Biochemical Pharmacology, Vol. 26, pp. 595–600. Pergamon Press, 1977. Printed in Great Britain.

EFFECT OF TETRACYCLINE ON CHONDROCYTE MITOCHONDRIA—AN EXPLANATION OF TETRACYCLINE-INDUCED DEFECTS OF MINERALIZED TISSUES*

IRVING M. SHAPIRO†, ANITA C. BURKE and NAM HEA LEE

Department of Biochemistry and Center for Oral Health Research, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA 19174, U.S.A.

(*Received* 24 *April* 1976; *accepted* 2 *August* 1976)

Abstract—To investigate the mechanism of tetracycline (TC)-induced defects of mineralization, the effect of the antibiotic on Ca^{2+} transport by chick chondrocyte mitochondria was studied. Multiple injections of TC resulted in a profound drop in the intramitochondrial Ca^{2+} concentration and an increase in the serum Ca^{2+} level. The antibiotic caused a reduction in Ca^{2+} uptake and an increase in Ca^{2+} efflux from the mitochondria *in vitro*; in the presence of 1 mM TC, the effect on efflux was greater than uptake. The addition of ATP to TC-treated mitochondria did not prevent Ca^{2+} release. Using rat liver mitochondria, it was found that, while the antibiotic had an oligomycin-like effect on respiration, this inhibitory action could be prevented by preincubating the organelles with 10 mM Mg^{2+}. The results of the studies both *in vitro* and *in vivo* suggest that TC interferes with mitochondrial Ca^{2+} translocation. It is concluded that critical levels of intramitochondrial Ca^{2+} are prerequisite for the normal mineralization process and that interference with Ca^{2+} accumulation leads to defective mineralization.

Numerous studies have shown that tetracyclines can induce skeletal hypoplasia and hypomineralization [1–5]. However, the mechanism by which the antibiotic interferes with the mineralization process is poorly understood. Histochemical studies by Milch *et al.* [6] suggested that tetracycline binds to collagen fibrils and these workers inferred that the drug blocked nucleation sites. Kaitila [7] expressed the view that the antibiotic delayed the phase transformation of amorphous calcium phosphate into apatite. West and Storey [8] showed that tetracyclines inhibited the phase transformation *in vitro*, but no attempt was made to study the effects *in vivo* of the antibiotic on bone crystallinity.

A different approach to explaining the effects of tetracycline on calcifying tissues is through its effects on tRNA. Thus, because tetracycline inhibits the binding of tRNA to the ribosome, some investigators consider that the principal site of tetracycline action is on protein synthesis. Indeed, tetracycline-induced inhibition of collagen formation [9] has been reported. However, these experiments were performed with elevated antibiotic levels and the investigators did not determine whether the tetracycline inhibited the ribosomal synthesis of collagen directly, or interfered with protein synthesis by blocking cellular respiration.

Antibiotics like tetracycline have been shown to decrease mitochondrial protein synthesis in mammalian cells. Indeed, in the presence of tetracycline, there is a diminution in mitochondrial cytochrome synthesis [10]. Whether tetracyclines cause changes in mitochondrial cation transport is not known with certainty. However, as an ionophore, it could conceivably inhibit the accumulation of Ca^{2+} in mitochondria of cells preparing for mineralization and interfere with cation-activated enzyme systems of the cytosol [11]. If, as Lehninger [12] has postulated, the intramitochondrial accumulation of calcium is an initial first step in the calcification process, impairment of Ca^{2+} accumulation could result in hypoplasia of teeth, poor mineralization of bone, and delayed osteogenesis. This investigation explores this possibility by studying the effect of tetracycline on Ca^{2+} transport by epiphyseal plate mitochondria.

MATERIALS AND METHODS

Materials. Bovine serum albumin, HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid), ATP, ADP, succinate Na_2, tetracycline HCl, oxytetracycline, chlortetracycline, DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)], DTT (dithiothreitol), DNP (2,4-dinitrophenol), ruthenium red, oligomycin and rotenone were all purchased from Sigma Chemical Co. $^{45}CaCl_2$ (1 mCi $^{45}Ca/\mu$mole of Ca^{2+}) was obtained from New England Nuclear Corp. This was diluted to achieve a final activity of 0.1 μCi ^{45}Ca/ml of reaction mixture.

Tissues. White Rock chicks, weighing between 4 and 5 kg, were maintained on a normal diet *ad lib.* For experiments on rachitic chicks, 5-day-old birds were maintained in the dark on a rachitogenic diet (Nutritional Biochemical Corp.) and distilled water. At 8–10 weeks, both normal and rachitic birds were killed by a pentobarbital overdose (i.v.). The legs were disarticulated at the pelvic joint and the epiphyseal

* This work was supported by National Institute of Dental Research Grant DE-02623.

† To whom reprint requests should be sent.

B.P. 26/7-C

joint was exposed by cutting through the capsular cartilage. Resting and proliferating cartilages were removed in thin slices and chopped finely with sharp tissue scissors.

Isolation of mitochondria. This was similar to the method previously published [13]. In studies *in vivo* of chick mitochondria, to block Ca^{2+} uptake during the isolation procedure and to inhibit the subsequent redistribution of Ca^{2+}, during the centrifugation steps, the mitochondrial fraction was isolated in the presence of 50 μM DTNB [14] and 73 μM ruthenium red [15]. The mitochondrial pellet was further purified by centrifugation in a continuous sucrose gradient (1.5 to 2.0 M). Centrifugation at 35,000 rev/min was performed in an SW 35 rotor in a Beckman model L-2 ultracentrifuge. Liver mitochondria were isolated from 150-g Sprague–Dawley rats by a previously described method [14].

Chemical analysis. Ca^{2+} analysis was performed by atomic absorption spectroscopy (Perkin Elmer model 360). Mitochondrial protein was measured using the method described by Lowry *et al.* [16].

Tetracycline solutions. Tetracycline, freshly prepared for each experiment, was dissolved in buffered saline or reaction medium and the pH was adjusted to 7.4.

Effect of tetracycline on endogenous Ca^{2+} levels on chondrocyte mitochondria. Rachitic chicks were given i.p. injections of tetracycline suspended in saline (100 mg/kg body weight) 24, 36, 60 and 72 hr prior to sacrifice. As a control, at the appropriate times, rachitic chicks were injected with saline. Prior to sacrifice, about 1 ml of venous blood from a wing vein was drawn into a syringe containing heparin, and the blood Ca^{2+} content was determined in the presence of lanthanum chloride (0.1 per cent). The mitochondria isolated from the epiphyseal plate cartilage were analyzed for Ca^{2+}, and the protein content was determined.

Effect of tetracycline on Ca^{2+} efflux from chondrocyte mitochondria. Mitochondria were isolated from normal chicks. To pre-load with Ca^{2+}, the organelles were incubated at 18° for 10 min with 0.5 ml of a reaction mixture consisting of 80 μM Ca^{2+} (containing 0.05 μCi ^{45}Ca), 38 mM HEPES, 8.5 mM $MgCl_2$, 3.8 mM P_i, 8 mM succinate and 0.2 μg rotenone. The osmolarity of the mixture was adjusted to 250 mOsM with 1.0 M KCl and the pH was 7.4. Tetracycline was then added to the reaction mixture such that the final concentration was 1.0 mM. At intervals of time, up to 20 min, 0.1-ml aliquots of the suspension were removed and immediately filtered through a 0.45 μm Millipore filter. The filter was then washed with 10 ml of 0.125 M KCl and dried, and the ^{45}Ca content was measured by liquid scintillation counting (Packard Tri-Carb model 3310). In a second series of experiments, the effect of tetracycline on the Ca^{2+} concentration of chondrocyte mitochondria was measured in the presence of 8 mM ATP in the reaction medium. To compare the effects of different tetracyclines, mitochondria were pre-incubated with the reaction medium described above and then exposed for 10 min to 1 mM tetracycline, oxytetracycline or chlortetracycline. The Ca^{2+} content of the mitochondria was then determined. Finally, the effect of different concentrations of tetracyclines on Ca^{2+} uptake was investigated. Mitochondria were preincubated with the reaction mixture described above for 10 min. Tetracycline (0.1 to 10 mM) was then added to the mitochondrial suspension and after 10 min it was filtered and the ^{45}Ca content of the filter was measured.

Effect of tetracyclines on Ca^{2+} uptake by chondrocyte mitochondria. The effect of tetracycline on Ca^{2+} uptake was studied by preincubating 60 μl of the mitochondrial suspension (0.5 mg protein) for 1 min with a Ca^{2+}-free reaction medium containing 1 mM tetracycline, 38 mM HEPES, 8 mM succinate, 8.5 mM $MgCl_2$, 3.8 mM P_i, 0.2 μg rotenone and the osmolarity adjusted to 250 mOsM with 1.0 M KCl. In some experiments, the reaction mixture was further supplemented with 8 mM ATP. Radioactive $CaCl_2$ was then added to the suspension to give a final concentration of 80 μM Ca^{2+}. At intervals of time up to 20 min, 0.1 ml of mitochondria was removed and filtered, and the Ca^{2+} content was determined. In another series of experiments using the same incubation medium, the effect of tetracycline, at concentrations ranging from 0.01 to 10 mM, on Ca^{2+} uptake was ascertained.

Effect of tetracyclines on mitochondrial respiration. Difficulties in isolating large quantities of cartilage mitochondria preclude the use of this tissue in studying oxidative phosphorylation. However, as liver and cartilage mitochondria have similar respiratory characteristics [17], rat liver mitochondria were utilized for these studies. Liver mitochondria were incubated with a reaction mixture containing 40 mM HEPES, and 1.25 mM P_i; the osmolarity was adjusted to 250 mOsM with 1.0 M KCl and the pH was 7.4. The effect of tetracycline (155 μM) and Mg^{2+} (0–10 mM) on the oxidation of glutamate and succinate (8 mM) was determined using a Clark oxygen electrode (No. 522, Yellow Springs Instruments, Yellow Springs, Ohio) linked to a 10 mV recorder, Sargent model SRL).

RESULTS

Centrifugation of the 14,000 *g* pellet on a continuous sucrose gradient yields two to three density bands. The high density mitochondrial band contained 2.25 ± 0.84 μmoles Ca^{2+}/mg of protein. (The composition of the low density bands will be described elsewhere.) The Ca^{2+} content of mitochondria isolated from the tetracycline-treated chicks was considerably lower than the controls (0.38 ± 0.2 μmoles Ca^{2+}/mg of protein). In contrast to its effects on mitochondria, tetracycline caused an increase in the serum Ca^{2+} concentration (Table 1). No change was noted in the serum Mg^{2+} concentration.

Table 1. Ca^{2+} analysis of chick blood*

	Ca^{2+} (mg/100 ml blood)
Control	5.28 ± 0.41
Tetracycline-treated	7.48 ± 0.37

* Seven-week-old rachitic animals were injected with tetracycline as described in the text. About 1 ml blood was drawn from a vein and analyzed for Ca^{2+} by atomic absorption spectroscopy. All analyses were performed in triplicate. The results shown are the mean ± S. E. M. of seven determinations.

The relationship between mitochondrial Ca^{2+} efflux and tetracycline concentration is shown in Fig. 1. The presence of the antibiotic results in a profound loss of Ca^{2+} from the mitochondria; the extent of Ca^{2+} release is dependent on the concentration of tetracycline. Incubation with 1 mM tetracycline results in efflux of over 30 per cent of the pre-loaded Ca^{2+}. At low antibiotic concentrations there is a slight increase in the mitochondrial Ca^{2+} level. Tetracycline-stimulated release of Ca^{2+} from chondrocyte mitochondria, expressed as a function of time, is shown in Fig. 2. Between 5 and 10 min, mitochondria pre-loaded with Ca^{2+} exhibit a rapid rate of release of accumulated Ca^{2+}. Mitochondria loaded with Ca^{2+} in the absence of added nucleotide exhibit an almost linear rate of Ca^{2+} efflux for the first 15 min. Figure 2 also reveals that mitochondria loaded with Ca^{2+} in the presence of ATP show an initial higher Ca^{2+} concentration than organelles that are provided with succinate as the sole energy source. However, irrespective of the mode of Ca^{2+} loading, after 10 min the Ca^{2+} efflux rates are similar.

The effects of different types of tetracyclines on mitochondrial Ca^{2+} efflux are shown in Fig. 3. The presence of tetracycline or oxytetracycline results in a dramatic release of Ca^{2+} from chondrocyte mitochondria. Chlortetracycline does not cause Ca^{2+} efflux from pre-loaded mitochondria.

The effect of tetracycline concentration on Ca^{2+} uptake by chondrocyte mitochondria is shown in Fig. 4. When the tetracycline level is below 1 mM, the antibiotic causes an increase in the Ca^{2+} content of the organelles. With 1 mM tetracycline, there is no change in the Ca^{2+} content of the mitochondria. However, concentrations of the antibiotic in excess of 1 mM result in a concentration-dependent net Ca^{2+} decrease.

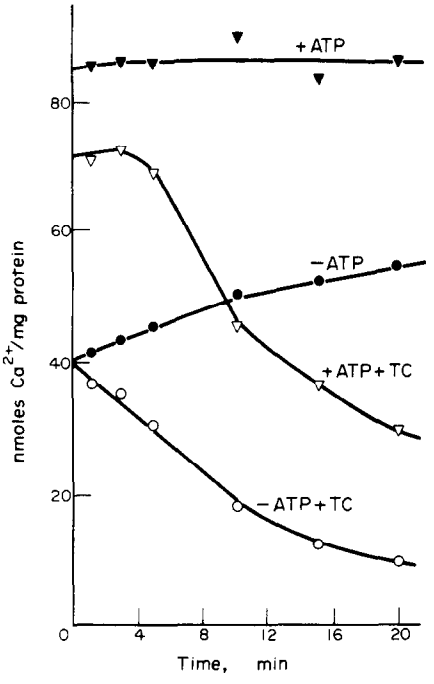

Fig. 2. Effect of tetracycline (1 mM) on Ca^{2+} efflux by chondrocyte mitochondria. Chondrocyte mitochondria were pre-incubated with a reaction medium in the presence and absence of 8 mM ATP. After 10 min, tetracycline (1 mM) was added to the suspension (0 time on the graph). At fixed intervals of time, aliquots were removed and the Ca^{2+} content of the mitochondria was determined. Key: plus ATP, ▼——▼; plus ATP and tetracycline, ▽——▽; minus ATP, ●——●; and minus ATP and tetracycline, ○——○. Each point is the mean of four repeated experiments; each experiment was performed in duplicate.

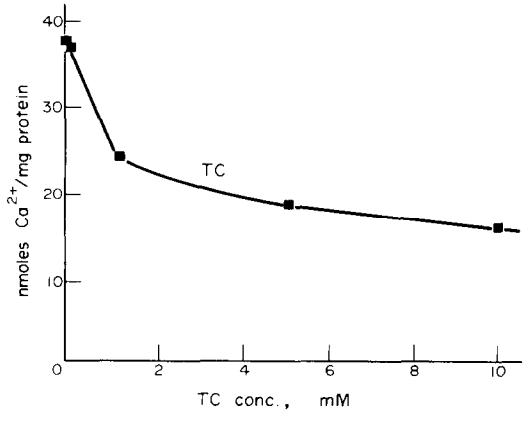

Fig. 1. Effect of tetracycline concentration on Ca^{2+} efflux from chondrocyte mitochondria. Chondrocyte mitochondria were pre-incubated with a reaction medium containing 80 μM Ca^{2+}, 38 mM HEPES, 8.5 mM $MgCl_2$, 3.8 mM P_i, 8.0 mM succinate and 0.2 μg rotenone. The medium contained 0.05 μCi ^{45}Ca and the osmolarity was adjusted to 250 mOsM with 1.0 M KCl. After 10 min, tetracycline (TC) (0.01 to 10 mM) was added and the suspension incubated for a further 10 min. The mitochondria were then filtered and the Ca^{2+} content of the filter was determined. Each point is the mean of three repeated experiments; each experiment was performed in duplicate.

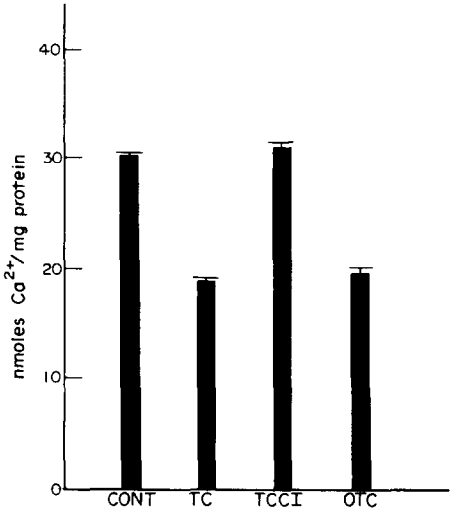

Fig. 3. Comparison of tetracycline, chlortetracycline and oxytetracycline on Ca^{2+} efflux from chondrocyte mitochondria. Mitochondria were pre-incubated with reaction media and loaded with Ca^{2+}. Tetracycline (TC), chlortetracycline (TCCl) and oxytetracycline (OTC) (1 mM) were then added to the mitochondrial suspension, and the Ca^{2+} content of the mitochondria was measured after a 10-min incubation period. Cont. = control. Results shown are the mean ± S. E. M. of six duplicate experiments.

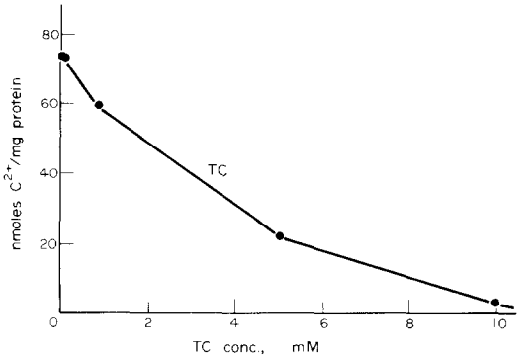

Fig. 4. Effect of tetracycline concentration on Ca^{2+} uptake by chondrocyte mitochondria. Chondrocyte mitochondria were pre-incubated with the Ca^{2+}-free reaction medium described in Fig. 1. This was supplemented with 0–10 mM tetracycline (TC). After 1 min, 80 μM Ca^{2+} was added to the mitochondrial suspension and incubated with the suspension for 10 min. The mitochondria were then filtered and the Ca^{2+} concentration was determined. Each point is the mean of four separate experiments: each experiment was performed in duplicate.

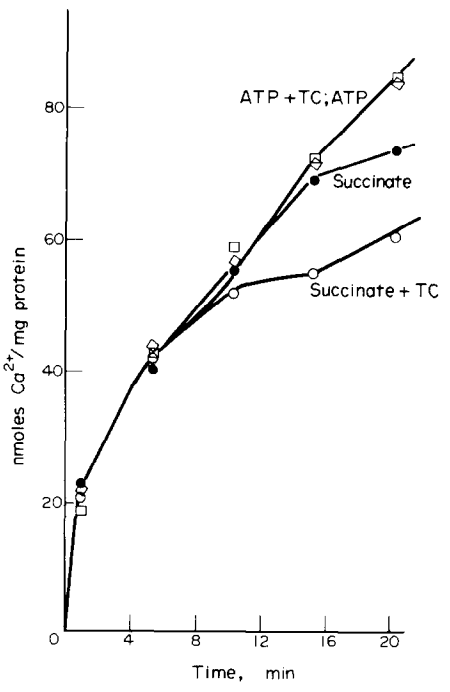

Fig. 5. Ca^{2+} uptake by chondrocyte mitochondria in the presence of 1 mM tetracycline. Chondrocyte mitochondria were pre-incubated for 1 min with a Ca^{2+}-free reaction mixture containing 1 mM tetracycline (TC). In some experiments the reaction mixture was supplemented with 8 mm ATP. Subsequently, Ca^{2+} was added to make a final concentration of 80 μM. At intervals of time, 0.1 ml of the mitochondrial suspension was removed and analyzed for Ca^{2+}. Key: succinate, ●–●; succinate plus TC, ○–○; ATP, □–□; and ATP plus TC, ◇–◇. Each point is the mean of three separate experiments; each experiment was performed in duplicate.

Ca^{2+} uptake by mitochondria, preincubated with tetracycline, expressed as a function of time, is shown in Fig. 5. For the first 10 min, irrespective of the energy source, there is little difference in the rate of Ca^{2+} uptake. However, after 10 min, tetracycline decreases the rate of succinate-supported uptake. The presence of ATP counteracts this effect such that the rates of ATP-supported Ca^{2+} uptake, in the presence and absence of tetracycline, are similar.

The effect of tetracycline on the oxidation of glutamate by mitochondria is shown in Table 2. At low Mg^{2+} concentrations (up to 6 mM) tetracycline decreases state 3 respiration. At the highest Mg^{2+} concentration studied (10 mM) state 3 respiration increases and cutoff is seen. Tetracycline also inhibits the state 3 respiratory rate of succinate. This inhibitory effect can be counteracted by the addition of Mg^{2+} to the mitochondrial suspension. However, in the presence of succinate, the inhibitory effect is not as severe as with glutamate. Thus, addition of ADP, even at low Mg^{2+} concentrations, elicits an increase in O_2 consumption that is always above the state 4 rate.

These experiments indicate that Mg^{2+} exerts a protective effect on respiration if the ion is added to the mitochondria prior to the addition of the antibiotic. However, if mitochondria are exposed to the antibiotic first, the tetracycline exerts an oligomycin-like

Table 2. Effect of tetracycline and Mg^{2+} on oxidative phosphorylation by rat liver mitochondria*

		Succinate			Glutamate		
		(μatoms O/mg protein/min)			(μatoms O/mg protein/min)		
Tetracycline (μM)	Mg^{2+} (mM)	State 3	State 4	ADP/O	State 3	State 4	ADP/O
155	0	0.096	0.038	1.7	0.016	0.028	
155	2	0.099	0.035	1.8	0.017	0.034	
155	4				0.022	0.032	
155	6	0.127	0.030	1.6	0.035	0.026	
155	10	0.129	0.034	1.7	0.054	0.035	2.7
0	10	0.140	0.040	1.9	0.064	0.026	3.02
0	0	0.142	0.037	2.0	0.059	0.029	2.91

* Rat liver mitochondria (3 mg/ml) were incubated with a reaction medium containing 40 mM HEPES, 1.25 mM P_i and 8 mM glutamate or succinate. The osmolarity of the solution was adjusted to 250 mOsM with 1 M KCl. Oxygen uptake was measured in the presence and absence of tetracycline (155 μM) and in the presence of varying concentrations of Mg^{2+} (0–10 mM). The results are the mean of four repeated experiments.

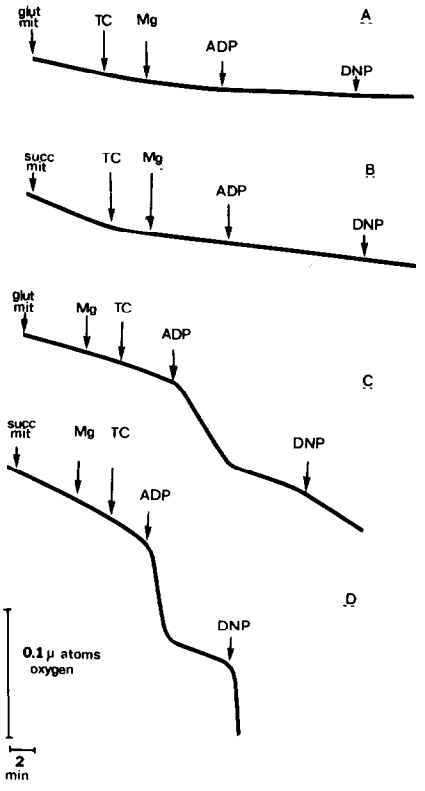

Fig. 6. Effect of Mg^{2+}, tetracycline (155 μM) and DNP on oxygen uptake by mitochondria. Conditions of incubation were the same as those described in Table 2. Where indicated on the tracings, tetracycline (TC), Mg^{2+} (10 mM), ADP (125 μmoles) and DNP (2 μg) were added to the reaction mixture. Values shown are the mean of four experiments.

effect; there is inhibition of ADP-stimulated respiration in the presence of both succinate and glutamate, and further addition of Mg^{2+} fails to increase the respiratory rate (Fig. 6, A and B). Figure 6 (C and D) illustrates the effect of DNP on respiration. The uncoupled rate of O_2 utilization with glutamate as a substrate in the presence of DNP is about the same or slightly less than ADP-stimulated respiration in the presence of tetracycline. However, with succinate as a substrate (Fig. 6D), the uncoupled rate is faster than the state 3 rate of O_2 uptake.

DISCUSSION

While previous workers have used tetracycline as a fluorescent probe to study anionic transport processes [18], the ionophoretic activities of these antibiotics have not been related to their reported inhibitory effects on mineralization. The results of this investigation suggest that tetracycline-induced defects of calcification may be a result of a direct action of the antibiotic on mitochondria of cells concerned with the mineralization process. If this is a valid explanation of this type of hypomineralization, then this finding also throws light on the mechanism by which tissues undergo normal biological mineralization. With respect to this mechanism, if as has been previously suggested, mitochondria are concerned initially with concentrating Ca^{2+} ions [12,19,20], then any interference with the accumulation mechanism will result in slowed, defective or irregular calcification. The findings *in vivo* and *in vitro* reported in this study clearly support the concept that tetracyclines are causing these aberrations by influencing cation transport, and the results lend credence to the view that mitochondrial ion accumulation is a necessary prerequisite to matrix mineralization.

While these experiments did not address themselves to studying the uptake of tetracycline by chondrocytes, the localization of tetracyclines in forming bone [3,21,22] and the slow rate of removal of tetracycline from bone would suggest that *in vivo* the chondrocytes are exposed to elevated tetracycline levels. Furthermore, the low molecular weight of the antibiotic and its lipophilic properties would facilitate its entry into chondrocytes. Indeed, Aaron and Pautard [23] showed that tetracycline is taken up by bone cells and is seen as a "generalized network" within the cell, while tetracycline localization within mitochondria of living cells has also been observed [24].

The effects *in vitro* of tetracycline on isolated chondrocyte mitochondria supported the observation *in vivo* that the antibiotic lowered the mitochondrial Ca^{2+} concentration. This study revealed that 1 mM tetracycline caused a net Ca^{2+} efflux from the mitochondria and at this concentration there was little change in Ca^{2+} uptake. Of the antibiotics studied, both tetracycline and oxytetracycline appeared to be active in promoting efflux; with tetracycline, the efflux rate was almost linear for the first 10 min of exposure, and Ca^{2+} release was proportional to the tetracycline concentration. Whether tetracycline activated a latent mitochondrial ATPase was not ascertained. However, in the presence of ATP, a marked decrease in the total amount of mitochondrial Ca^{2+} was observed.

The effect of Mg^{2+} on the respiration of tetracycline-treated mitochondria is disputed. In an earlier study, Brody *et al.* [25] indicated that the effect of tetracycline could be reversed by the addition of excess Mg^{2+}. De Jonge [26], using 2 mM Mg^{2+}, showed that the effect of oxytetracycline could be "prevented to a large extent" by preincubating the mitochondria with Mg^{2+}. To investigate in detail the extent of Mg^{2+} protection, mitochondria were titrated with concentrations of Mg^{2+} varying from 0 to 10 mM. This study confirmed that Mg^{2+} did give protection to mitochondria, but that Mg^{2+} levels in excess of 6 mM were needed to reverse the tetracycline inhibition. Whether there is sufficient Mg^{2+} at calcifying sites to protect the mitochondria from the antibiotic is not known. However, it was noted that 6 mM Mg^{2+} protects mitochondria against the deleterious effects of 155 μM tetracycline (see Table 2). If respiratory chain protection is proportional to the relative concentration of Mg^{2+}, then in mineralizing tissues where the extracellular Mg^{2+} concentration is only 0.4 mM [27], the organelles would be protected from the damaging effects of 10 μM of the antibiotic. Ignoring any corrections for the known accumulation of tetracycline at calcifying sites, it is clear that the therapeutic blood tetracycline level of 23 μM would exceed the protection afforded by 0.4 mM Mg^{2+} and there would be impairment of respiratory chain function. It is interesting to speculate that

the low Mg^{2+} concentration of the extracellular fluid of calcifying cartilage may well be the reason why this tissue is so susceptible to the effects of this antibiotic.

With respect to the site of action of tetracycline, the inhibitory action of the antibiotic on the oxidation of glutamate confirmed that NADH-linked substrates were more susceptible to the effects of the antibiotic [26]. In addition, because DNP released the tetracycline inhibition of succinate oxidation, then electron transfer between succinate and oxygen must be intact. However, as DNP only elicited a partial release of glutamate oxidation, it is probable that the antibiotic inhibits the activity of the respiratory chain lying between the site of entry of NADH-linked substrate into the chain and the entry of reducing equivalents from succinate.

If the experiments on the effects of tetracycline on oxidative phosphorylation by liver mitochondria are related to the changes in cation translocation of chondrocyte mitochondria, then it is apparent that the antibiotic can affect Ca^{2+} transport in a number of different ways. First, the investigation clearly reveals that tetracycline selectively impairs normal functioning of the respiratory chain. This impairment alone could markedly decrease the ability of chondrocyte mitochondria to translocate Ca^{2+}. Second, it is known that pre-loading mitochondria with Ca^{2+} produces pathological changes in mitochondria [28]. Thus, the addition of tetracycline to organelles that may have already been damaged through Ca^{2+} accumulation could exacerbate any Ca^{2+}-induced defects in function as well as affecting other as yet unimpaired sites on the respiratory chain necessary for Ca^{2+} accumulation. The possibility that Ca^{2+} loading causes disturbances in mitochondrial function may also explain why tetracycline has a greater effect on Ca^{2+} efflux than Ca^{2+} uptake. A third explanation for Ca^{2+} efflux could be associated with the oligomycin-like effects of tetracycline. Thus, if the antibiotic interferes with the phosphorylation of ADP and reduces the rate of P_i–ATP exchange, the high energy intermediate necessary for the maintenance of Ca^{2+} within the organelle will not be generated at the requisite rate. In that instance, extrusion will occur.

Finally, the observed increase in serum Ca^{2+} levels of chicks treated with large doses of tetracycline lends support to the concept that *in vivo*, the antibiotic is causing a release of Ca^{2+} from mineralizing cartilage. A possible alternative explanation, in line with the suggestion by Baker [29] that tetracyclines cause a transient hyperparathyroidism, is that the antibiotic is initially chelating Ca^{2+} and the resulting serum Ca^{2+} elevation is due to secondary hyperparathyroidism. This study did not address itself to studying this relationship; however, at the concentrations used, a serum tetracycline level of about 10 μg/ml would be expected [10]. Assuming that 1 mole of the antibiotic binds 1 mole Ca^{2+}, then less than 2 per cent of the total serum Ca^{2+} could be bound by the antibiotic. It is unlikely that this small change in serum Ca^{2+} level would be sufficient to markedly alter the secretion rate of the parathyroid hormone. It therefore must be concluded that the serum Ca^{2+} changes noted are due primarily to the tetracycline acting directly on the bone cells. Nevertheless, it must be admitted that the possible associations between this class of antibiotics and the factors controlling Ca^{2+} homeostasis are poorly understood. It is suggested, therefore, that more detailed investigation should be carried out to ascertain the relationship between serum Ca^{2+} concentrations, parathyroid hormone secretion and tetracycline dose.

Acknowledgements—The critical comments of Dr. N. Haugaard and Dr. P. S. Leboy are gratefully acknowledged.

REFERENCES

1. G. Bevelander, H. Nakahara and G. K. Rolle, *Dev. Biol.* **2,** 298 (1960).
2. C. G. Lofgren, K. A. Omnell and M. U. Nylen, *Calcif. Tissue Res.* **2,** 145 (1968).
3. I. Kaitila, J. Wartiovaara, O. Laitinen and L. Saxen, *J. Embryol. exp. Morph.* **23,** 185 (1970).
4. L. Saxen, *Science, N.Y.* **149,** 870 (1965).
5. W. H. Harris, J. Lovorgna, D. L. Hambless and E. A. Hayword, *Clin. Orthop.* **61,** 52 (1968).
6. R. A. Milch, J. E. Robie and R. A. Robinson, *J. Histochem. Cytochem.* **9,** 261 (1961).
7. I. Kaitila, *Calcif. Tissue Res.* **7,** 46 (1971).
8. V. C. West and E. Storey, *Calcif. Tissue Res.* **9,** 207 (1972).
9. J. E. Halme and J. Aer, *Biochem. Pharmac.* **17,** 1479 (1968).
10. H. R. De Jonge and W. C. Hülsmann, *Eur. J. Biochem.* **32,** 356 (1973).
11. T. Spencer and F. L. Bygrave, *Bioenergetics* **4,** 347 (1973).
12. A. L. Lehninger, *Biochem. J.* **119,** 129 (1970).
13. N. H. Lee and I. M. Shapiro, *Analyt. Biochem.* **58,** 117 (1974).
14. N. Haugaard, N. H. Lee, R. Kostrezewa and E. S. Haugaard, *Biochem. Pharmac.* **18,** 2385 (1969).
15. F. D. Vasington, P. Gazzotti, R. Tiozzo and E. Carafoli, *Biochim. biophys. Acta* **256,** 43 (1972).
16. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
17. N. H. Lee and I. M. Shapiro, *Calcif. Tissue Res.* **16,** 277 (1974).
18. S. M. Schuster and M. S. Olson, *J. biol. Chem.* **249,** 7151 (1974).
19. I. M. Shapiro and J. S. Greenspan, *Calcif. Tissue Res.* **3,** 100 (1969).
20. J. H. Martin and J. L. Matthews, *Clin. Orthop.* **68,** 273 (1970).
21. G. K. Rolle and G. Bevelander, *J. Morph.* **118,** 317 (1966).
22. L. Saxen, *J. exp. Zool.* **162,** 269 (1966).
23. J. E. Aaron and F. G. E. Pautard, in *Calcium Metabolism, Bone and Metabolic Bone Diseases* (Eds. F. Kuhlencordt and H. P. Kruse), pp. 211–17. Springer, New York (1973).
24. H. G. Dubuy and J. L. Showacre, *Science, N.Y.* **133,** 196 (1961).
25. T. M. Brody, R. Hurwitz and J. A. Bain, *Antibiot. Chemother.* **4,** 864 (1954).
26. H. R. De Jonge, *Biochem. Pharmac.* **22,** 2659 (1973).
27. W. F. Neuman and W. K. Ramp, in *Cellular Mechanisms for Calcium Transfer and Homeostasis* (Eds. G. Nichols and R. H. Wasserman), pp. 197–206. Academic Press, New York (1971).
28. F. D. Vasington and J. W. Greenawalt, *J. Cell Biol.* **39,** 661 (1968).
29. K. L. Baker, *Archs oral Biol.* **19,** 717 (1974).