Increase in fatty acid oxidation in calvaria cells cultured with diphosphonates

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1. Cultured calvaria cells oxidized palmitate and octanoate to CO_2 and water-soluble products. 2. When these cells were treated for 6 days with 0.025 and 0.25 mM-dichloromethanediphosphonate, oxidation of palmitate was increased, whereas that of octanoate was influenced less. 3. When the rate of oxidation was raised by increasing the palmitate concentration in the medium, the effect of the diphosphonate was decreased and finally disappeared. 4. 1-Hydroxyethane-1,1-diphosphonate had only minor effects. 5. The increase in palmitate oxidation appeared 2 days after the addition of dichloromethanediphosphonate, simultaneously with a fall in lactate production. (Inhibition of glycolysis by diphosphonates has already been shown.) 6. Cycloheximide, an inhibitor of protein synthesis, did not influence the effect of dichloromethanediphosphonate on the oxidation of palmitate and the production of lactate. 7. Cells cultured with dichloromethanediphosphonate showed a faster uptake of palmitic acid than did control cells. However, this observation did not explain the increased palmitate oxidation, since uptake was much faster than oxidation, and was therefore not the rate-limiting step. 8. 2-Bromopalmitate, an inhibitor of fatty acid oxidation, did not influence the inhibition of glycolysis by the diphosphonates. This inhibition, therefore, did not result from the increased oxidation of palmitate. It is also unlikely that the increased oxidation of palmitate is connected with the inhibition of glycolysis.

Diphosphonates 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 mineral dissolution *in vitro*. They prevent ectopic calcification and the resorption of bone *in vivo* (Fleisch *et al.*, 1969, 1970; Francis *et al.*, 1969; Russell *et al.*, 1970). Recently these effects have been made use of clinically. Thus, 1-hydroxyethane-1,1-diphosphonate and dichloromethanediphosphonate have been proved useful in the management of Paget's disease, a disease in which bone turnover is increased (Altman *et al.*, 1973; Gunčaga *et al.*, 1974; Russell *et al.*, 1974; Meunier *et al.*, 1979) and of tumoural bone disease (Siris *et al.*, 1980).

Until recently the effects of diphosphonates *in vivo* have been mainly attributed to physico-chemical interactions with calcium phosphate crystals. However, the diphosphonates also influence cellular metabolism. Their effect on bone might therefore be not only a result of physico-chemical interaction. Thus the release of calcium from mitochondria is delayed by dichloromethanediphosphonate and by 1-hydroxyethane-1,1-diphosphonate *in vitro* (Guilland *et al.*, 1974) and the latter

compound is also effective when given *in vivo* (Guilland & Fleisch, 1974). In cultured mouse calvaria, dichloromethanediphosphonate not only inhibits the release of calcium, but also inhibits the production of lactate (Morgan *et al.*, 1973). Both diphosphonates increase the production of $^{14}\text{CO}_2$ from $[1\text{-}^{14}\text{C}]\text{acetate}$, $[\text{U-}^{14}\text{C}]\text{leucine}$ and $[6\text{-}^{14}\text{C}]\text{-citrate}$, which suggests that the tricarboxylic acid cycle is stimulated (Ende & van Rooijen, 1979a,b). That the diminished production of lactate is not secondary to the impaired dissolution of apatite has been shown by studies on isolated cells. With calvaria or rabbit ear cartilage cells in cultures it has been shown that diphosphonates present in the medium for at least 6 days also inhibit glycolysis (Fast *et al.*, 1978). In bone cells cultured in the presence of 1-hydroxyethane-1,1-diphosphonate or dichloromethanediphosphonate for only 24 h, the production of lactate and of $^{14}\text{CO}_2$ from $[\text{U-}^{14}\text{C}]\text{-glucose}$ is slightly stimulated at low concentrations and inhibited at high concentrations (Ende, 1978). Dichloromethanediphosphonate also increases the cellular glycogen content (Felix *et al.*, 1980), alkaline phosphatase activity (Felix & Fleisch, 1979) of bone cells, and synthesis of glycosaminoglycans

and collagen in cartilage cells (Guenther *et al.*, 1979, 1981). Therefore, at least in culture, the diphosphonates are able to influence directly cell metabolism.

Glycolysis is the main pathway for energy production in cultured calvaria cells. Since it is decreased by treatment with diphosphonates (Fast *et al.*, 1978), the question arose whether a possible deficit of energy might be overcome by stimulating the pathways of oxidative metabolism. As mentioned above, an increased oxidation of acetate, leucine and citrate had been found when mouse calvaria were cultured in the presence of diphosphonates for 24 h.

We report in the present paper that treatment of cells with dichloromethanediphosphonate for 6 days induced an increase in the oxidation of long-chain fatty acids.

Experimental

Materials

Collagenase (*Clostridium histolyticum*, code CLS II) was obtained from Worthington; 0.25% trypsin solution and foetal calf serum were from Gibco; Eagle's minimum essential medium was from North American Biologicals Inc., Miami, FL, U.S.A.; disodium 1-hydroxyethane-1,1-diphosphonate and disodium dichloromethanediphosphonate were from Procter & Gamble Co., Cincinnati, OH, U.S.A.; [U - ^{14}C]palmitic acid (specific radioactivity 403 mCi/mmol), glycerol tri[1 - ^{14}C]palmitate (specific radioactivity 57 mCi/mmol) and sodium n -[1 - ^{14}C]octanoate (specific radioactivity 30 mCi/mmol) from The Radiochemical Centre; 2-bromopalmitate and cycloheximide were from Fluka; bovine albumin (Cohn fraction V, cat. no. A4503) and bovine albumin (essentially fatty acid free, cat. no. 6003) were from Sigma. Triton X-100 was from Siegfried AG, Zofingen, Switzerland and lactate dehydrogenase (rabbit muscle) was from Boehringer. All other reagents were analytical grade and were obtained from Merck. The scintillation liquid used to determine the radioactivity was composed of 0.6 litre of toluene, 0.4 litre of methoxyethanol (Methylcellosolve) and 7 g of butyl-PBD [5-(biphenyl-4-yl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole] (Ciba-Geigy). For aqueous solutions the solvent was a mixture of 0.75 litre of toluene and 0.25 litre of Triton X-100.

Cell culture

The cells were prepared as previously described from the calvaria of 1-day-old Wistar rats. They were cultured in minimum essential medium with Earle's salt solution (Eagle, 1959) containing 10% (v/v) foetal calf serum (Fast *et al.*, 1978). Diphosphonates were usually present from days 1 to 8, the end of the experiment. For experiments where $^{14}CO_2$

production was measured, 100 000 cells in 0.5 ml of medium were plated in glass ampoules with an inner diameter of 1.7 cm. In other cases the same number of cells in the same amount of medium was grown in 24-well tissue culture cluster-dishes 3524 (diameter 1.6 cm; Costar, Cambridge, MA, U.S.A.) and in one kind of experiment, 300 000 cells in 1.5 ml of medium were grown in plastic dishes of 3.5 cm diameter (Corning Glass Works, Corning, NY, U.S.A.). The number of cells at the end of the experiments was determined with a Coulter Counter (Fast *et al.*, 1978).

Oxidation of [U - ^{14}C]palmitate and n -[1 - ^{14}C]octanoate

Usually fatty acid oxidation is measured in medium containing no serum but containing fatty-acid-free albumin to which palmitate is added. Under these conditions, however, the cell number decreased drastically and the rate of oxidation was not linear with time. Furthermore, we were interested to study the cells under the same conditions in which we had studied the effect of diphosphonates on glycolysis (Fast *et al.*, 1978). Therefore the fatty acid oxidation was measured in minimum essential medium (Eagle, 1959) containing 10% (v/v) foetal calf serum. When the fatty acid concentration was increased, palmitate from a stock solution, prepared as described by Lorch & Gey (1966), containing 6 mM-palmitate and 8% (w/v) bovine serum albumin was added. [U - ^{14}C]Palmitate, after evaporation of hexane, was directly dissolved in the medium or added with ethanol [0.2 μ Ci/ml of medium; final concentration of ethanol, when present, 1% (v/v)]. For the calculation of the specific radioactivity of the palmitate, the fatty acid originally present in the serum and in the albumin was all regarded as palmitate. The serum contained 0.21 ± 0.01 ($n=3$) mM-fatty acid; the solution of 4% (w/v) albumin contained 0.30 ± 0.003 ($n=2$) mM-fatty acid (mean $\pm$ S.E.M.). Fatty acid oxidation was measured in three conditions: (a) 10% (v/v) serum only present, giving a final fatty acid concentration in the medium of 0.02 mM; (b) 10% (v/v) serum, 0.1 mM-palmitate and 0.133% (w/v) bovine serum albumin were added to the medium to give a final concentration of fatty acid of 0.13 mM; (c) 10% (v/v) serum, 0.6 mM-palmitate and 0.8% (w/v) bovine serum albumin were added to the medium to give a final fatty acid concentration of 0.68 mM. In the following text an approximation will be made and all fatty acid will be considered to be palmitate.

The fatty acid concentration of the serum and of the albumin solution was determined as described by Dole & Meinertz (1960). Since the serum contained a high concentration of lactate, the organic phase was extracted a second time as suggested by these authors.

To measure oxidation of octanoate, unlabelled *n*-octanoate and $0.1 \mu\text{Ci/ml}$ of *n*-[1- ^{14}C]octanoate were added to the minimum essential medium containing 10% (v/v) foetal calf serum. The fatty acid present in the serum was neglected for the calculation of the specific radioactivity of the octanoate.

To measure the oxidation of triacylglycerols, a solution ($0.2 \mu\text{Ci/ml}$) of glycerol tri[1- ^{14}C]palmitate dissolved in propan-2-ol was added to the minimum essential medium containing 10% (v/v) foetal calf serum, the final concentration of propan-2-ol being 1% (v/v). This concentration of propan-2-ol did not influence palmitate oxidation. It slightly decreased lactate production without changing the inhibitory action of dichloromethanediphosphonate.

The oxidation of fatty acid was measured usually on day 7 as follows. The old medium was sucked off and the new one containing the [^{14}C]fatty acid was added. The flask was flushed with air/ CO_2 (19:1) for 1 min and closed with a rubber stopper, from which was suspended a plastic centre well containing a piece of folded filter paper (Kontes Glass Co., Vineland, NJ, U.S.A.). The stopper was sealed with Balco 270 glue (Bally CTU, Schönenwerd, Switzerland) and incubated at 37°C for various time intervals. Then 0.2 ml of ethanolamine and 0.2 ml of 0.33 M-HClO_4 were injected into the centre well and flask, respectively. The flasks were shaken for 1 h at room temperature to trap the $^{14}\text{CO}_2$. The ethanolamine was then transferred to 10 ml of scintillation fluid and the radioactivity was determined. The production of $^{14}\text{CO}_2$ was linear with respect to time up to 16 h. In the acidified medium the protein was then completely precipitated by adding 0.2 ml of 2.65 M-HClO_4 and, after centrifugation, the supernatant was neutralized with 2.5 M-KHCO_3 . One part of the neutralized extract was used for determination of lactate, the other one was brought to pH 4.0 with 3 M-acetate buffer. This solution was extracted twice with petroleum benzene and the radioactivity left in the aqueous phase was determined. In the experiments where only the production of $^{14}\text{CO}_2$ and lactate were measured, the reaction was stopped with 0.3 ml of 1.2 M-HClO_4 . With $\text{NaH}[^{14}\text{C}]\text{CO}_3$ the recovery of $^{14}\text{CO}_2$ was 90%.

Thin-layer chromatography

Lipids were extracted from the medium with methanol/chloroform (2:1, v/v) (Kates, 1972). The extract was separated on silica gel 60 with a layer thickness of 0.25 mm (Merck) using as solvent light petroleum (b.p. $50\text{--}70^\circ\text{C}$)/diethyl ether/acetic acid (70:30:1, by vol.). The R_F values were 0.30 for palmitic acid and 0.62 for glycerol tripalmitate. After staining in iodine vapour, the silica gel was scraped into scintillation vials and the radioactivity was determined.

Lactate determination

The frozen acidified medium left from the $^{14}\text{CO}_2$ determination was centrifuged, the supernatant was neutralized with K_2CO_3 and the lactate was determined with lactate dehydrogenase (Engel & Jones, 1978).

Uptake of [^{14}C]palmitate

The cells were grown in 24-well tissue culture cluster-dishes (diameter 1.6 cm) in the usual conditions of air/ CO_2 (19:1). The uptake, however, was measured at 37°C in air. To keep the pH at 7.4 the medium was replaced with minimum essential medium (Eagle, 1959), with and without diphosphonate, containing 10% (w/v) foetal calf serum, 2.7 mM-NaHCO_3 , 5 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] adjusted to pH 7.4, and 0.14 M-NaCl . The uptake was started by sucking off the medium and by adding 0.25 ml of the same medium containing [^{14}C]palmitate. Three concentrations of palmitate were used: (a) no addition of unlabelled palmitate, (b) $+0.1 \text{ mM-palmitate}$ and 0.133% (w/v) bovine serum albumin, (c) $+0.6 \text{ mM-palmitate}$ and 0.8% (w/v) bovine serum albumin. The uptake was stopped by sucking off the medium and by washing 3 times with 0.5 ml of cold Hanks solution (Hanks & Wallace, 1949) containing 5 mM-Hepes , adjusted to pH 7.4 and 0.5% (w/v) bovine serum albumin. For 0.6 mM-palmitate , 0.8% (w/v) bovine serum albumin was added to the Hanks solution. The cell layer was dissolved in 0.2 ml of 1% (w/v) Triton X-100 and transferred along with two rinses of 0.4 ml of 0.1 M-NaOH to 10 ml of scintillation fluid. The specific radioactivity of palmitate was calculated as described for $^{14}\text{CO}_2$ production.

Uptake of *n*-[1- ^{14}C]octanoate

This was done in the same way as for palmitate, except that the medium contained no serum, no serum albumin and no Hepes buffer. The washing solution contained 0.5 mM-octanoate .

Statistics

Statistical significance was determined by Student's *t* test. When several experiments were combined, the results are expressed as percentages of control values for the individual experiments.

Results

It was first tested whether any hydrolysis of the triacylglycerols and phospholipids added with the serum could occur. This was not the case, as shown in the following two ways. (1) Cells ($\pm 0.25 \text{ mM-dichloromethanediphosphonate}$) were incubated for 16 h and the fatty acid concentration of the medium

was determined after the volume had been decreased by freeze-drying. No change in the concentration during incubation was observed. (2) When glycerol tri[1- ^{14}C]palmitate was added to the medium no hydrolysis could be detected in the lipid extract separated on thin-layer plates. In conclusion, the specific radioactivity of the fatty acids did not change during incubation. This agrees with the finding that $^{14}\text{CO}_2$ production was linear with time. No significant amount of triacylglycerols was oxidized, as measured by incubation with glycerol tri[1- ^{14}C]palmitate.

As seen in Table 1, the production of $^{14}\text{CO}_2$ from [U- ^{14}C]palmitate was higher in cells treated with 0.25 or 0.025 mM-dichloromethanediphosphonate than in control cells. The effect was dependent on palmitate concentration. When 0.6 mM-palmitate was added, no increase was observed. In cells treated with 1-hydroxy-ethane-1,1-diphosphonate the effect

was either small, absent, or there was even an inhibition.

In the previous experiments the ratio palmitate/albumin (serum not included) was kept constant at 5.5. When 0.6 mM-palmitate was added, it could not be excluded that the high concentration of albumin might have blocked the stimulation by dichloromethanediphosphonate. The influence of albumin was therefore tested. As seen in Table 2, the relative stimulation by dichloromethanediphosphonate was decreased to some degree by 0.8% (w/v) albumin, but the effect was too small to explain the absence of an increased oxidation at 0.6 mM-palmitate.

In the experiments presented in Table 1, labelled palmitate was added dissolved in ethanol. Since ethanol might be utilized as substrate and itself change metabolism, the influence of this compound was studied (Table 3). The production of $^{14}\text{CO}_2$ was

Table 1. *Effect of diphosphonates on the production of $^{14}\text{CO}_2$ from [U- ^{14}C]palmitate*

Cells were grown in glass vials from day 1 to 7 in the presence or absence of dichloromethanediphosphonate or 1-hydroxyethane-1,1-diphosphonate. They were then incubated ($\pm$ diphosphonate) in a medium containing (a) 10% (v/v) foetal calf serum and tracer amounts of [U- ^{14}C]palmitate, (b) 10% (v/v) foetal calf serum, 0.133% bovine serum albumin and 0.1 mM-[U- ^{14}C]palmitate or (c) 10% (v/v) foetal calf serum, 0.8% bovine serum albumin and 0.6 mM-[U- ^{14}C]palmitate. [U- ^{14}C]Palmitate was added dissolved in ethanol. $^{14}\text{CO}_2$ production was measured during 16 h. The results are expressed as percentage of control and are the means $\pm$ S.E.M. for the number of vials shown in parentheses. In the control, the cells oxidized the following amounts of palmitate to CO_2 : (a) 66.2 ± 2.2 (18) pmol/ 10^6 (b) 2.04 ± 0.19 (13) nmol/ 10^6 cells and (c) 4.22 ± 0.34 (8) nmol/ 10^6 cells *, significantly higher than control ($P < 0.001$).

Incubation	Concentration of [U- ^{14}C]palmitate added (mM)	Addition ...	[U- ^{14}C]palmitate oxidized to $^{14}\text{CO}_2$ (% of control)				
			None	Dichloromethanediphosphonate		1-Hydroxyethane-1,1-diphosphonate	
			(control)				
			Concn. (mM) ...	0.025	0.25	0.025	0.25
(a)	Tracer		100 ± 2.5 (18)	$178.8 \pm 6.3^*$ (10)	$479.3 \pm 44.7^*$ (16)	113.0 ± 8.0 (8)	$126.1 \pm 7.2^*$ (6)
(b)	0.1		100 ± 3.5 (13)	$196.1 \pm 20.2^*$ (7)	$274.8 \pm 20.2^*$ (9)	$138.5 \pm 11.8^*$ (4)	$147.2 \pm 0.8^*$ (3)
(c)	0.6		100 ± 3.7 (8)	93.5 ± 5.6 (7)	113.1 ± 7.6 (4)	$74.0 \pm 4.2^*$ (8)	92.2 ± 2.4 (4)

Table 2. *Effect of albumin on the production of $^{14}\text{CO}_2$ from [U- ^{14}C]palmitate*

Cells were grown in glass vials from day 1 to 7 in the presence or absence of 0.25 mM-dichloromethanediphosphonate. Then they were incubated ($\pm$ dichloromethanediphosphonate) in a medium containing 10% (v/v) foetal calf serum, different concentrations of bovine serum albumin, [U- ^{14}C]palmitate and 1% (v/v) ethanol. The production of CO_2 from palmitate during 16 h was measured and is given as the mean $\pm$ S.E.M. for four vials.

Conditions	[U- ^{14}C]palmitate oxidized to $^{14}\text{CO}_2$		
	Control (pmol/ 10^6 cells)	+0.25 mM-dichloromethanediphosphonate	
		(pmol/ 10^6 cells)	(% of control)
0.01 mM-Palmitate			
No albumin	46.0 ± 8.6	309.2 ± 11.7	672 ± 26
0.8% Albumin	138.2 ± 8.5	731.4 ± 40.4	529 ± 29
0.1 mM-Palmitate			
0.13% Albumin	1021 ± 67	4520 ± 121	443 ± 12
0.8% Albumin	851 ± 85	2770 ± 150	325 ± 18

Table 3. *Effect of dichloromethanediphosphonate on the production of CO₂ and of acid-soluble radioactivity in the presence or absence of ethanol*

The cells were grown in glass vials from day 1 to 7 in the presence or absence of 0.25 mM-dichloromethanediphosphonate and incubated as described in the legend to Table 1. The results are given as means $\pm$ S.E.M. for the number of vials given in parentheses. (a) Without ethanol, (b) with ethanol. *, Significantly different from control ($P < 0.001$).

Concentration of [U- ¹⁴ C]palmitate added (mM)	Oxidation of [U- ¹⁴ C]palmitate to:					
	¹⁴ CO ₂			Water-soluble radioactive products		
	Control		Treated (%)	Control		Treated (%)
	(nmol/10 ⁶ cells)	(%)		(nmol/10 ⁶ cells)	(%)	
(a) Tracer	0.039 $\pm$ 0.002 (4)	100 $\pm$ 6 (4)	472* $\pm$ 44 (4)	0.077 $\pm$ 0.004 (4)	100 $\pm$ 6 (4)	566* $\pm$ 33 (4)
0.1	1.23 $\pm$ 0.08 (8)	100 $\pm$ 7 (8)	284* $\pm$ 9 (8)	4.15 $\pm$ 0.17 (8)	100 $\pm$ 4 (8)	213* $\pm$ 13 (8)
0.6	4.81 $\pm$ 0.27 (8)	100 $\pm$ 5 (8)	118 $\pm$ 6 (8)	13.7 $\pm$ 0.9 (8)	100 $\pm$ 3 (8)	108 $\pm$ 7 (8)
(b) Tracer	0.037 $\pm$ 0.002 (4)	100 $\pm$ 5 (4)	434* $\pm$ 51 (4)	0.067 $\pm$ 0.003 (4)	100 $\pm$ 4 (4)	636* $\pm$ 34 (4)
0.1	0.67 $\pm$ 0.07 (4)	100 $\pm$ 10 (4)	382* $\pm$ 27 (4)	2.04 $\pm$ 0.04 (8)	100 $\pm$ 2 (8)	327* $\pm$ 21 (8)
0.6	2.79 $\pm$ 0.17 (8)	100 $\pm$ 5 (8)	220* $\pm$ 15 (7)	7.25 $\pm$ 0.38 (8)	100 $\pm$ 4 (8)	205* $\pm$ 18 (8)

indeed higher, and the increase due to dichloromethanediphosphonate was smaller, in the absence of ethanol. Ethanol, however, influenced the effect of dichloromethanediphosphonate only indirectly, since comparison of the relative increase in treated cells with the absolute amount in control cells indicated that this increase was inversely dependent on the absolute amount of ¹⁴CO₂ produced by the control cells. The effect of ethanol on the action of dichloromethanediphosphonate was therefore due to the change in oxidation rate in the control cells. Lactate production was not influenced by ethanol (results not shown).

Table 3 also shows that cultured calvaria cells formed, besides ¹⁴CO₂, water-soluble radioactive products from [U-¹⁴C]palmitate. The formation of the latter was about 2–3-fold larger than that of CO₂. The effect of dichloromethanediphosphonate on the production of water-soluble radioactive products was the same as on that of ¹⁴CO₂.

Fig. 1 shows the time course of the effect of dichloromethanediphosphonate. An increase of the oxidation of palmitate (the effect on the production of water soluble radioactivity was the same as that on the production of CO₂ and is not shown) and a decrease in lactate production was observed after 40 h. Cycloheximide, an inhibitor of protein synthesis, added at the same time as dichloromethanediphosphonate or later, influenced the absolute values to some degree, but it did not change the effect of the diphosphonate. In a separate experiment it was shown that cycloheximide indeed inhibited protein synthesis, inhibition being more than 90% by 2 h after addition.

Uptake of palmitate

The rate of uptake was rapid from 0 to 1 min and

Fig. 1. *Time course of the effect of dichloromethanediphosphonate on the oxidation of palmitate and on the production of lactate, and the influence of cycloheximide*

After 5 days of culture 0.25 mM-dichloromethanediphosphonate was added to half of the culture at time 0 h. Cycloheximide (1 μ g/ml) was added for 16 h at time 0, 24 and 48 h. The productions of ¹⁴CO₂ (upper parts) and of lactate (lower parts) were measured during 16 h by adding new medium containing 10% (v/v) foetal calf serum and tracer amounts of [U-¹⁴C]palmitate at time 0, 24 and 48 h. The means $\pm$ S.E.M. for four vials are given; S.E.M. values smaller than the symbols were not drawn. Open symbols and solid line, in the absence of the diphosphonate; closed symbols and broken line, in the presence of the diphosphonate. $\circ$, $\bullet$, cycloheximide not present; $\square$, $\blacksquare$, cycloheximide present during the incubation; Δ , $\blacktriangle$, cycloheximide given from time 0–16 h.

slower later on. Two rates of uptake have also been observed in cultured cardiac cells by Samuel *et al.* (1976). As seen in Table 4, uptake measured for 1 min was increased by about 50% in the dichloro-

Table 4. *Effect of dichloromethanediphosphonate on the uptake of palmitate*

Cells were grown in plastic dishes (diameter of 1.6 cm) in the presence or absence of 0.25 mM-dichloromethanediphosphonate from day 1 to 7. For the measurement of the uptake the cells were incubated at the same concentrations of palmitate and albumin as described in Table 1. For more details see the Experimental section. The mean $\pm$ S.E.M. for eight dishes is given. *, significantly different from control ($P < 0.005$); †, significantly different from control ($P < 0.001$).

Concentration of [U- ¹⁴ C]palmitate added (mM)	Uptake at:					
	1 min			10 min		
	Control		+Dichloromethane-diphosphonate (% of control)	Control		+Dichloromethane-diphosphonate (% of control)
	(nmol/10 ⁶ cells)	(%)		(nmol/10 ⁶ cells)	(%)	
Tracer	0.071 $\pm$ 0.002	100 $\pm$ 3.3	153.0 $\pm$ 6.1†	0.763 $\pm$ 0.011	100 $\pm$ 1.2	142.1 $\pm$ 5.9†
0.1	0.348 $\pm$ 0.019	100 $\pm$ 5.0	142.0 $\pm$ 8.8*	2.26 $\pm$ 0.05	100 $\pm$ 1.4	112.4 $\pm$ 2.5†
0.6	1.10 $\pm$ 0.12	100 $\pm$ 7.1	161.0 $\pm$ 13.5*	5.12 $\pm$ 0.13	100 $\pm$ 2.6	101.7 $\pm$ 3.9

Table 5. *Inhibition of glycolysis by the diphosphonates in the presence or absence of 2-bromopalmitate*

Cells were grown in plastic dishes from day 1 to 7 in the presence or absence of dichloromethanediphosphonate or 1-hydroxyethane-1,1-diphosphonate. Then they were incubated for 16 h in the presence or absence of 0.5 mM-2-bromopalmitate in medium containing 10% (v/v) foetal calf serum, 0.66% bovine serum albumin, with or without the diphosphonates. The production is given as percentages of control (means $\pm$ S.E.M. for four dishes). The lactate production for control in the absence of 2-bromopalmitate was 3.68 ± 0.07 ($n = 4$) μ mol/10⁶ cells and in the presence of 2-bromopalmitate was 5.60 ± 0.20 ($n = 4$) μ mol/10⁶ cells.

Diphosphonate added	Concn. (mM)	Production of lactate (%)	
		No 2-bromopalmitate	+0.5 mM-2-Bromopalmitate
Control	0	100.0 $\pm$ 2.0	100.0 $\pm$ 3.6
Dichloromethanediphosphonate	0.025	56.0 $\pm$ 2.7	54.3 $\pm$ 2.3
	0.25	11.8 $\pm$ 0.6	17.0 $\pm$ 1.5
1-Hydroxyethane-1,1-diphosphonate	0.025	87.1 $\pm$ 0.8	79.5 $\pm$ 1.5
	0.25	52.6 $\pm$ 0.4	61.6 $\pm$ 1.8

methanediphosphonate-treated cells. When the uptake was measured for 10 min, this increase was smaller, and it was not present at high palmitate concentration.

Effect of 2-bromopalmitate

The inhibition of glycolysis by diphosphonates could be secondary to increased oxidation of fatty acids. To test this hypothesis the cells were incubated with 2-bromopalmitate, which decreased the production of ¹⁴CO₂ to 31.0 ± 2.0 ($n = 4$) % at 0.15 mM, to 22.5 ± 4.6 ($n = 4$) % at 0.3 mM and to 12.1 ± 3.5 ($n = 4$) % at 0.5 mM (mean $\pm$ S.E.M.). As seen in Table 5, although 2-bromopalmitate increased the absolute production of lactate, the relative inhibition of glycolysis by diphosphonates was not different whether 2-bromopalmitate was present or not. Thus, the effect of the diphosphonates on glycolysis appears to be independent of fatty acid oxidation.

Effect of 2-deoxy-D-glucose

Conversely, it is possible that the stimulating

effect of dichloromethanediphosphonate on fatty acid oxidation is secondary to the inhibition of glycolysis. Therefore another inhibitor of glycolysis, 2-deoxy-D-glucose, was given to the cells, to see whether under these conditions the oxidation of fatty acids was also higher. As seen from Table 6, this was not the case, although the production of lactate was diminished to 30–40%.

Oxidation of n-octanoate

The production of ¹⁴CO₂ with respect to time was studied at 0.5, 0.1, 0.02 mM and at tracer amounts (3.3 μ M) of *n*-[1-¹⁴C]octanoate. At the first three concentrations, production of ¹⁴CO₂ was linear with time up to 7 h in cells treated with dichloromethanediphosphonate, whereas the rate decreased somewhat after about 4 h in the control cells. When tracer amounts of *n*-[1-¹⁴C]octanoate were used, the rate decreased slightly after 2 h in cells treated with dichloromethanediphosphonate, and the decrease was even greater in control cells. Table 7 summarizes the effect of dichloromethanediphosphonate on the oxidation of different concentrations of

n-[1-¹⁴C]octanoate measured during the linear period of 4 h, and after 7 h, a time when the rate in the control cells had decreased. Dichloromethane-diphosphonate influenced the production of both

¹⁴CO₂ and the water-soluble radioactive product to the same degree. The increase, compared with the oxidation of palmitate, was small, the highest value being around 150%.

Table 6. *Effect of 2-deoxy-D-glucose*

After 7 days of growth in glass vials, the cells were incubated for 16 h in the presence or absence of 1 mM-2-deoxy-D-glucose in a medium containing 10% (v/v) foetal calf serum and either (a) tracer amounts of [U-¹⁴C]palmitate or (b) 0.1 mM-[U-¹⁴C]-palmitate and 0.133% bovine serum albumin and 1% (v/v) ethanol. The mean $\pm$ S.E.M. for four vials is given.

[2-Deoxy-D-glucose] added (mM)	Lactate production (μ mol/10 ⁶ cells)	[U- ¹⁴ C]Palmitate oxidized to ¹⁴ CO ₂ (pmol/10 ⁶ cells)
(a) 0	5.03 $\pm$ 0.22	72.6 $\pm$ 12.0
1.0	1.38 $\pm$ 0.07	84.7 $\pm$ 6.4
(b) 0	4.38 $\pm$ 0.46	1806 $\pm$ 215
1.0	1.93 $\pm$ 0.16	1949 $\pm$ 33

Effect of the diphosphonates on the uptake of n-octanoate

The time course of uptake showed, as for palmitate, a fast period during the first 5 min and afterwards a period of lower rate. Therefore the uptake for 5 and 15 min was measured. 1-Hydroxyethane-1,1-diphosphonate had no influence, but dichloromethanediphosphonate significantly decreased the uptake, being 73.8 $\pm$ 5.9% at 5 min and 82.0 $\pm$ 4.2% at 15 min (mean $\pm$ S.E.M. for eight dishes) of the control values.

Discussion

Cultured calvaria cells have been found to convert fatty acids to CO₂ and water-soluble products, a

Table 7. *Effect of dichloromethanediphosphonate on the oxidation of n-[1-¹⁴C]octanoate*

Cells were grown in glass vials from day 1 to 7 in the presence or absence of 0.25 mM-dichloromethanediphosphonate and were then incubated for 4 h or 7 h with medium containing 10% (v/v) foetal calf serum, *n*-[1-¹⁴C]octanoate, different concentrations of non-radioactive *n*-octanoate and $\pm$ 0.25 mM-dichloromethanediphosphonate. The production of ¹⁴CO₂ and water-soluble radioactive products from *n*-[1-¹⁴C]octanoate is given as means $\pm$ S.E.M. for the number of dishes in parentheses in nmol/10⁶ cells or as percentages of control values (no dichloromethanediphosphonate). *, significantly different from control ($P < 0.05$); †, significantly different from control ($P < 0.001$).

[Dichloromethane-diphosphonate] (mM)	Oxidation of <i>n</i> -[1- ¹⁴ C]octanoate at a concentration of:			
	3.3 μ M	0.02 mM	0.1 mM	0.5 mM
(a) to ¹⁴ CO ₂				
(nmol/10 ⁶ cells)				
After 4 h				
0	0.302 $\pm$ 0.01 (7)	2.84 $\pm$ 0.11 (4)	10.04 $\pm$ 0.66 (17)	20.4 $\pm$ 2.3 (8)
		(% of control)		
0	100.0 $\pm$ 3.1 (7)	100.0 $\pm$ 4.6 (4)	100.0 $\pm$ 2.4 (17)	100.0 $\pm$ 8.2 (8)
0.25	134.4 $\pm$ 4.6 (7)†	119.4 $\pm$ 6.3* (4)	114.0 $\pm$ 8.1 (16)	115.9 $\pm$ 14.4 (8)
After 7 h				
0	0.380 $\pm$ 0.017 (3)	3.40 $\pm$ 0.05 (4)	14.40 $\pm$ 0.9 (13)	30.9 $\pm$ 2.2 (4)
		(% of control)		
0	100.0 $\pm$ 4.3 (3)	100.0 $\pm$ 1.4 (4)	100.0 $\pm$ 2.0 (13)	100.0 $\pm$ 7.3 (4)
0.25	140.2 $\pm$ 9.5* (3)	151.3 $\pm$ 1.4† (3)	155.7 $\pm$ 11.6† (11)	116.2 $\pm$ 13.1 (4)
(b) to water-soluble radioactive products				
(nmol/10 ⁶ cells)				
After 4 h				
0	—	2.33 $\pm$ 0.10 (4)	14.23 $\pm$ 0.16 (4)	18.53 $\pm$ 2.14 (4)
		(% of control)		
0	—	100.0 $\pm$ 4.3 (4)	100.0 $\pm$ 1.1 (4)	100.0 $\pm$ 11.5 (4)
0.25	—	147.6 $\pm$ 2.6† (4)	119.8 $\pm$ 9.9 (4)	102.8 $\pm$ 3.4 (4)
After 7 h				
0	—	3.51 $\pm$ 0.11 (4)	19.41 $\pm$ 0.76 (4)	34.52 $\pm$ 1.06 (4)
		(% of control)		
0	—	100.0 $\pm$ 3.1 (4)	100.0 $\pm$ 3.9 (4)	100.0 $\pm$ 3.1 (4)
0.25	—	129.3 $\pm$ 3.1† (4)	133.8 $\pm$ 3.6† (4)	91.5 $\pm$ 2.9 (4)

process which has to our knowledge not yet been reported. Treatment of cells with dichloromethanediphosphonate augmented the oxidation of fatty acids. This cannot be explained by the fact that cells treated with dichloromethanediphosphonate took up palmitate more rapidly than did untreated cells since the rate of uptake is about 100–1000 times faster than the rate of oxidation. Ende & van Rooijen (1979a,b) found in mouse calvaria that the two diphosphonates increased the production of CO_2 from $[1-^{14}\text{C}]$ acetate, $[\text{U}-^{14}\text{C}]$ leucine and from $[6-^{14}\text{C}]$ citrate and they suggested that the tricarboxylic acid cycle might be stimulated. This finding does not contradict our observation, since this cycle is involved in the oxidation of fatty acids to CO_2 . The data presented here, however, show that the diphosphonates acted mainly on β -oxidation itself, since the production of the acid-soluble products was also increased.

Glycolysis in cultured calvaria cells is decreased by diphosphonates, as has already been shown (Fast *et al.*, 1978). The increase of the fatty acid oxidation might be related to this inhibition. As shown in Fig. 1, both effects appeared at the same time and neither is abolished by cycloheximide. Glycolysis produces most of the energy of these cells, and an inhibition of it might stimulate another pathway, e.g. β -oxidation. The amount of ATP produced by fatty acid oxidation compared with that produced by glycolysis was, however, small. Calculating from Table 3, the higher oxidation of palmitate yielded only about 1–15% of the ATP that was lost due to a lower rate of glycolysis at 0.25 mM-dichloromethanediphosphonate. Furthermore, inhibition of glycolysis by 2-deoxy-D-glucose to a similar degree as with 0.25 mM-dichloromethanediphosphonate did not influence the oxidation of palmitate, which shows that a diminution of glycolysis does not automatically lead to a higher oxidation of fatty acids. On the other hand, the comparison of the two inhibitors might not be relevant, since the diphosphonates act after 2 days, whereas 2-deoxy-D-glucose acts immediately. The fact that 1-hydroxyethane-1,1-diphosphonate inhibits glycolysis strongly (Fast *et al.*, 1978), but has minor effects on palmitate oxidation, suggests also that inhibition of lactate production is not the only reason for the effect of dichloromethanediphosphonate on palmitate oxidation.

When fatty acid oxidation was blocked with 2-bromopalmitate, which probably acts by inhibiting fatty acyl-CoA synthetase, carnitine acyltransferase and by interfering with binding of fatty acids and acyl-CoA species to intracellular binding proteins (Mahadevan & Sauer, 1971; Chase & Tubbs, 1972; Ockner & Manning, 1976), the inhibition of glycolysis by the diphosphonates was not changed. This suggests that the lower rate of glycolysis is not

connected to the increased oxidation of fatty acids.

The oxidation of octanoate was much less increased than that of palmitate in the cells treated with dichloromethanediphosphonate. Since the oxidation of long-chain fatty acids, but not that of octanoate, is dependent on the transport of these acids as carnitine esters into the mitochondria (Bremer, 1977), it is possible that dichloromethanediphosphonate interacts with this system. Another possibility might be that the diphosphonate interacts with the fatty-acid-binding protein, which seems to participate in the cellular transport of hydrophobic long chain fatty acids, but has low affinity for medium-chain fatty acids (Ockner & Manning, 1976). The synthesis of this binding protein, as also of the carnitine acyltransferase was, however, not influenced, because inhibition of protein synthesis did not abolish the increase in fatty acid oxidation.

The role of fatty acid oxidation in bone metabolism is not yet known. The fact that dichloromethanediphosphonate, a powerful inhibitor of bone resorption, influences this process raises the possibility that fatty acid oxidation could perhaps be involved in bone destruction. Studies with other compounds known to influence this process might throw some light on this hypothesis.

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References

- Altman, R. D., Johnston, C. C., Khairi, M. R. A., Wallman, H., Serafini, A. N. & Sankey, R. R. (1973) *New Engl. J. Med.* **289**, 1379–1384.
- Bremer, J. (1977) *Trends Biochem. Sci.* **2**, 207–209
- Chase, J. F. A. & Tubbs, P. K. (1972) *Biochem. J.* **129**, 55–65
- Dole, V. P. & Meinertz, H. (1960) *J. Biol. Chem.* **235**, 2595–2599
- Eagle, H. (1959) *Science* **130**, 432–437
- Ende, J. J. (1978) *Proc. Kon. Ned. Akad. Wet.* **C-81**, 252–264
- Ende, J. J. & van Rooijen, H. J. M. (1979a) *Proc. Kon. Ned. Akad. Wet.* **C-82**, 43–54
- Ende, J. J. & van Rooijen, H. J. M. (1979b) *Proc. Kon. Ned. Akad. Wet.* **C-82**, 55–63
- Engel, P. C. & Jones, J. B. (1978) *Anal. Biochem.* **88**, 475–484
- Fast, D. K., Felix, R., Dowse, C., Neuman, W. F. & Fleisch, H. (1978) *Biochem. J.* **172**, 97–107

- Felix, R. & Fleisch, H. (1979) *Biochem. J.* **183**, 73–81
- Felix, R., Fast, D. K., Sallis, J. D. & Fleisch, H. (1980) *Calcif. Tissue Int.* **30**, 163–166
- Fleisch, H., Russell, R. G. G. & Francis, M. D. (1969) *Science* **165**, 1262–1264
- Fleisch, H. A., Russell, R. G. G., Bisaz, S., Mühlbauer, R. C. & Williams, D. A. (1970) *Eur. J. Clin. Invest.* **1**, 12–18
- Francis, M. D., Russell, R. G. G. & Fleisch, H. (1969) *Science* **165**, 1264–1266
- Guenther, H. L., Guenther, H. E. & Fleisch, H. (1979) *Biochem. J.* **184**, 203–214
- Guenther, H. L., Guenther, H. E. & Fleisch, H. (1981) *Biochem. J.*, in the press
- Guilland, D. F. & Fleisch, H. (1974) *Biochem. Biophys. Res. Commun.* **61**, 906–911
- Guilland, D. F., Sallis, J. D. & Fleisch, H. (1974) *Calcif. Tissue Res.* **15**, 303–314
- Gunčaga, J., Lauffenburger, T., Lentner, C., Dambacher, M. A., Haas, H. G., Fleisch, H. & Olah, A. J. (1974) *Horm. Metab. Res.* **6**, 62–69.
- Hanks, J. H. & Wallace, R. E. (1949) *Proc. Soc. Exp. Biol. Med.* **71**, 196–200
- Kates, M. (1972) *Techniques of Lipidology. Isolation, Analysis and Identification of Lipids*, pp. 347–353, North-Holland, Amsterdam
- Lorch, E. & Gey, K. F. (1966) *Anal. Biochem.* **16**, 244–252
- Mahadevan, S. & Sauer, F. (1971) *J. Biol. Chem.* **246**, 5862–5867
- Meunier, P. J., Chapuy, M. C., Alexandre, C., Bressot, C., Edouard, C., Vignon, E., Mathieu, L. & Trechsel, U. (1979) *Lancet* **ii**, 489–492
- Morgan, D. B., Monod, A., Russell, R. G. G. & Fleisch, H. (1973) *Calcif. Tissue Res.* **13**, 287–294
- Ockner, R. K. & Manning, J. A. (1976) *J. Clin. Invest.* **58**, 632–641
- Russell, R. G. G., Mühlbauer, R. C., Bisaz, S., Williams, D. A. & Fleisch, H. (1970) *Calcif. Tissue Res.* **6**, 183–196
- Russell, R. G. G., Smith, R., Preston, C., Walton, R. J. & Woods, C. G. (1974) *Lancet* **i**, 894–898
- Samuel, D., Paris, S. & Ailhaud, G. (1976) *Eur. J. Biochem.* **64**, 583–595
- Siris, E. S., Sherman, W. H., Baquiran, D. C., Schlatterer, J. P., Ossermann, E. F. & Canfield, R. E. (1980) *New Engl. J. Med.* **302**, 310–315