Preliminary studies do not support the idea that plasma proteins absent from the perfusate are responsible for this difference.

Ultrastructurally, the perfused-superfused ventricles of all 3 species did not appear to have been significantly altered by long perfusion times. The endothelial cells lining the continuous capillaries were found to be intact with no separation at the junctions. More importantly, the myocardial cells appeared intact and healthy. The mitochondria were neither swollen nor condensed and the myofibrillar pattern was regular. The absence of blood elements in our micrographs confirm the adequacy of myocardial muscle perfusion via the coronary vasculature.

These results support the view that when rat and guinea-pig hearts are perfused-superfused with a 36 °C Ringer's solution, unlike skeletal muscle[14], the anion permeability of these tissues are not influenced by the absence of plasma proteins in the perfusate. This would confirm the reliability of the Ringer's perfused heart preparation as described by Polimeni and Page[5] for measuring anion fluxes in vitro which approximates close to that in situ.

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The effect of bisphosphonates on glycolysis in cultured calvaria cells and their homogenate

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Summary. Rat calvaria cells previously cultured for 7 days in the presence of 1-hydroxyethylidene-1,1-bisphosphonate (HEBP) or dichloromethylenebisphosphonate (Cl_2MBP), showed a decrease in the glycolytic pathway. When glycolysis was analyzed under anaerobic conditions, this effect was not observed. The inhibition by the bisphosphonates occurred to a similar degree regardless of whether lactate production was measured in whole cells or in cell homogenates. Bisphosphonates added directly to the homogenate had no inhibitory effect. Thus, the effect is not a direct one and is unlikely to be due to a soluble mediator in the cytoplasm.

Bisphosphonates are compounds which contain a P–C–P bond and are thus related to pyrophosphate, but are resistant to metabolic destruction. They inhibit mineral formation and dissolution in vitro and prevent ectopic calcification and resorption of bone in vivo[2–5]. These effects have been made use of clinically. Thus, 1-hydroxyethylidene-1,1-bis-phosphonate (HEBP) has been found to decrease the development of ectopic ossification after total hip replacement[6] and in paraplegia[7]. Furthermore, various bisphosphonates have proved useful in the management of Paget's disease, a disease in which bone turnover is increased[8–10] and in tumoral bone disease[11,12].

Initially, these in vivo effects were mainly attributed to physico-chemical interaction of the bisphosphonates with calcium phosphate crystals. In recent years, however, it has been shown that bisphosphonates also influence cellular metabolism[13]. Recently, it has been demonstrated that HEBP and dichloromethylenebisphosphonate (Cl_2MBP) are taken up by calvaria cells in culture, where intracellular concentrations of up to three times that in the medium were measured[14,15]. 75% of the accumulated bisphosphonate is in the cytosol, and apart from other effects[13] these compounds decrease the production of lactate[14]. The mechanism by which they act on the glycolytic pathway is as yet unknown. To elucidate the mechanism of action, the effect of HEBP and Cl_2MBP on the production of lactate under anaerobic conditions was studied. Furthermore, the rate of glycolysis was measured in homogenates of cells treated with these bisphosphonates.

Materials and methods. The bisphosphonates 1-hydroxyethylidene-1,1-bisphosphonate (HEBP) and dichloromethylenebisphosphonate (Cl_2MBP) were provided by the Procter & Gamble Co., Cincinnati, USA.

Cell culture. Calvaria cells of 1-day-old rats were isolated and cultured in petri dishes with a diameter of 5.5 cm (Corning) or in 24-well tissue culture cluster dishes 3542, diameter 1.6 cm (Costar, Cambridge, USA) as previously described[14]. The medium was changed on days 1, 4 and 7 (day of plating: day 0). The bisphosphonates were added from days 1–8. To determine lactate production, cells were incubated in 95% air, 5% CO_2 or in 95% N_2, 5% CO_2 at days 7–8 for 16 h[14]. Medium was then collected, protein precipi-

Table 1. Effect of HEBP and Cl_2MBP on the lactate production of calvaria cells in culture under aerobic and anaerobic conditions

Condition	Control		HEBP		Cl_2MBP	
	μmoles/10^6 cells	%	0.025 mM (%)	0.25 mM (%)	0.025 mM (%)	0.25 mM (%)
Aerobic	5.27 ± 0.23 (16)	100.0 ± 2.6 (16)	83.9 ± 3.0 (15)	54.6 ± 2.4 (15)	64.2 ± 3.0 (15)	25.8 ± 2.5 (15)
Anaerobic	7.43 ± 0.25 (16)	100.0 ± 1.9 (16)	103.5 ± 2.8 (12)	96.3 ± 2.7 (16)	103.3 ± 2.9 (16)	85.4 ± 6.9 (16)

The cells were cultured from days 1–8 in the presence or absence of bisphosphonates. Lactate production of 16 h (days 7–8) was measured under aerobic and anaerobic conditions and is expressed as percentage of the control value (no bisphosphonate) as mean ± SEM of n dishes (4 separate experiments).

Table 2. Effect of HEBP and Cl_2MBP on the lactate production of cultured calvaria cells and of their homogenate

Bisphosphonate	Lactate production per h in Homogenate			Cultured cells		
	Control μmoles/10^6 cells	Treated	%	Control μmoles/10^6 cells	Treated	
250 μM Cl_2MBP 1st experiment	0.69 ± 0.03	0.34 ± 0.01	49.3	0.299 ± 0.03	0.089 ± 0.008	29.7
250 μM Cl_2MBP 2nd experiment	0.50 ± 0.03	0.14 ± 0.03	28.0	0.300 ± 0.006	0.108 ± 0.008	35.8
25 μM Cl_2MBP	0.58 ± 0.03	0.34 ± 0.02	58.9	0.261 ± 0.008	0.179 ± 0.007	68.5
250 μM HEBP 1st experiment	0.71 ± 0.04	0.50 ± 0.04	70.6	0.343 ± 0.014	0.235 ± 0.003	68.6
250 μM HEBP 2nd experiment	0.58 ± 0.03	0.42 ± 0.02	72.2	0.261 ± 0.008	0.166 ± 0.009	63.5

Calvaria cells were cultured from days 1–8 in the presence or absence of bisphosphonates. Lactate produced overnight (days 7–8) by the cultured cells was determined in the medium. Cells were harvested and homogenized and the lactate production of the homogenate was measured. The values are the mean ± SEM of 4 individual media or homogenates.

tated with $HClO_4$ and lactate determined using lactate dehydrogenase[16]. The cell number was determined with a Coulter Counter[14].

Measurement of lactate production in homogenate of cultured cells. The method employed was as described by Diamond et al.[17]. The cell layer was washed 3 times with cold 0.14 M NaCl. The cells were then scraped off with a rubber policeman and transferred to a test tube with 3 lots of 0.5 ml of 0.14 M NaCl. The cells of 3 dishes (in the case of 0.25 mM Cl_2MBP, 5 dishes) were pooled in one test tube and immediately centrifuged at 750 × g for 5 min. Following centrifugation they were disrupted in 1 ml of 10 mM Tris-HCl, pH 7.2; 10 mM KCl; 2 mM $MgSO_4$, by 100 strokes in a Dounce homogenizer. The lactate production was measured by incubating 90 μl of homogenate for 1 h at 37 °C in a final volume of 100 μl containing 2 mM $MgSO_4$, 2 mM ATP, 5 mM potassium phosphate, 1 mM NAD, 50 mM Hepes, pH 7.5, 100 mM glucose. The reaction was stopped by adding 0.9 ml of cold H_2O and lactate was determined as described above. The production of lactate was proportional to the amount of homogenate and the time of incubation. Protein was determined according to Lowry et al.[18].

Results. As shown in table 1 and as described previously[14] HEBP and Cl_2MBP inhibit the lactate production in cultured calvaria cells, when incubated under aerobic conditions. The consumption of glucose and the production of $^{14}CO_2$ from [U-^{14}C]glucose (the latter being 2% of the glucose metabolized) were inhibited to a similar degree, whereas the oxidation of pyruvate was not influenced (not shown). Anaerobic conditions, however, increased the rate of glycolysis in the untreated cells by about 40% and eliminated completely the inhibition caused by the bisphosphonates.

As it can be seen in table 2, lactate production was likewise inhibited, and to a similar extent as in the intact cells, when it was measured with cell homogenates obtained from cultures with or without bisphosphonates. Expressing the data for the homogenate per mg protein instead of per 10^6 cells yielded similar results (not shown). When bisphosphonates were added directly to the homogenates (up to 1 mM, which is a higher concentration than is found in cells in culture[14]) no inhibition was observed (not shown).

Discussion. HEBP and Cl_2MBP inhibit the production of lactate in cultured calvaria cells, as has been shown previously[14]. This effect develops and disappears slowly. It starts to appear about 2 days after addition of the bisphosphonates and is not altered by cycloheximide, an inhibitor of protein synthesis[19]. In cells treated with bisphosphonates for 6 days lactate production returned to normal 7 days after removal of HEBP but remained at 55% of the control value 14 days after removal of Cl_2MBP (not published). The effect seems therefore to be quite a stable modification. Under anaerobic conditions, however, this inhibition was not observed. This has previously also been shown for rabbit ear cartilage cells in culture. These cells, however, are less sensitive to bisphosphonate treatment than calvaria cells[14]. Under anaerobic conditions glycolysis is the only pathway which provides the cells with the necessary energy, which ultimately results in a higher lactate production also in cells not treated with bisphosphonates. It seems that in this extreme situation, signals stimulating glycolysis overcome the inhibition by the bisphosphonates.

Glycolysis is diminished in homogenates of treated cells to a similar degree as it is in cells in culture. It is therefore unlikely that soluble factors are responsible for the inhibition, since they become highly diluted in the homogenate, and thus are unlikely still to be effective. Furthermore, it appears that the effect is not a direct one, since bisphosphonates added to homogenates did not inhibit the production of lactate. The state of some enzyme(s), e.g. degree of phosphorylation, might be altered by the bisphosphonates, whereas in anaerobic conditions this alteration does not seem to occur. A change in the amount of enzyme is a possibility, but rather unlikely, since it would have to be converted rapidly within hours in the absence of oxygen.

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Characterization studies of glucose dehydrogenase[1]

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Summary. Porcine liver β-D-glucose dehydrogenase has been isolated using Triton X-114 to release it from the endoplasmic reticulum. The purified enzyme contains a limited amount (1.7%) of lipid material, including cholesterol, fatty acids, mono and diglycerides, phosphatidylcholine, phosphatidylethanolamine, and cholesterol esters. This enzyme is a tetrameric protein containing an extensive number of hydrophobic residues. This form of glucose dehydrogenase is capable of turning over both β-D-glucose and α-D-glucose-6-phosphate in vivo as indicated from a steady state kinetic analysis at 37 °C.

Glucose dehydrogenase (E.C.1.1.1.47) is a membrane bound protein that catalyzes the oxidation of β-D-glucose and α-D-glucose-6-phosphate to their corresponding 1,5-gluconolactones along with the simultaneous reduction of NAD and NADP, respectively[2]. This enzyme is reported to be located on the luminal side of the endoplasmic reticulum, as is glucose-6-phosphatase[3].

In this work, we report the results of a quantitative lipid and amino acid analysis of the purified enzyme. These results, together with other evidence suggest that this hydrophobic protein may not be limited to the cisternal side of the endoplasmic reticulum. Furthermore, a steady-state kinetic study indicates the ability of this enzyme to function in 2 catabolic pathways simultaneously. This latter result is consistent with glucose dehydrogenase providing NADH and NADPH for the microsomal electron transport systems[4,5].

Materials and methods. Glucose dehydrogenase was purified by the method of Campbell et al.[2], with the following modifications. Triton X-114 was substituted for Triton X-100 in view of its ability to solubilize membrane proteins and its convenient separation from these proteins[1]. Finally, a Whatman SE-53 cation exchange column, previously equilibrated with 5 mM pH 6.0 phosphate buffer, was used prior to the DEAE column. The enzyme was eluted from the SE-53 column with 5 mM pH 7.0 phosphate buffer, added to the DEAE column, and eluted with 50 mM pH 7.0 phosphate buffer.

Spectrophotometric assays were made at pH 7.5 in 0.05 M tris/HCl buffer and at pH 10.0 in 0.05 M glycine/NaOH buffer. All buffers were 1 mM in EDTA and 10^{-4} M in dithioerythritol. Absorbance readings at 340 nm were recorded after initiation of the reaction by addition of the sugar solution.

Total lipid extracts were made with 2:1 chloroform-methanol. 20 vols of 2:1 chloroform-methanol were added to the sample, mixed well and allowed to stand for 30 min. The sample was then centrifuged for 20 min at 12,000 × g. The organic layer containing lipid material was removed and evaporated with dry nitrogen. The various lipids were separated by TLC using Adsorbisil plates in a chloroform/methanol/acetic acid/water (75:45:12:6) mixture and silica gel G plates in a hexane/ethyl ether/acetic acid (80:20:1) solvent mixture. The plates were charred and scanned on a Helena Quick Scan Densitometer with cholesterol acetate incorporated as an internal standard.

Hydrolysis for amino acid analysis was carried out in vacuo at 110 °C in 6 N HCl with 0.2% (v/v) 2-mercaptoethanol for 24, 48, 72, and 96 h. Amino acid analyses were determined on a Dionex D-300 amino acid analyzer with a ninhydrin detection system. Threonine, serine and tryptophan values were obtained by extrapolation to zero time. Cysteine was determined separately[7] as was tyrosine[8].

Sodium dodecyl sulfate electrophoresis[9] was carried out on 12% gels using Coomassie brilliant blue R as a stain. Standards used included bovine serum albumin (67,200), catalase (60,000) and mol.wt markers (14,300, 28,600, 42,900, 57,200 and 71,500) furnished by the Gallard-Schlesinger Chemical Corp. SDS electrophoresis of glucose dehydrogenase gives a single subunit band (not shown) of mol.wt 58,000 (±1100). In view of the previously determined mol.wt of 235,000[10], porcine liver glucose dehydrogenase is apparently a tetrameric protein consisting of similar subunits.