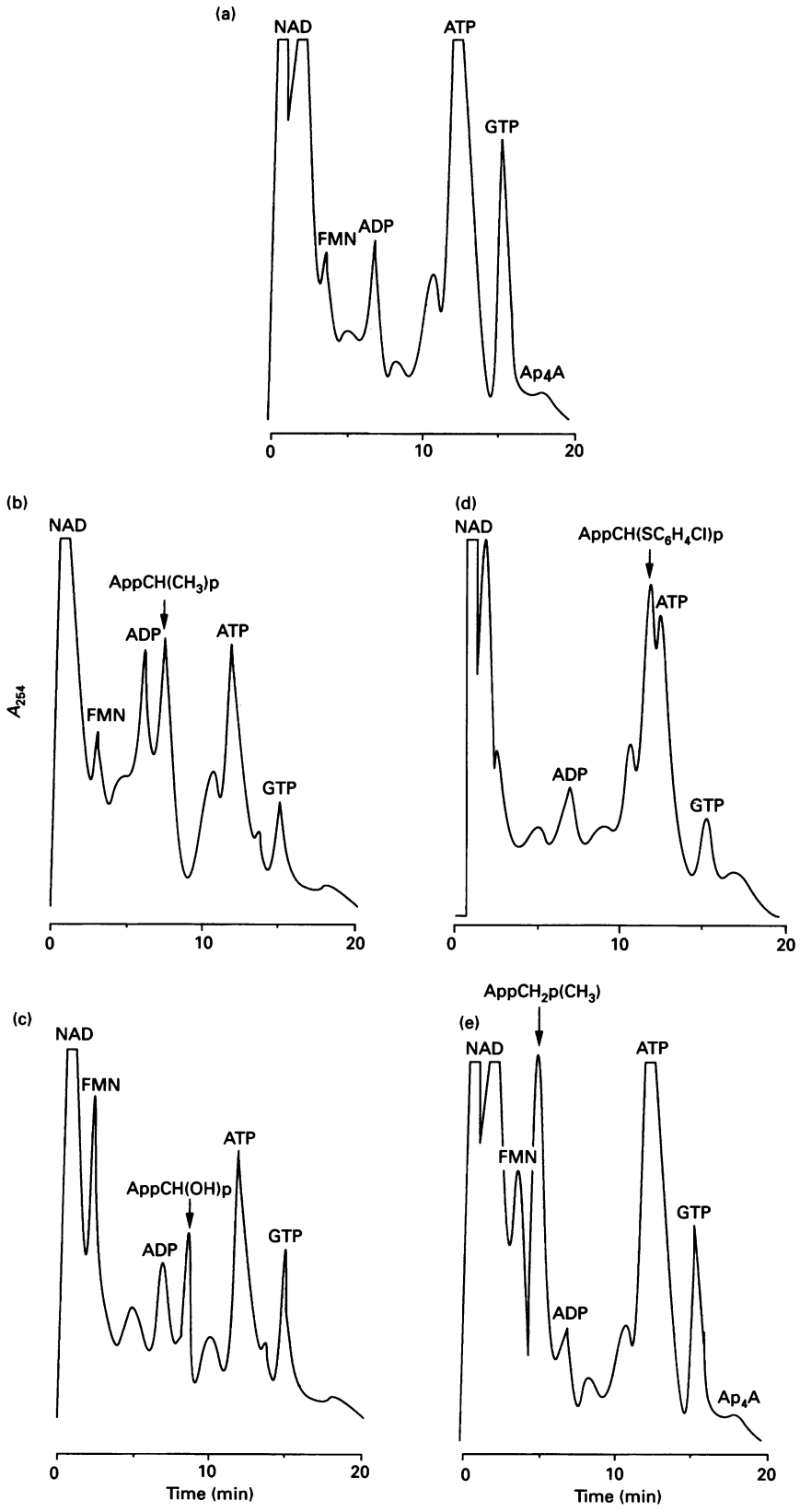

Figure 2 F.p.l.c. elution profiles of perchloric acid extracts from *Dictyostelium* amoebae incubated with bisphosphonates

Extracts were prepared from approx. 10^{10} intact amoebae that had been incubated for 2 h in the absence of bisphosphonate (**a**), with 5 mM EBP (**b**), with 5 mM HMBP (**c**), with 5 mM CPTMBP (**d**) or with 5 mM PMMP (**e**). After incubation, protein was precipitated by the addition of perchloric acid and the supernatants were neutralized with a saturated solution of $KHCO_3$. The extracts were then eluted from a Mono Q anion-exchange column in a gradient of NH_4HCO_3. The identity of physiological nucleotides was determined by co-elution with authentic standards, whereas bisphosphonate metabolites (for which no standards were available) were identified by the appearance of new peaks (indicated by arrows).

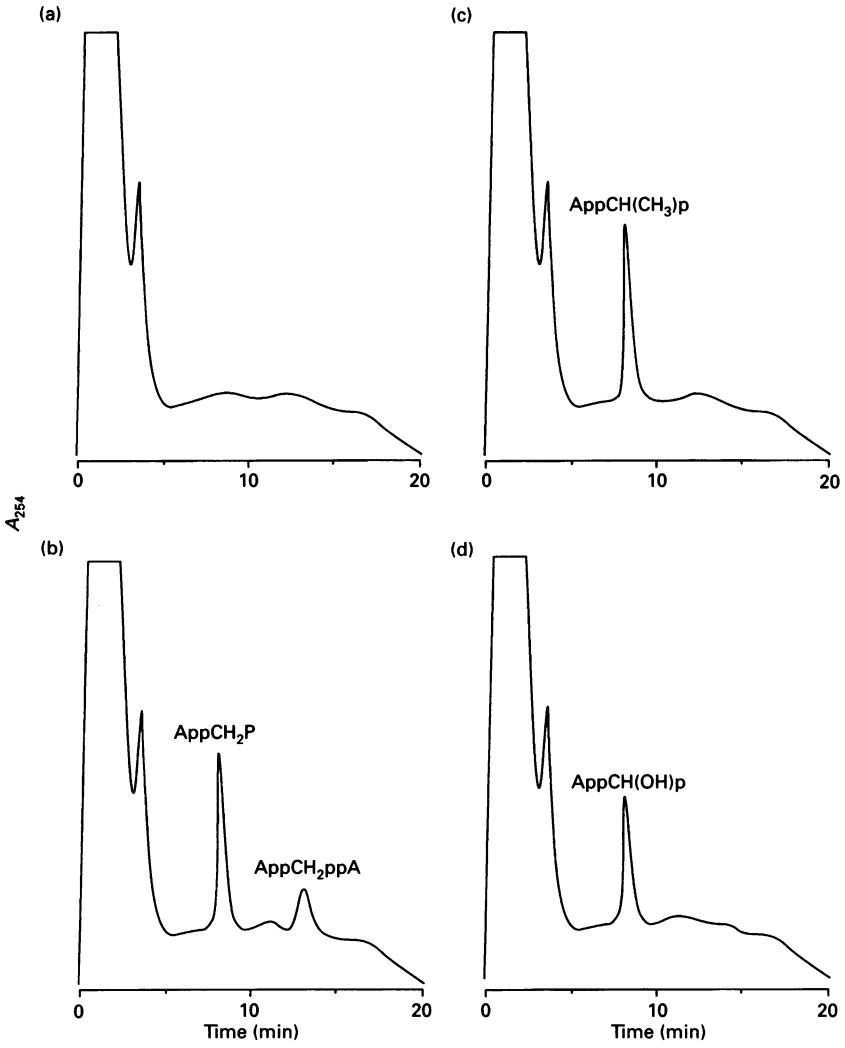

Figure 3 F.p.l.c. elution profiles of cell-free extracts of *Dictyostelium* amoebae incubated with bisphosphonates

Cell-free extracts of *Dictyostelium* were incubated for 2 h in the absence of bisphosphonate (**a**), with 5 mM MBP (**b**), with 5 mM EBP (**c**) or with 5 mM HMBP (**d**). After incubation at 22 °C for 2 h, apyrase was added and the mixtures were incubated for a further 1 h after which proteins were removed by precipitation with perchloric acid. The supernatants were neutralized with a saturated solution of $KHCO_3$ and eluted from a Mono Q anion-exchange column in a gradient of NH_4HCO_3. The large AMP peak eluted at 3 min is off-scale. Bisphosphonate metabolism was indicated by the presence of a new peak. The elution times of the $AppCH_2p$ and $AppCH_2ppA$ metabolites of MBP were identical with those of authentic standards. No standards were available for the AppCp metabolites of EBP or HMBP.

coupling of the γ-phosphonate to the β-phosphonate but not to the α-phosphate.

Phosphonomethylenemethylphosphinate (PMMP; PO_3H_2-CH_2-PO_2HCH_3) was also incorporated into an AppCp-type analogue, containing the phosphinate group only at the γ-position, i.e. $AppCH_2pCH_3$. Peaks in the ^{31}P n.m.r. spectrum at +13 p.p.m. and +39 p.p.m. were identified as coming from the phosphonate and phosphinate groups respectively of unincorporated PMMP (Figure 1d). An additional peak at +9 p.p.m. and an increase in peak height at around −10 p.p.m. corresponded to the β-phosphonate and α-phosphate groups respectively of an AppCp-type nucleotide. The peak at +35 p.p.m. appeared to arise from the γ-phosphinate group. In confirmation of these peak assignments it was found, from one-dimensional coupling experiments, that the β-phosphonate (+9 p.p.m.) was coupled to the α-phosphate (−10 p.p.m.) but not to the γ-methylphosphinate of the $AppCpCH_3$ nucleotide.

PCCP did not appear to be incorporated into an AppCCp nucleotide, but, after amoebae had been incubated with PCPCP, six minor clusters of new peaks (in addition to the peaks at +32 p.p.m. and +14 p.p.m. resulting from PCPCP itself), at around −10 p.p.m., +9 p.p.m., +10.5 p.p.m., +13 p.p.m., +26 p.p.m. and +29 p.p.m., could be identified in the n.m.r. spectrum. These probably represent the resonances of the nucleotide AppCpCp.

Identification of bisphosphonate metabolites by ion-exchange f.p.l.c.

Extracts prepared from amoebae that had been incubated in EBP (Figure 2b) or VBP appeared to contain new nucleotides that were eluted from the ion-exchange column after 8 min and were not present in control cell extracts (Figure 2a). As the retention times of the new nucleotides in these extracts very

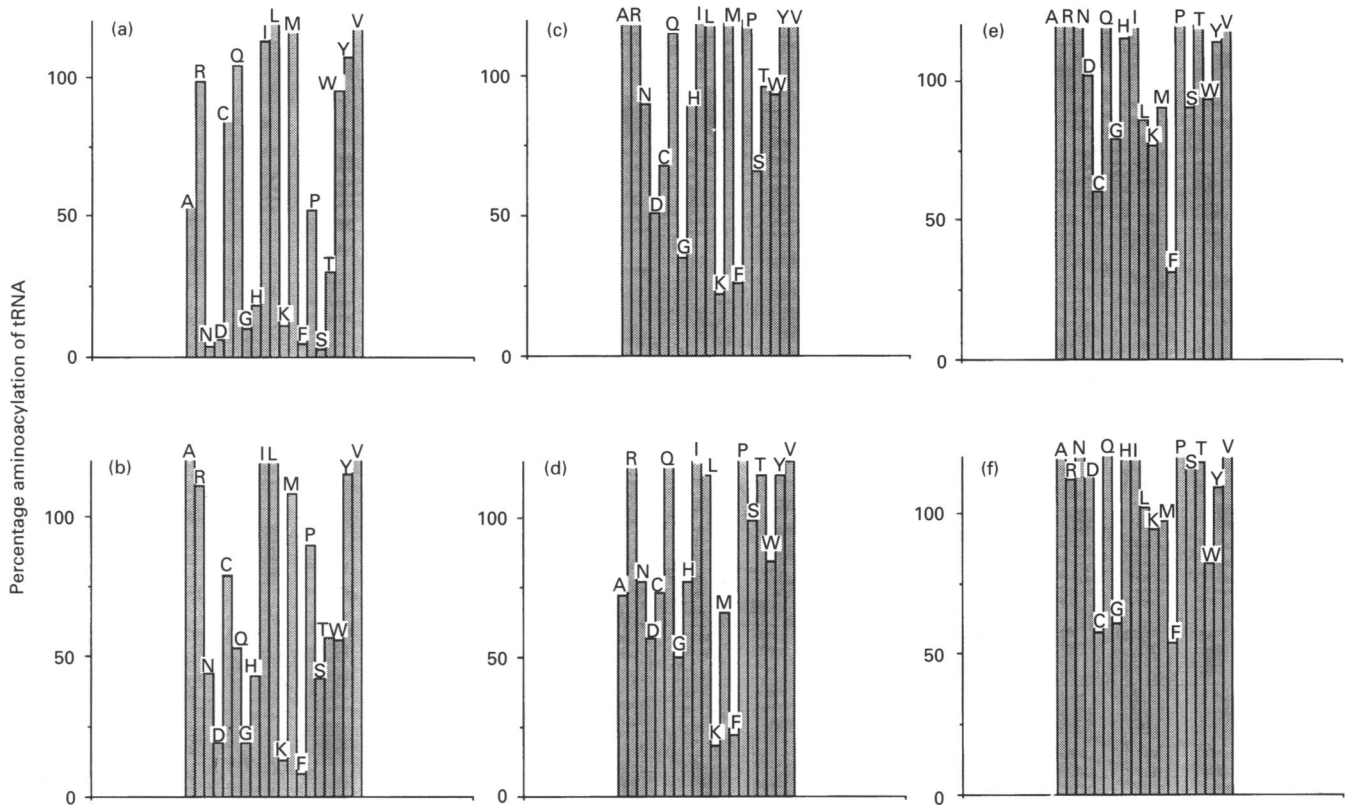

Figure 4 Percentage aminoacylation of tRNA in the presence of bisphosphonates by aminoacyl-tRNA synthetases in a cell-free extract of *Dictyostelium* amoebae

After incubation of cell-free extract with a radiolabelled amino acid, tRNA and 500 μM MBP (**a**), F_2MBP (**b**), EBP (**c**), HEBP (**d**), AHBuBP (**e**) or 3-PHEBP (**f**), aminoacyl-tRNA was precipitated on to filters with trichloroacetic acid. Inhibition of aminoacyl-tRNA synthetase activity was determined by calculating the amount of radiolabelled aminoacyl-tRNA synthesized in the presence of bisphosphonate as a percentage of the amount of radiolabelled aminoacyl-tRNA synthesized in the absence of bisphosphonate. Each amino acid is represented by the conventional one-letter code.

closely resembled the retention time of $AppCH_2p$ (Rogers et al., 1992a), and ^{31}P n.m.r. had also indicated the presence of an AppCp-type metabolite of EBP, the new nucleotides were most probably $AppCH(CH_3)p$ and $AppC(=CH_2)p$. The authentic compounds, with which the retention times could be compared, were not available.

Further evidence for the incorporation of HMBP into the nucleotide AppCH(OH)p, which had been tentatively identified by ^{31}P n.m.r., was obtained by f.p.l.c. The metabolite AppCH(OH)p was eluted as a distinct peak after 8.5 min (Figure 2c). HEBP, however, did not appear to be incorporated into an AppCp-type nucleotide.

Of the bisphosphonates with larger side chains, only CPTMBP appeared to be incorporated into an AppCp-type nucleotide, i.e. $AppCH(SC_6H_4Cl)p$. The new peak was eluted after 12.6 min, slightly earlier than ATP (Figure 2d). Isolation of the peak, followed by m.s. analysis, confirmed that the material had the expected molecular mass.

The $AppCH_2pCH_3$ metabolite of PMMP (PO_3H_2-CH_2-PO_2HCH_3) was eluted as a distinct new peak after 5 min (Figure 2e). No metabolites of PCCP or PCPCP could be detected by f.p.l.c.

Bisphosphonate metabolism in cell-free extracts of *Dictyostelium*

All the bisphosphonates (MBP, EBP, VBP, HMBP, F_2MBP, Cl_2MBP and CPTMBP) found during this study or previously (Rogers et al., 1992a) to be incorporated into nucleotide analogues by intact amoebae also appeared to be incorporated into nucleotide analogues by cell-free extracts of *Dictyostelium* amoebae (Figure 3). Detection of these nucleotide analogues by f.p.l.c. was aided by removing ADP and ATP from the extracts with apyrase. Furthermore, the bisphosphonates that did not appear to be metabolized by intact amoebae also appeared not to be metabolized in the cell-free extracts of *Dictyostelium*.

Bisphosphonates inhibit the activity of some aminoacyl-tRNA synthetases in cell-free extracts *in vitro*

Incorporation of 19 of the 20 amino acids examined into aminoacyl-tRNA by aminoacyl-tRNA synthetases could be detected in a cell-free extract of *Dictyostelium*. The activity of glutaminyl-tRNA synthetase, however, could not be detected. The ability of the 19 synthetase enzymes to catalyse aminoacyl-tRNA formation was determined in the presence of HEBP [which was reported by Pelorgeas et al. (1992) to be metabolized by *Dictyostelium*], three other bisphosphonates that are metabolized by *Dictyostelium* (MBP, F_2MBP and EBP) and two that are not metabolized (AHBuBP and 3-PHEBP).

MBP (Figure 4a) or F_2MBP (Figure 4b), both at 500 μM, inhibited by at least 50% the same seven aminoacyl-tRNA synthetases (those specific for Asn, Asp, Gly, His, Lys, Phe or Ser). In some cases, tRNA aminoacylation was almost completely

inhibited. EBP (Figure 4c), also at 500 μM, caused more than 50% inhibition of only four of these synthetases (Asp-, Gly-, Lys- and Phe-tRNA synthetases), whereas 500 μM HEBP (Figure 4d) inhibited only three (Gly-, Lys- and Phe-tRNA synthetases) by more than 50%. Thus the bisphosphonates that we and others (Pelorgeas et al., 1992) have found to be metabolized by *Dictyostelium* are also potent inhibitors of several aminoacyl-tRNA synthetases.

Bisphosphonates not metabolized by *Dictyostelium* (AHBuBP and 3-PHEBP) were poor inhibitors of the aminoacyl-tRNA synthetases *in vitro*. AHBuBP (Figure 4e) and 3-PHEBP (Figure 4f), both at 500 μM, somewhat inhibited synthesis of Phe-tRNA and Cys-tRNA, and 3-PHEBP also inhibited synthesis of Gly-tRNA. However, the extent of inhibition was much less than that observed in the presence of MBP, F_2MBP, EBP or HEBP. At a concentration of 100 μM, neither AHBuBP nor 3-PHEBP had any obvious effect on any of the 19 synthetases assayed.

DISCUSSION

It has previously been reported that MBP and simple halogenated derivatives of MBP inhibit growth of *Dictyostelium* amoebae and are incorporated into AppCp analogues of ATP and AppCppA analogues of Ap_4A (Klein et al., 1988; Rogers et al., 1991, 1992a; Rogers, 1993). Further investigations of a range of bisphosphonates with widely different potencies for inhibiting *Dictyostelium* growth (summarized in Table 1) have now shown that some, but not all, are also metabolized by *Dictyostelium* to AppCp and AppCppA nucleotides, and it is clear that the widely held view that bisphosphonates are metabolically inert is incorrect. Two other compounds containing a phosphonate group were also metabolized by *Dictyostelium*. PMMP was incorporated into an AppCp nucleotide but the incorporation occurred only so that the methylphosphinate group was in the γ-position. PCPCP was also metabolized and, although the metabolite could not be identified with certainty from its n.m.r. spectrum, it is probable that it was AppCpCp, as the similar compound tripolyphosphate reacts readily in the back reaction of lysyl-tRNA synthetase to form Apppp (Zamecnik and Stephenson, 1969).

Bisphosphonates previously found to be metabolized are all relatively poor inhibitors of *Dictyostelium* growth, the IC_{50} values (Rogers, 1993; Rogers et al., 1994) for growth inhibition being high (MBP, 2200 μM; FMBP, 2000 μM; F_2MBP, 2700 μM; Cl_2MBP, 480 μM). Other bisphosphonates that have now been found to be metabolized also have low potency (CPTMBP, 3100 μM; EBP, 1100 μM; VBP, 1000 μM; HMBP, 600 μM; PCPCP, > 2000 μM). By contrast, no metabolites of more potent bisphosphonates (HEBP, 380 μM; AHPrBP, 167 μM; ABuBP, 44 μM; AHBuBP, 32 μM; 3-PHEBP, 14 μM) could be detected. This was not because of an inability of *Dictyostelium* amoebae to take up the potent bisphosphonates as they could be easily detected in extracts prepared from amoebae that had been incubated for 2 h in bisphosphonate and then thoroughly washed to remove extracellular bisphosphonate (Rogers et al., 1992b; Rogers, 1993). Furthermore, the potent bisphosphonates were not metabolized in cell-free extracts of *Dictyostelium* amoebae whereas all of the bisphosphonates that were metabolized by intact amoebae were also metabolized by cell-free extracts. It is possible that the potent bisphosphonates are metabolized but that the nucleotide products are unstable. Pelorgeas et al. (1992) tentatively identified a metabolite of HEBP that was highly unstable. Our inability to detect such a metabolite of HEBP may therefore be due to breakdown of the metabolite during analysis, even though assays were used to detect the ATP analogues that were more sensitive than those used by Pelorgeas et al. (1992).

As the bisphosphonates that are apparently not metabolized to AppCp or AppCppA nucleotides generally have a large side chain (such as an aminoalkyl chain or a heterocyclic group), it might appear that they are not incorporated into nucleotide analogues because they cannot act as analogues of pyrophosphate. However, bisphosphonate metabolism appears to be controlled by factors apparently unrelated to the size of the side chain because CPTMBP is metabolized even though it has a large side chain. Comparison of the metabolism of HMBP with that of MBP or HEBP with that of EBP suggests that possession of a hydroxy group on the geminal carbon also decreases bisphosphonate metabolism. It is therefore possible that it is the lack of a hydroxy group that allows CPTMBP to be metabolized. However, ABuBP, which also lacks a hydroxy group, behaves in a similar manner to AHBuBP, neither being metabolized, although this may be due to the influence of the nitrogen atom in the side chains which appears to be an essential feature of all potent bisphosphonates (Sietsema et al., 1989; Rogers et al., 1994).

Zamecnik and Stephenson (1969) demonstrated that MBP could react rapidly and irreversibly with lysyladenylate to form $AppCH_2p$ in a reaction catalysed by purified lysyl-tRNA synthetase, and accumulation of lysyl-tRNA was then completely inhibited. It has therefore been proposed that incorporation of bisphosphonates into analogues of ATP in *Dictyostelium in vivo* is also catalysed by the back reactions of aminoacyl-tRNA synthetases (Klein et al., 1988). Furthermore, from the observations of Zamecnik and Stephenson (1969), it would also be expected that bisphosphonate incorporation into adenine nucleotides by the aminoacyl-tRNA synthetases would be accompanied by inhibition of aminoacyl-tRNA synthesis. By measuring tRNA aminoacylation in the presence of bisphosphonates *in vitro*, using a cell-free extract of *Dictyostelium* amoebae as a source of aminoacyl-tRNA synthetases, it has been demonstrated that some bisphosphonates do indeed inhibit tRNA aminoacylation.

In *Dictyostelium*, the activities of up to seven aminoacyl-tRNA synthetases (specific for the amino acids Asn, Asp, Gly, His, Lys, Phe and Ser) were substantially inhibited in cell-free extracts by 500 μM MBP, F_2MBP, EBP or HEBP. By contrast, the non-metabolized AHBuBP and 3-PHEBP had little effect at concentrations of 100–500 μM on the activities of these seven synthetases. MBP, F_2MBP, EBP and possibly HEBP are all metabolized by *Dictyostelium* and it would appear that metabolizable bisphosphonates are the only ones able to inhibit significantly some of the *Dictyostelium* aminoacyl-tRNA synthetases. The inhibition of several aminoacyl-tRNA synthetases by HEBP appears to support the evidence of Pelorgeas et al. (1992) that HEBP is metabolized to an AppCp nucleotide. It is therefore likely that, in *Dicytostelium*, there are at least seven aminoacyl-tRNA synthetases (i.e. those that are inhibited by bisphosphonates) that are responsible for the incorporation of bisphosphonates into AppCp and AppCppA nucleotides. As AHBuBP and 3-PHEBP had little effect on the aminoacyl-tRNA synthetases, this is further evidence that these bisphosphonates are not metabolized.

It was suggested 20 years ago (Loftfield, 1973) that MBP ought to be a potent inhibitor of cell growth because of its ability to inhibit the activity of aminoacyl-tRNA synthetases and thus ultimately protein synthesis. We have shown that metabolism of some bisphosphonates and inhibition of some aminoacyl-tRNA synthetases can be detected *in vitro* at a bisphosphonate concentration (500 μM) even lower than that required to affect growth of intact *Dictyostelium* amoebae in culture. It is therefore

possible that the inhibition of amoebal growth by these compounds could be the result of accumulation of nucleotide analogues, as proposed by Klein et al. (1988). However, our study indicates that inhibition of the activity of several aminoacyl-tRNA synthetases may be a more likely mechanism, the need for relatively high bisphosphonate concentrations to inhibit growth presumably being due to their poor cellular uptake. Bisphosphonates that are the most potent inhibitors of growth, such as AHBuBP and 3-PHEBP, are not metabolized by *Dictyostelium* and little inhibition of aminoacyl-tRNA synthetase activity *in vitro* occurred in the presence of concentrations (100–500 μM) of these bisphosphonates that are much higher than those required to inhibit growth of amoebae in culture completely. These bisphosphonates therefore appear to inhibit growth by mechanisms that do not depend on their incorporation into nucleotide analogues or on inhibition of the aminoacyl-tRNA synthetase enzymes.

Bisphosphonates, including two that are metabolized by *Dictyostelium* (Cl_2MBP and F_2MBP), are inhibitors of osteoclast-mediated bone resorption and have been shown to have cytotoxic and antiproliferative effects on mammalian cells such as osteoclasts, macrophages and connective tissue cells (Rowe and Hausmann, 1976; Fast et al., 1978; Rowe and Hays, 1983; Stevenson and Stevenson, 1986; Cecchini et al., 1987; Flanagan and Chambers, 1989; Van Rooijen and Kors, 1989; Carano et al., 1990). As bisphosphonates appear to have cellular targets that are common to both osteoclasts and *Dictyostelium* (Rogers et al., 1994), it is possible that those of low potency such as Cl_2MBP affect mammalian cells, including osteoclasts, by inhibiting aminoacyl-tRNA synthetases or by incorporation into nucleotide analogues. Indeed, our preliminary experiments have confirmed that those bisphosphonates that are metabolized by *Dictyostelium* are also metabolized to AppCp-type nucleotides and inhibit aminoacyl-tRNA synthetase activity in cell-free extracts of human cells (Hodkin et al., 1993; Brown et al., 1994). The mechanism of action of bisphosphonates with high potency as inhibitors of bone resorption and growth of *Dictyostelium* does not appear to involve inhibition of aminoacyl-tRNA synthetases or incorporation of these bisphosphonates into nucleotides. *Dictyostelium*, however, appears to be an excellent model system in which to investigate further the mechanism of action of potent bisphosphonates.

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