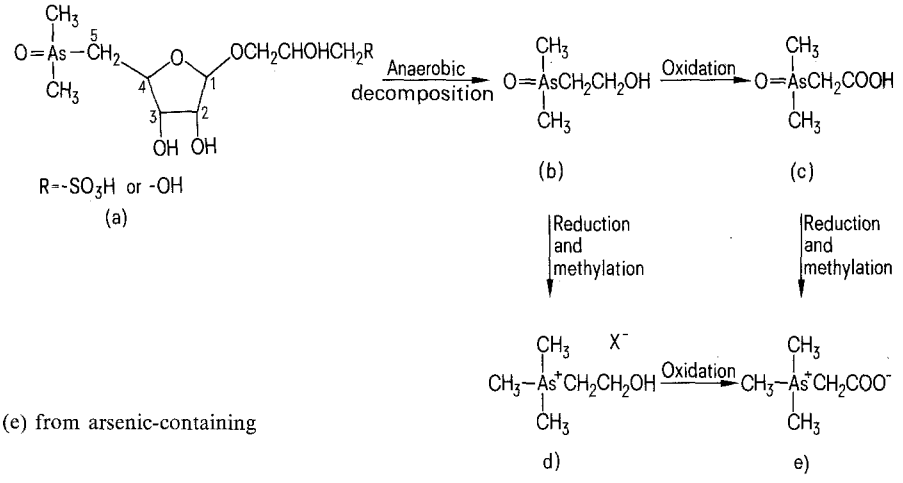

Biosynthetic scheme for arsenobetaine (e) from arsenic-containing sugars (a).

pler end-products. Specific fermentation end-products are then used as carbon substrates in anaerobic respiration by methanogenic and sulphate reducing bacteria. Because fermenters can metabolize a diverse and complex range of substrates, the formation of dimethyloxarsylethanol may be a product of their activity. However, McBride and Wolfe[10] have shown reduction and methylation of arsenate by Methanobacterium strain M.o.H. and, possibly, by *Desulfovibrio gigas*. Sulphate reducing and methanogenic species may therefore be involved at this step or other steps in arsenobetaine formation.

It is yet to be established if the route to arsenobetaine from dimethyloxarsylethanol is via arsenocholine or dimethyloxarsylacetic acid (fig. c). It is also not yet clear at what stage in the food chain (bacteria → detritovores → arsenobetaine-containing animals of higher trophic levels) the final conversions occur. We are currently studying these processes.

1 C, H microanalyses were performed by the Australian Microanalytical Service.
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Effect of diphosphonates on ATP and Pi content, Pi uptake and energy charge of cultured calvaria cells[1]

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Summary. In previous studies we have shown that glycolysis is strongly inhibited in cultured calvaria cells treated with ethane-1-hydroxy-1,1-diphosphonate (EHDP) or dichloromethanediphosphonate (Cl_2MDP). This study shows that the energy charge of the adenylate pool, and the ATP and Pi content, were not changed by treatment with diphosphonates except for a slight decrease of ATP and Pi at 0.25 mM Cl_2MDP. The uptake of Pi was diminished by 50% and 20% in cells treated for 6 days with Cl_2MDP or EHDP, respectively, but not when diphosphonates were present only during the uptake studies.

Diphosphonates are compounds which contain a P-C-P bond and are thus related to pyrophosphate, but they are resistant to metabolic destruction. They inhibit both formation and dissolution of calcium phosphate in vitro; in vivo they prevent ectopic calcification and bone resorption[3,4]. Recently these effects have been utilized clinically. Thus EHDP[2] has been found to decrease the development of ectopic ossification after total hip replacement[5] and in paraplegia[6]. Furthermore, various diphosphonates have proved useful in the management of Paget's disease[7], a disease in which bone turnover is increased, and of tumoral bone disease[8,9]. In high doses, however, certain diphosphonates also lead to an inhibition of normal mineralization. These effects of diphosphonates in vivo have been mainly attributed to their physicochemical interactions with calcium phosphate crystals. Recently, however, it has been found that the diphosphonates also influence cellular metabolism[10–15]. Thus, we found that in cultured rat calvaria and rabbit ear cartilage cells, both Cl_2MDP[2] and EHDP drastically decrease the production of lactate[10]. Since in these cells glycolysis is an important metabolic pathway for the production of ATP, the question arose whether the ATP content and the energy charge[16] might consequently be lowered. Another possibility might be that yet other metabolic pathways may be activated or that the cells utilize less energy. In this paper we describe the effect of diphosphonates on the ATP content, the energy charge and the content and uptake of Pi, in cultured calvaria cells.

Materials and methods. EHDP and Cl_2MDP were obtained as the sodium salts from Procter & Gamble Co., Cincinnati,

Ohio, USA; ATP from Sigma, St. Louis, Missouri, USA; ADP, AMP and luciferase (fire-fly) from Boehringer GmbH, D-6800 Mannheim 31, Fed. Rep. Germany; [2,8-^{3}H]adenosine 5′-triphosphate-tetrasodium salt from New England Nuclear, Dreieichenhain, Fed. Rep. Germany; [2-^{3}H]adenosine from the Radiochemical Centre, Amersham, Bucks., UK; [^{32}P]sodium phosphate from the Federal Institute for Reactor Research, CH-5303 Würenlingen, Switzerland. The scintillation liquid used to determine the radioactivity was mixed as follows: 0.25 litre of triton X-100, 0.75 litre of toluene, 7 g of butyl-PBD [5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] (Ciba-Geigy, Basle, Switzerland) and 80 g naphthalene.

Cell culture. The procedure described earlier[10] was employed. The diphosphonates were added from day 1 until the end of the experiment.

ATP and Pi content. The dish (3.5 cm diameter) was taken from the incubator, the medium was immediately removed, and the cells were washed 3 times with 1.5 ml of ice-cold 0.15 M NaCl, frozen in an ethanol-dry ice mixture and kept at −80 °C. There was no difference in the ATP content whether the cells had or had not been washed with NaCl. For the extraction, 0.5 ml of ice-cold 0.5 M $HClO_4$ was added to the frozen cells. They were scraped off with a rubber policeman, sonicated for 20 sec (Sonifier B-12, Branson, Danbury, Colorado, USA) and 0.2 ml of the suspension was transferred to a 2-ml Eppendorf tube. After neutralization with 20 µl of 5 M K_2CO_3 and 20 µl of 0.05 M Tris-acetate, pH 8.0, and centrifugation at $10000 \times g_{av}$ for 1 min, the supernatant was frozen at −20 °C until it was used for the ATP determination. By adding [2,8-^{3}H]-adenosine-5′-triphosphate to the cells before extraction and by separating the radioactivity of the extract on a thin layer plate (see below) it was found that less than 4% of the ATP was destroyed during the extraction procedure. ATP was determined with the luciferase assay[17]. The neutralized cell extract was diluted 1:20 with 0.1 M Na_2HAsO_4, pH 7.4, and 0.1 ml of this diluted solution was added to a glass scintillation tube, to which 1.7 ml of H_2O and 50 µg of luciferase dissolved in 0.2 ml of 0.05 M Na_2HAsO_4, 0.02 M $MgSO_4$, pH 7.4, had been added. The light emission was immediately measured in a Tri-Carb Packard scintillation counter Mod. 3330, in which the coincidence was switched off. To determine Pi the remainder of the acid cell extract described above was centrifuged at $10000 \times g_{av}$ for 1 min to precipitate the protein. In 50 µl of supernatant Pi was then precipitated according to Sugino and Myoshi[18] and was determined with malachite green according to the method of Altmann et al.[19].

Ratio ATP:ADP:AMP. Since a relatively small amount of cells was available, it was not possible to determine ADP and AMP chemically. The ratio ^{3}H-ATP:^{3}H-ADP:^{3}H-AMP was therefore determined by TLC after the cells had been labeled with ^{3}H-adenosine. This was done in the same batch of cells which was used for ATP and Pi measurement. 15 h before harvesting the cells, 2 µCi of [2-^{3}H]-adenosine (sp. act. 24 Ci/mmole) was added to each culture dish (3.5 cm diameter). After the extraction procedure as described above, 3.5 µl of the solution, which had also been used for the ATP determination, were mixed with 0.5 µl of a solution containing 5 mM ATP, ADP and AMP and 7.5 mM adenosine. The mixture was applied to a thin layer plate silica gel F_{254}, layer thickness 0.25 mm (Merck AG, Darmstadt, FRG) and developed as described by Norman et al.[20]. The R_f-values were ATP: 0.21, ADP: 0.32, AMP: 0.46 and adenosine: 0.69. The spots, visible in light of 254 nm, were marked, and the silica gel scraped off and transferred to scintillation tubes to which 1 ml of 12.5% (w/v) ammonia was added. After 1 h, scintillator was added and the radioactivity measured. The ratio ATP:ADP:AMP did not change whether [2-^{3}H]-adenosine was incubated for 6, 12 or 24 h, although the total radioactivity extracted decreased with time.

Uptake of Pi. The cells were grown in dishes with a diameter of 1.6 cm under the usual conditions with 5% CO_2. 1 h before the experiment the dishes (1.6 cm diameter)

Table 1. The effect of diphosphonates on Pi and ATP content of the cells

	Pi or ATP content (%) Control	EHDP 0.25 mM	Cl_2MDP 0.025 mM	0.25 mM
After 7 days:				
Pi	100.0±2.0 (10)	95.8±3.2 (10)	104.3±2.1 (11)	88.9±2.6[b] (9)
ATP	100.0±3.6 (10)	91.4±3.7 (10)	104.5±5.8 (5)	81.1±2.0[c] (10)
After 8 days:				
Pi	100.0±5.9 (5)	102.2±6.1 (5)	93.2±4.1 (5)	101.0±4.2 (5)
ATP	100.0±2.7 (5)	94.3±4.7 (5)	99.7±5.3 (5)	81.9±4.9[a] (5)

Cells were grown in dishes with a diameter of 3.5 cm (Corning Glass Works, Corning, USA) in the presence or absence of diphosphonates from day 1 to 7 or 1 to 8 respectively. The media were changed at day 1, 4 and 7. The cells were harvested either on day 7 or 8. The mean of n dishes ± SEM is given as percentage of the control. The absolute values (mean ± SEM (n)) for the control were: Pi after 7 days of culture 11.75±0.24 (10) nmoles/10^6 cells and after 8 days: 12.98±0.77 (5) nmoles/10^6 cells; ATP after 7 days of culture: 8.13±0.33 (10) nmoles/10^6 cells and after 8 days: 7.97±0.22 (5) nmoles/10^6 cells. [a]Significantly different from control, $p < 0.05$; [b]significantly different from control, $p < 0.005$; [c]significantly different from control, $p < 0.001$.

Table 2. The effect of diphosphonates on uptake of Pi

Substrate	Control (nmoles/10^6 cells)	EHDP (nmoles/10^6 cells)	(%)	Cl_2MDP (nmoles/10^6 cells)	(%)
0.1 mM Pi	0.59±0.04; 0.80±0.03	0.59±0.03; 0.67±0.03	99.7; 83.6	0.34±0.02; 0.35±0.03	57.0; 44.2
1.0 mM Pi	2.73±0.19; 3.94±0.17	2.44±0.21; 3.23±0.14	89.4; 81.7	1.57±0.12; 2.34±0.20	57.4; 59.3

Cells were grown in dishes with a diameter of 1.6 cm in the presence or absence of 0.25 mM diphosphonate from day 1 to 7. On day 7, the uptake of [^{32}P]Pi within 10 min at 0.1 and 1.0 mM Pi was determined. The values of 2 separate experiments are given as mean ± SEM of 4 dishes.

were taken out from the incubator. To keep the pH at 7.4, the medium was replaced with 0.5 ml of Hanks solution adjusted to pH 7.4 (± diphosphonates). The uptake itself was then studied at 0.1 and 1.0 mM Pi in the manner described below.

0.1 mM Pi: After preincubation the cells were first washed once with Hanks solution (37 °C) containing 0.1 mM Pi. Then, 0.5 ml of Hanks solution containing 0.1 mM [^{32}P]Pi was added. After 0 or 10 min the medium was removed and the cells were washed 3 times with ice-cold Hanks solution. The cell layer was dissolved overnight in 0.5 ml of formic acid and the solution transferred together with 2 0.5-ml rinses of water to a scintillation tube. The radioactivity was determined by measuring Cerenkov radiation.

1.0 mM Pi: This was done as for 0.1 mM Pi except that the cells were not washed before the incubation with 1.0 mM [^{32}P]Pi. The solution used to wash the cell layer after incubation contained 1.0 mM Pi. The uptake was linear with respect to incubation time at least up to 30 min.

Results. The effect of the diphosphonates on the Pi and ATP content is shown in table 1. In cells treated with 0.25 mM Cl_2MDP, both ATP and Pi decreased by 10–20%. EHDP and 0.025 mM Cl_2MDP had no effect. There was no difference whether the determinations were made at day 7, which was 3 days after the last change of medium, or at day 8, about 16 h after the last change of medium, except that the Pi content of the cells treated with 0.25 mM Cl_2MDP was significantly decreased at day 7, but not at day 8.

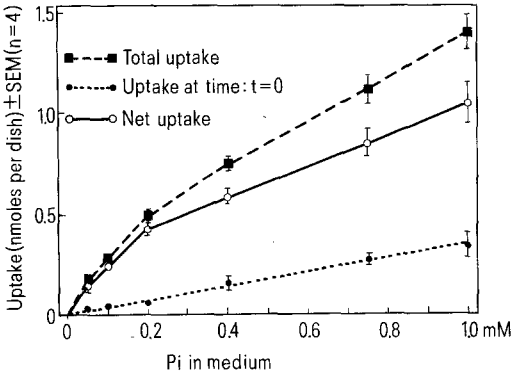
The uptake of Pi showed saturation kinetics up to about 0.1–0.2 mM Pi, and at a higher concentration it was proportional to the Pi concentration of the medium (fig.). In normal cells this uptake was not influenced when the diphosphonates were added to the incubation medium at a concentration of 0.25 mM (not shown). In cells which had been treated for 6 days with diphosphonates, however, inhibition was observed (table 2).

The ratio ^{3}H-ATP:^{3}H-ADP:^{3}H-AMP and therefore the adenylate energy charge[16] were not influenced by treatment with diphosphonates. The energy charge: ATP + ½ ADP/(ATP + ADP + AMP) was in the range 0.927–0.933.

Discussion. The ATP content and adenylate energy charge found in untreated cultured calvaria cells were very similar to the values found by Schwartz and Johnson[21] for fibroblasts. Although both diphosphonates strongly inhibit the glycolysis[11] which is the main energy source of cultured calvaria cells, they had no influence on the energy charge and only 0.25 mM Cl_2MDP slightly decreased the ATP content. This suggests that another metabolic pathway might be responsible for energy production. Indeed, we found that the oxidation of ^{14}C-palmitate is increased in cells treated with Cl_2MDP[13]. In mouse calvaria cultured for 48 h in the presence of EHDP or Cl_2MDP, the formation of $^{14}CO_2$ from ^{14}C-labelled acetate, leucine and citrate was increased, suggesting increased activity of the citric acid cycle[22,23]. However, it cannot be excluded that the cells consume less energy under the treatment with diphosphonates.

Since Pi had been found to be a regulator of glycolysis in ascites tumor cells[24], the inhibition of lactate production by diphosphonates[10] could have been due to a decrease of Pi content. This, however, is not the case since EHDP did not influence this parameter and Cl_2MDP did so only slightly.

High concentrations of Pi in the plasma decrease the urinary excretion of EHDP in the rat[25]. One interpretation could be that Pi and diphosphonates share the same secretory transport system. The data in this paper do not indicate that diphosphonates compete directly with the Pi transport, since in untreated cultured cells, diphosphonates added only during the uptake study had no effect. They produced an inhibition only when given continuously for 6 days, suggesting an indirect action.



Pi uptake. The cells were grown in dishes with a diameter of 1.6 cm (24-well tissue culture cluster-dish 3524, Costar, Cambridge, USA). On day 7 the uptake of [^{32}P]Pi within 10 min was measured at different concentrations of Pi.

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2 Abbreviations: EHDP, ethane-1-hydroxy-1,1-diphosphonate; Cl_2MDP, dichloromethanediphosphonate.

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