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INFLUENCE OF DIPHENYLHYDANTOIN ON ISOTOPE RELEASE AND BONE ENZYMES *IN VITRO*

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The acute effect of diphenylhydantoin on isotope release in an *in vitro* system using mouse calvaries was studied. A depressive effect on bone resorption was found involving radioactive calcium as well as tritiated proline. Bone resorption and formation were further investigated by semiquantitative histochemistry. Depressed activity of both alkaline and acid phosphatase was found, indicating a direct inhibitory effect of diphenylhydantoin on bone turnover.

Key words: bone; diphenylhydantoin; enzymes; histocytochemistry; isotopes

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The influence of diphenylhydantoin (DPH) on calcium homeostasis in patients on anti-convulsant therapy is well known, and explained partially by interaction of the drug with the metabolism of the biologically active vitamin D metabolites (Christensen et al. 1981). The direct action of DPH on bone resorption in fetal rat bone in culture has also been the subject of investigation in recent years (Harris et al. 1974, Hahn et al. 1978). In the intact human organism a high turnover bone disease has been found (Mosekilde & Melsen 1976). The purpose of the present study was to evaluate the effect of DPH on calcium metabolism in mouse calvaria maintained in culture. We have directed our attention to bone turnover evaluated by Ca^{45} and H^3 proline release. Uptake studies are difficult to interpret, while semiquantitative bone cell histochemistry offers a better chance to study osteoclastic and osteoblastic function separately. The action of DPH on bone might be attributed to its influence on turnover rather than on resorption alone.

MATERIAL AND METHOD

Isolation and culture. Calvaries from 4-day-old mice injected subcutaneously with Ca^{45} (Radiochemical Center, Amersham) or H^3 proline (NEN) were removed and cultured for 48 hours according to the method of Reynolds & Dingle (1970). The parietal bones were cut into two pieces, one used as control, the other for experiments. The isolated bone pieces were cultured in test tubes containing 2 ml Medium 199 (Gibco 115 EE), to which bovine serum albumin fraction V (5 g/l) and Ampicillin (40 mg/l) had been added. The culture was incubated in an atmosphere of 5% CO_2, 75% N_2 and 20% O_2 at 37°C and regassed after 24 h. Each calvaria was decalcified in 1 ml 1 M hydrochloride acid at 90°C. 0.5 ml of this solution and 0.5 ml of medium were processed by standard liquid scintillation counting techniques. In uptake studies non-labelled calvaries were incubated in the same medium with isotopes added. After 48 h the bones were rinsed in fresh medium and thoroughly dried before being treated with hydrochloride acid.

Release was expressed as a percentage of total isotope found in the medium. The values were then calculated as the ratio between treated and control bones.

Histological analysis. Non-labelled calvaries grown for 48 h were used for histological and semiquantitative histochemical examinations. The specimens were fro-

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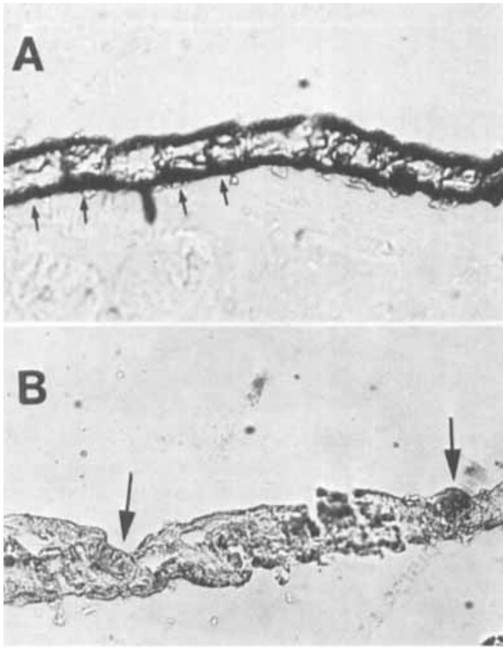

Figure 1. A: Photograph of frozen section, 6 μm thick from a mouse calvaria stained for alkaline phosphatase. The reaction (black color) is seen at the surface in the osteoblasts and preosteoblasts (arrows). Magnification ×100. B: Frozen section, 6 μm thick, stained for acid phosphatase. The reaction (black color) is seen at the surface in the osteoclasts (arrows). Magnification ×100.

zen at −20°C immediately after removal from the medium and 6-μm-thick sections were cut in a cryostat. Twelve sections were prepared from each specimen, six for determination of alkaline phosphatase and six for acid phosphatase activity. For histochemical demonstration of non-specific alkaline phosphatase activity, the simultaneous coupling method described by Burstone (1959) was used, with naphthol-AS-BI-phosphate (Sigma N 22508) as substrate (concentration 0.2 mg/ml) and Fast Red Violet LB (Difco 8194-12) as indicator (1.4 mg/ml). Acid phosphatase activity was demonstrated by Burstone's modification of the simultaneous coupling method described by Barka & Anderson (1963) with naphthol-AS-BI-phosphate (Sigma N 22509) as substrate (concentration 0.5 mg/ml) and hexazotised pararosaniline (Sigma P 37507) as indicator.

The examinations were performed by light microscopy (Leitz Orthoplan) at a magnification 100×, at 22°C, pH 8.3 for alkaline phosphatase and pH 5.0 for acid phosphatase.

In order to obtain a semiquantitative estimation of the enzyme activity, the initial time was recorded, i.e. the interval between incubation and occurrence of the first staining reaction. For alkaline phosphatase the first sign of this reaction was a red color at the surface of the bone located in osteoblasts and preosteoblasts (Figure 1A). For acid phosphatase the first color was observed at the surface in the osteoclasts corresponding to the lysosomes (punctate reaction) (Figure 1B).

For control purposes incubation without substrate was carried out simultaneously. In order to ensure that the substrate concentration was sufficient to achieve zero-order kinetics, the initial time was measured on serial sections with different concentrations of the substrate.

As a control for enzyme diffusion in the incubation medium serial sections were incubated in pairs with and without preincubation for varying periods of time with the respective buffer. The control for enzyme diffusion showed that within the times obtained in this study, there was no or inconsiderably decreased activity for both alkaline and acid phosphatase. Statistical analyses were performed on the results using the Wilcoxon rank sum test for paired and unpaired data.

RESULTS

Isotope studies. Table 1 shows the mean values (± s.d.) of the percentage resorbed, compared to controls for Ca^{45} and H^3 proline expressed as ratios. The ratio of control to control is 1.02 ± 0.10 and of dead bone to control 0.45 ± 0.06. The Ca^{45} release is inhibited by DPH at a concentration of 175 mg/l giving a ratio of 0.60 ± 0.09, and the same concentration significantly depresses H^3 proline release. The reduction in Ca^{45} release by DPH was significant at concentrations of 40 mg/l and above. A dose dependent inhibition of Ca^{45}

Table 1. Ratios and initial times for calvaries treated with DPH in concentration of 175 mg/l and for controls

Ca^{45} release T_{175}/C	0.60±0.09
H^3 proline release T_{175}/C	0.75±0.12
Ca^{45} release D_{175}/D	0.99±0.08
Alkaline phosphatase C	21.4±1.6
T_{175}	26.2±3.0
Acid phosphatase C	101.2±12.8
T_{175}	224.6±36.7
Ca^{45} release C/C	1.02±0.10
H^3 proline release C/C	1.02±0.16
Ca^{45} release D/C	0.45±0.06
H^3 proline release D/C	0.43±0.05

C, control bone. D, dead bone. T_{175}, treated bone, DPH 175 mg/l. D_{175}, dead treated bone, DPH 175 mg/l
Release expressed in ratios. $n = 12$.
Phosphatase activity expressed as initial time in seconds.
Alkaline phosphatase: $n = 12$, acid phosphatase: $n = 6$.
All values from treated bones except treated dead bones significantly different from controls at $P < 0.001$.

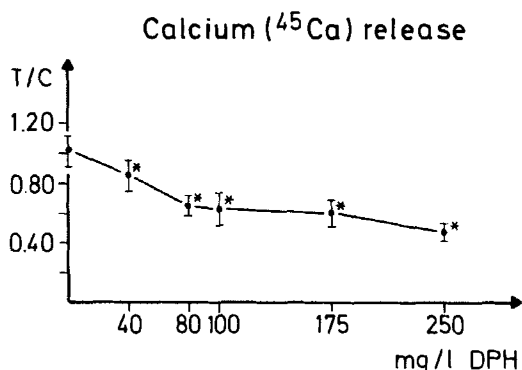

*Figure 2. Dose dependent release curve of radioactive calcium from calvaries cultured for 2 days. T/C, ratio between treated and control bones. Each value represents mean ± s.d. of 12 incubations. * Significantly different from control group at P < 0.001.*

release is shown in Figure 2. The efflux of Ca^{45} from dead bone was not affected by DPH at a concentration of 175 mg/l (Table 1).

Phosphatase activity studies. Figure 3 shows the DPH dose dependent depression of alkaline phosphatase activity expressed in terms of the initial time. Significant inhibition of enzyme activity is seen at concentrations of 175–250 mg/l. Even the acid phosphatase activity was affected by the drug, showing a highly significant increase in initial time at 175 mg/l DPH from 101.2 s ± 12.8 s to 224.6 s ± 36.7 s in a study with six paired specimens.

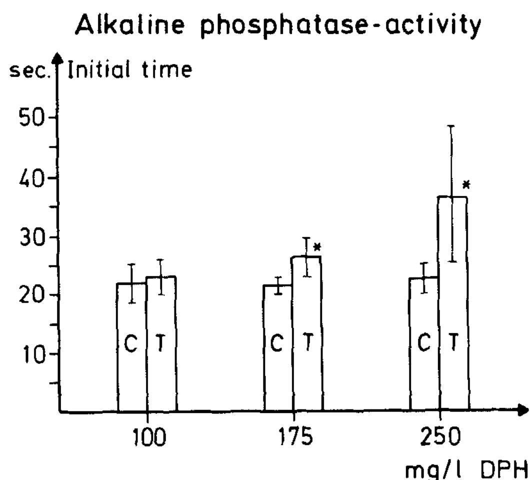

*Figure 3. Relationship between initial time for alkaline phosphatase activity measured by histochemistry on mouse calvaries cultured for 2 days and DPH concentration. Each value represents mean ± s.d. of 12 incubations. T, treated. C, control. * Significantly different from control group at P < 0.001.*

DISCUSSION

The present results indicate that DPH exhibits a depressive effect on bone formation as well as on bone resorption. The effect of DPH on the basal bone resorption process involves inhibition of both Ca^{45} and H^3 proline release, confirming the results of Hahn et al. (1978). This suggests that DPH acts on both mineral bone and bone matrix. The anticipated result of long term anti-epileptic treatment must thus be primarily an osteosclerotic response, which has been confirmed in clinical studies (Harris & Goldhaber 1974, Kattan 1970). However, the most common change in bone metabolism is osteopenia, which may be complicated by fractures (Wallöe 1978). A reduced bone mineral content is very often found (Christiansen et al. 1973, Hahn et al. 1975, Wallöe 1978).

To study bone formation the uptake of Ca^{45} and H^3 proline was measured. About 20% of the isotopes were absorbed by the bones. This uptake was, however, not affected by DPH or by killing the bone cells. Total calcium content in the calvaries was therefore measured at the end of a release study. Even these results showed no detectable differences when the data had been corrected for bone size and different release. Should bone formation be found in an *in vitro* system, our results indicate that the amount of new bone would be very small. The 20% uptake registered represents passive absorption.

Routine histological sections stained with hematoxylin-eosin did not prove useful for quantitation of differences between treated and control bones.

A semiquantitative histochemical technique described by Burstone (1959) and Hopsu & Glenner (1965) was considered applicable for the present purpose. The use of alkaline and acid phosphatase activity as marker enzymes for bone formation and bone resorption, respectively, is generally accepted (Bourne 1972, Vaes 1968, Reimann & Christensen 1979).

The relatively long initial times observed in the acid phosphatase study compared to alkaline phosphatase might influence the determination of the exact initial time. This problem is, however, of no importance, when the great difference be-

tween the treated and the control values is taken into account. The results of our enzyme studies show a possible inhibitory effect of DPH on bone formation as well as resorption. The anticipated effect on the formation of bone combined with the direct depressive effect on intestinal Ca absorption (Kock et al. 1972) might counteract the osteosclerotic response mentioned earlier, and thus provide part of the explanation for the predominant clinical feature of osteopenia in humans treated with DPH.

The present results support Hahn et al.'s (1978) hypothesis that the depressive effects of DPH and calcitonin on bone resorption are mediated through different mechanisms, since DPH depresses the cell enzymes studied, while calcitonin causes an increase in enzyme activity (Morgan et al. 1973). It can be concluded that the influence of DPH on bone cells is complex and still not clarified, but the drug might act directly on bone cells and inhibit bone turnover in *in vitro* systems.

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