*Br. J. Pharmac.* (1974), **50**, 405-408

# PHENYTOIN INHIBITION OF PARATHYROID HORMONE INDUCED BONE RESORPTION *in vitro*

M. HARRIS
Department of Oral Surgery, King's College Hospital Dental School, London, S.E.5

M.V. JENKINS & M.R. WILLS
Department of Chemical Pathology, Royal Free Hospital, Gray's Inn Road, London, W.C.1

1 A study is reported of the effects of phenytoin and phenobarbitone on bone calcium mobilization by parathyroid hormone *in vitro*.

2 In a therapeutic concentration (15 μg/ml), phenytoin significantly inhibited parathyroid hormone-induced calcium release from bone.

3 The inhibitory effect of phenytoin on bone calcium mobilization could play a role in the maintenance of hypocalcaemia in epileptic patients on long-term anticonvulsant drug therapy.

## Introduction

Hypocalcaemia with associated rickets or osteomalacia are recognized complications of long-term therapy with anticonvulsant drugs (Kruse, 1968; Dent, Richens, Rowe & Stamp, 1970; Richens & Rowe, 1970; Flury, 1972; Sotaniemi, Hakkarainen, Puranen & Lahti, 1972; Christiansen, Kristensen & Rødbro, 1972). The induction of hepatic microsomal enzyme activity by the anticonvulsant drugs with an alteration in cholecalciferol metabolism has been proposed as the mechanism for the disturbance in calcium homeostasis (Richens & Rowe, 1970; Hunter, Maxwell, Stewart, Parsons & Williams, 1971; Schaefer, Flury, Herrath, Kraft & Schweingruber, 1972). Phenobarbitone, a potent inducer of hepatic hydroxylation enzymes, when administered both to man and animals, has been shown to accelerate the conversion of cholecalciferol to 25-hydroxycholecalciferol (25-HCC) and to biologically inactive metabolites (Herrath, Kraft, Schaefer & Koeppe, 1972; Hahn, Birge, Scharp & Avioli, 1972a; Silver, Neale, Davies, Breckenridge & Thompson, 1972) while phenytoin had a negligible effect (Herrath *et al.*, 1972). In a dental survey by one of the authors (M.H.) of the epileptic patients on long-term treatment with anticonvulsant drugs, who were studied by Richens & Rowe (1970), it was noted that there was a significant incidence of stunted root formation. Such dental findings are characteristic of idiopathic hypoparathyroidism and pseudo-hypoparathyroidism, conditions in which there is either a lack of parathyroid hormone or a defect in the end-organ, or tissue, response to the hormone. These observations suggested that in patients with anticonvulsant osteomalacia or rickets the drugs have some effect on parathyroid hormone. This effect could be on either the release of the peptide hormone, or its direct action on bone or a more complex involvement interfering with its role in the hydroxylation of 25-hydroxycholecalciferol by the kidney to the biologically active 1,25-dihydroxycholecalciferol. This would give rise to low plasma levels of the active dihydroxysteroid impairing not only the intestinal absorption of calcium but also parathyroid hormone induced bone resorption which is cholecalciferol-dependent (De Luca, 1973). The study reported here was undertaken to investigate the effect of anticonvulsant drugs *in vitro* on bone calcium mobilization by parathyroid hormone.

## Methods

The bone-tissue culture system used was based on the paired half-calvaria technique described by Reynolds & Dingle (1970). Calvaria were removed aseptically from five- or six-day-old albino mice that had been injected subcutaneously with 5 μCi of $^{45}Ca$ (sterile $^{45}CaCl_2$ solution, Radiochemical Centre, Amersham) in 0.025 ml 0.154 M sodium chloride four days previously. After removal the calvaria, consisting of frontal and parietal bones, were divided along the line of the sagittal suture. The half-calvaria were placed on stainless steel grids in separate 30 mm Petri dishes with 3 ml of defined medium (Reynolds, 1966) which contained 5% (v/v) heat-inactivated rabbit serum

(TC 69, Wellcome Reagents Limited). The cultures were incubated at 37.5° C in an atmosphere of 5% carbon dioxide in air and the medium was changed after 48 hours. One half-calvarium was used for experimental purposes, the other half as a control. During the initial 48 h culture period the medium contained no test substances; this initial period was used as a base-line to correct the response obtained in the following 48 h when the test substances were added to the medium in which the experimental bone was cultured. These were either parathyroid extract or parathyroid hormone or one of these with either phenytoin or phenobarbitone. The effect of these test compounds was determined by the changes in the amount of $^{45}Ca$ released from the explants into the medium. The change in release of $^{45}Ca$ was calculated according to the formula:

$$\%\text{ change in } {}^{45}Ca \text{ release} = 100\left[\frac{e_t}{c_t} \times \frac{c_o}{e_o}\right] - 100$$

where $e_t$ and $c_t$ were the counts/min in the culture medium from the experimental ($e$) and control ($c$) half-calvaria respectively during the second 48 h culture period and $e_o$ and $c_o$ were the counts/min during the initial base-line period. The result expressed by this formula gives the corrected percentage change in $^{45}Ca$ release of the treated explant relative to the control. Radioactivity was measured, in a liquid scintillation counter, from 1 ml of medium mixed with 8 ml of a toluene : triton X 100 mixture (66 : 33 v/v) containing 10 g of butyl PBD/litre.

Parathyroid extract (Para-Thor-Mone, Lilly and Co.) 100 U.S.P. units/ml was diluted with 0.154 M sodium chloride to a concentration of 3 U.S.P. units/ml, and 0.1 ml used in 3.0 ml of medium (final concentration 0.1 U.S.P. unit/ml of medium).

Parathyroid hormone (bovine sephadex 72/286, Medical Research Council, Division of Biological Standards): the contents of an ampoule (0.2 mg peptide with a potency of 500 units) were dissolved in 2 ml of M/50 acetic acid (containing 1% heated rabbit serum and 10 mg/100 ml of cysteine) and then diluted with 0.154 M sodium chloride to a concentration of 3 units/ml, and 0.1 ml used in 3.0 ml of medium (final concentration 0.1 unit/ml of medium).

Phenytoin sodium B.P. (Epanutin parenteral, Parke Davis and Co.) 12.5 mg was dissolved in 5 ml M/100 sodium hydroxide; this was diluted with culture medium to obtain final concentrations in the range 5 to 100 μg/ml.

Phenobarbitone sodium B.P. (200 mg/ml) was diluted with 0.154 M sodium chloride to a concentration of 2 mg/ml; this was diluted with culture medium to obtain final concentrations of 5 and 25 μg/ml.

The addition of either phenytoin or phenobarbitone to the defined medium caused no marked change in the pH of the medium.

## Results

The effect of parathyroid extract on bone calcium mobilization, and the alteration of this effect by the addition of either phenytoin or phenobarbitone to parathyroid extract-containing culture media are shown in Table 1. At phenytoin concentrations of 15 μg/ml and above there was significant inhibition of bone calcium mobilization

**Table 1** Effects of substances studied on % change in $^{45}Ca$ release from the treated bone relative to the untreated control.

| *Group* | *Additions (final concentrations/ml of media)* | *No.* | *Mean % $^{45}Ca$ increase* | *s.e. mean* | P* |
|---|---|---|---|---|---|
| 1 | P.T.E. 0.1 U.S.P. units | 18 | 61.8 | 6.35 | – |
| 2 | P.T.E. 0.1 U.S.P. units + phenytoin (5 μg) | 13 | 60.7 | 7.8 | N.S. |
| 3 | P.T.E. 0.1 U.S.P. units + phenytoin (15 μg) | 16 | 43.5 | 4.3 | <0.05 |
| 4 | P.T.E. 0.1 U.S.P. units + phenytoin (25 μg) | 14 | 25.8 | 8.6 | <0.005 |
| 5 | P.T.E. 0.1 U.S.P. units + phenytoin (50 μg) | 17 | 3.8 | 5.3 | <0.001 |
| 6 | P.T.E. 0.1 U.S.P. units + phenytoin (100 μg) | 18 | 3.2 | 4.4 | <0.001 |
| 7 | P.T.E. 0.1 U.S.P. units + phenobarbitone (5 μg) | 9 | 59.1 | 6.2 | N.S. |
| 8 | P.T.E. 0.1 U.S.P. units + phenobarbitone (25 μg) | 10 | 53.5 | 7.9 | N.S. |

* Comparisons of groups 2-7 with Group 1 by Student's t index and significance ($P$) of the $t$ values.
No. = number of calvaria.
P.T.E., parathyroid extract.

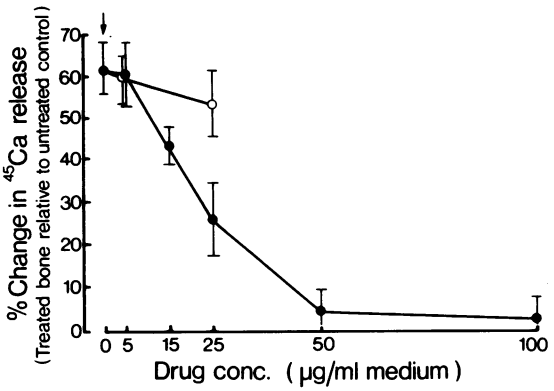


**Fig. 1** Effect of anticonvulsant drugs on parathyroid extract (P.T.E.) induced bone resorption. P.T.E. (arrowed); phenytoin (•); phenobarbitone (○).

by parathyroid extract. Similar results were obtained using parathyroid hormone. At a parathyroid hormone concentration of 0.1 unit/ml of medium the mean (±s.e. mean) % increase in $^{45}$Ca release from the treated bones relative to the untreated controls was 43.7 (±3.7) ($n = 7$). With the addition of phenytoin to the culture medium in a concentration of 50 μg/ml the mean (±s.e. mean) % change in $^{45}$Ca release induced by parathyroid hormone was significantly reduced ($P < 0.001$) to −8.9 (±1.1) ($n = 7$).

As phenytoin is avidly bound to albumin *in vivo* (approximately 90%) the effect of increasing the protein concentration of the defined medium on the phenytoin-inhibition of parathyroid extract induced bone calcium mobilization was studied. A batch of synthetic medium was prepared containing 50% (v/v) inactivated rabbit serum. Using this culture medium, parathyroid extract in a concentration of 0.1 unit/ml of medium caused a mean (±s.e. mean) percentage increase in $^{45}$Ca release of 37.3 (±4.7) ($n = 5$); with the addition of phenytoin to the medium in a concentration of 50 μg/ml, this was significantly reduced ($P < 0.001$) to a mean (±s.e. mean) of −7.9 (±5.7) ($n = 5$).

At the concentrations of phenobarbitone studied there was no significant inhibition of parathyroid extract-induced bone calcium mobilization (Figure 1).

## Discussion

Although it is debatable that observations of drug effects in tissue culture reflect the more complex *in vivo* situation, the phenytoin concentration of 15 μg/ml *in vitro* is comparable to the mean therapeutic plasma concentration of 25 μg/ml (Nordentoft-Jensen & Gryderup, 1966) and is particularly relevant to those patients with higher plasma phenytoin concentrations in which complications occur. A variety of adverse reactions are recognized complications of long-term anticonvulsant drug therapy (Calne, 1973). It has been established, in both man and experimental animals, that the administration of phenobarbitone accelerates the hepatic conversion of cholecalciferol both to 25-hydroxycholecalciferol (Herrath *et al.*, 1972; Silver *et al.*, 1972) and to other more polar metabolites (Hahn *et al.*, 1972a). The reports of decreased serum concentrations of 25-hydroxycholecalciferol in epileptic patients on long-term anticonvulsant therapy with drugs including phenobarbitone (Hahn, Hendin, Scharp & Haddad, 1972b; Stamp, Round, Rowe & Haddad, 1972) are consistent with a drug-induced alteration in cholecalciferol metabolism. In experimental animals, however, there is evidence that phenytoin does not cause an alteration in cholecalciferol metabolism (Herrath *et al.*, 1972). This evidence, and the reports that hypocalcaemia and the associated metabolic bone disease is most marked in patients either on phenytoin alone or in combination with drugs other than phenobarbitone (Richens & Rowe, 1970; Koch, Kraft, Herrath & Schaefer, 1972), suggests that other mechanisms are involved in either the genesis or maintenance of phenytoin-induced hypocalcaemia.

A defect in the intestinal absorption of either vitamin D (Flury, 1972) or calcium (Flury, 1972; Viukari, Kauko & Tammisto, 1972) has been proposed as a factor in the aetiology of anticonvulsant hypocalcaemia. It has been reported (Koch *et al.*, 1972) that, in experimental animals, the administration of phenytoin, but not phenobarbitone, caused a reduction in intestinal calcium uptake which was not due to a reduction in intestinal mucosal calcium-binding protein activity. Koch *et al.* (1972) concluded that phenytoin may exert a direct influence on intestinal calcium transport not involving cholecalciferol or its active metabolites. The findings reported in the present study suggest that phenytoin, but not phenobarbitone, has also a direct inhibitory effect on the mobilization of calcium from bone induced by parathyroid hormone. These observations would suggest that phenytoin exerts an inhibitory effect on cellular calcium transport.

The mechanism of action of anticonvulsant drugs such as phenytoin is by suppression of post-tetanic potentiation (Calne, 1973). The phenomenon of post-tetanic potentiation is associated with the movement of calcium ions into the presynaptic area during repetitive stimulation. The

suppression of post-tetanic potentiation by phenytoin appears to be due to an alteration in membrane permeability to sodium ions, during stimulation, which indirectly diminishes calcium influx (Pincus, 1972). There is evidence that sodium ions play a role in the cellular transport of calcium both in the intestinal tract and bone (Martin & De Luca, 1969; Birge, Gilbert & Avioli, 1972; Wills, 1973). It could be postulated therefore that the inhibitory effect of phenytoin on membrane permeability to sodium indirectly affects calcium transport and this also plays a role in the genesis and maintenance of phenytoin-induced hypocalcaemia. The inhibitory effect of phenytoin on cellular calcium transport undoubtedly warrants further evaluation with respect to its role in the hypocalcaemia and associated metabolic bone disease in patients on long-term anticonvulsant drug therapy.

We wish to thank the Endowment Fund of the Royal Free Hospital with whose financial support this project was undertaken.

## References

BIRGE, S.J., Jr, GILBERT, H.R. & AVIOLI, L.V. (1972). Intestinal transport: The role of sodium. *Science, N.Y.*, **176**, 168-170.

CALNE, D.B. (1973). The drug treatment of epilepsy. *Br. J. Hosp. Med.*, **9**, 171-175.

CHRISTIANSEN, C., KRISTENSEN, M. & RØDBRO, P. (1972). Latent osteomalacia in epileptic patients on anticonvulsants. *Br. med. J.*, **3**, 738-739.

DE LUCA, H.F. (1973). The kidney as an endocrine organ; production of 1,25-dihydroxyvitamin $D_3$. *New Engl. J. Med.*, **289**, 359-365.

DENT, C.E., RICHENS, A., ROWE, D.J.F. & STAMP, T.C.B. (1970). Osteomalacia with long-term anticonvulsant therapy in epilepsy. *Br. med. J.*, **4**, 69-72.

FLURY, W. (1972). Osteomalazie nach langdauernder antiepileptischer Behandlung. *Schweiz. med Wschr.*, **102**, 1333-1338.

HAHN, T.J., BIRGE, S.J., SCHARP, C.R. & AVIOLI, L.V. (1972a). Phenobarbital-induced alterations in vitamin D metabolism. *J. clin. Invest.*, **51**, 741-748.

HAHN, T.J., HENDIN, B.A., SCHARP, C.R. & HADDAD, J.G., Jr. (1972b). Effect of chronic anticonvulsant therapy on serum 25-hydroxycholecalciferol levels in adults. *New Engl. J. Med.*, **287**, 900-904.

HERRATH, D. VON, KRAFT, D., SCHAEFER, K. & KOEPPE, P. (1972). Influence of phenobarbitone and diphenylhydantoin on vitamin D metabolism and calcium retention in rats. *Z. ges. Exp. Med.*, **158**, 194-204.

HUNTER, J., MAXWELL, J.D., STEWART, D.A., PARSONS, V. & WILLIAMS, R. (1971). Altered calcium metabolism in epileptic children on anticonvulsants. *Br. med. J.*, **4**, 202-204.

KOCH, H.U., KRAFT, D., HERRATH, D. VON & SCHAEFER, K. (1972). Influence of diphenylhydantoin and phenobarbital on intestinal calcium transport in the rat. *Epilepsia*, **13**, 829-834.

KRUSE, R. (1968). Osteopathien bei antiepileptischer Langzeitherapie (Vorläufige Mitteilung). *Mschr. Kinderheilk.*, **116**, 378-381.

MARTIN, D.L. & DE LUCA, H.F. (1969). Influence of sodium on calcium transport by the rat small intestine. *Am. J. Physiol.*, **216**, 1351-1359.

NORDENTOFT-JENSEN, B. & GRYNDERUP, V. (1966). Studies on the metabolism of phenytoin. *Epilepsia*, **7**, 238-245.

PINCUS, J.H. (1972). Diphenylhydantoin and ion flux in lobster nerve. *Arch. Neurol. Psychiat., Chicago*, **26**, 4-10.

REYNOLDS, J.J. (1966). The effect of ascorbic acid on the growth of chick bone rudiments in chemically defined medium. *Exp. Cell. Res.*, **42**, 178-188.

REYNOLDS, J.J. & DINGLE, J.T. (1970). A sensitive *in vitro* method for studying the induction and inhibition of bone resorption. *Calcif. Tissue Res.*, **4**, 339-349.

RICHENS, A. & ROWE, D.J.F. (1970). Disturbance of calcium metabolism by anticonvulsant drugs. *Br. med. J.*, **4**, 73-76.

SCHAEFER, K., FLURY, W.H., HERRATH, D. VON, KRAFT, D. & SCHWEINGRUBER, R. (1972). Vitamin-D-Stoffwechsel und Antiepileptika. *Schweiz. med. Wschr.*, **102**, 785-791.

SILVER, J., NEALE, G., DAVIES, D., BRECKENRIDGE, A. & THOMPSON, G.R. (1972). The effect of phenobarbitone-induction on vitamin D metabolism. *Clin. Sci.*, **42**, 12P.

SOTANIEMI, E.A., HAKKARAINEN, H.K., PURANEN, J.A. & LAHTI, R.O. (1972). Radiologic bone changes and hypocalcaemia with anticonvulsant therapy in epilepsy. *Ann. intern. Med.*, **77**, 389-394.

STAMP, T.C.B., ROUND, J.M., ROWE, D.J.F. & HADDAD, J.G. (1972). Plasma levels and therapeutic effect of 25-hydroxycholecalciferol in epileptic patients taking anticonvulsant drugs. *Br. med. J.*, **4**, 9-12.

VIUKARI, N.M.A., KAUKO, K. & TAMMISTO, P. (1972). Anticonvulsant hypocalcaemia. *Br. med. J.*, **3**, 768-769.

WILLS, M.R. (1973). Calcium homeostasis in health and disease. In: *Recent Advances in Medicine*, ed. Baron, D.N., Compston, N. & Dawson, A.M., 16th ed, p. 65, London: Churchill Livingstone.

*(Received June 25, 1973)*