Inhibition of Protocollagen Proline Hydroxylase by Dilantin[1] (36999)

TSAN Z. LIU AND RAJENDRA S. BHATNAGAR[2]
(Introduced by I. Zipkin)

Laboratory of Connective Tissue Biochemistry, School of Dentistry, University of California, San Francisco Medical Center, San Francisco, California 94122

Dilantin (sodium 5,5′-diphenylhydantoinate) is considered to be one of the basic drugs in the treatment of convulsive disorders (1). One of the common side effects of Dilantin is the hyperplastic enlargement of gingivae (2, 3). Several lines of indirect evidence suggest that gingival hyperplasia may involve increased collagen [for review, see Ref. (4)]. In a recent report (5), Ebadi and Scott have reported enhanced synthesis of collagen in carrageenin-induced granulomas in guinea pigs treated with Dilantin. They suggested that increased protocollagen proline hydroxylase (PPH) activity may be involved in the increased synthesis of collagen. We have examined the effect of Dilantin on purified PPH and our observations suggest that Dilantin inhibits PPH, probably by complexing Fe^{2+} which is required in the hydroxylation reaction (6).

Materials and Methods. Dilantin was obtained from Sigma Chemical Co. Protocollagen substrate was prepared from cartilage explants from 10-day-old chick embryos. The tissues were preincubated for 15 min with 1.0 m*M*, a,a′-bipyridyl in Krebs' phosphate buffered medium (7). 3,4-^{3}H-proline (50 μCi, 4.8 Ci/mmole, New England Nuclear) was added in a final volume of 5.0 ml and incubation continued for 2 hr after which the tissues were washed with chilled water and homogenized in 0.5 *N* acetic acid and extracted overnight at 4° in 0.5 *N* acetic acid. The extracts were centrifuged at 100,000*g* for 1 hr and the supernatant was dialyzed against several changes of 0.05 *N* acetic acid. Aliquots of the dialyzed protocollagen extract, containing approximately 150,000 dpm were used in the enzyme assay.

PPH was purified by an adaptation of the procedure described by Halme, Kivirikko and Simons (8) through the DEAE-cellulose chromatography step. The most purified preparations obtained in this step had a specific activity 330 times greater than that of the 15,000*g* supernatant fraction of chick embryo homogenate used as the starting point. The enzyme assay was conducted as described previously (6).

Results and Discussion. Increasing concentrations of Dilantin caused increasing inhibition of the enzyme (Table I). In order to examine the nature of the interaction between Dilantin and PPH, a Hill plot (Fig. 1) (9) was constructed from the inhibition data. The interaction coefficient measured from the slope of the Hill plot was 0.97 suggesting that

TABLE I. Inhibition of Protocollagen Proline Hydroxylase by Dilantin.

Additions[a]	^{3}HHO[b] formed (dpm/ml aliquot)	% of control[b]
None	3960	100
+ Dilantin, 1 m*M*	3405	86
+ Dilantin, 2m*M*	2850	72
+ Dilantin, 4m*M*	2495	63
+ Dilantin, 8m*M*	1620	41
+ Dilantin, 8m*M* + Fe^{2+}, 1.0m*M*	3828	96

[a] Complete components for the enzyme assay included enzyme, 200 μg; substrate, 108,000 dpm; ascorbic acid, 0.5 m*M*; α-ketoglutaric acid, 0.1 m*M*; ferrous ammonium sulfate, 0.1 m*M*; Tris–HCl buffer, 100 m*M* and H_2O to a total volume of 2.0 ml. The total incubation time was 20 min.

[b] The values indicated are the average of two samples.

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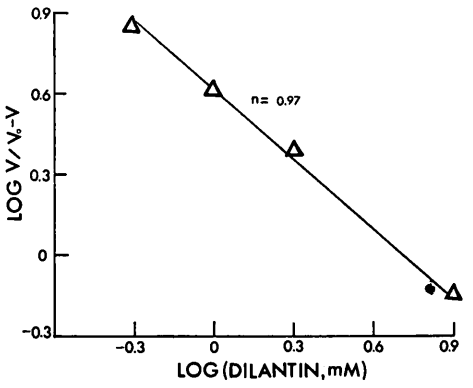

FIG. 1. Hill plot of the inhibition of protocollagen proline hydroxylase by Dilantin. The tritiated protocollagen used for each incubation tube was 231,600 dpm. V_o represents the initial velocity in the absence of Dilantin and V is the residual activity in the presence of Dilantin. Each experimental point represents an average value of two determinations.

the interaction followed first order kinetics and approximately one molecule of the drug was bound to one active molecule of the enzyme.

The inhibition constant (K_i) of Dilantin was determined from a Dixon plot at two substrate concentrations (Fig. 2). As shown in Fig. 2, the K_i for the inhibition of PPH by Dilantin is 8.3 mM.

The nature of the inhibition of PPH by Dilantin was examined by comparing the double-reciprocal plots of the inhibition data at two concentrations of Dilantin with the noninhibited system (Fig. 3). These plots

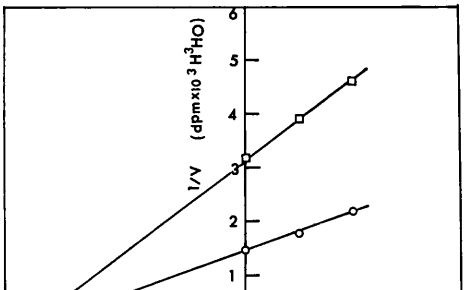

FIG. 2. Dixon plot of the inhibition of protocollagen proline hydroxylase by Dilantin. The tritiated protocollagen used for the incubations were: (□—) 75,200 and (○—) 225,600 dpm/tube.

shared a common intercept at the abscissa indicating that the inhibition is noncompetitive in nature. These observations suggest that Dilantin did not interfere with the binding of the substrate.

PPH is known to be inhibited by compounds which complex Fe^{2+} (6, 10). In order to examine if the inhibition by Dilantin was caused by complex formation between Fe^{2+} and the drug, excess Fe^{2+} was added after preincubating the enzyme with 8.0 mM Dilantin (approx. 1 K_i unit) for 10 min. As shown in Table I, the addition of Fe^{2+} reversed the inhibition of PPH by complexing Fe^{2+} which is required for the enzyme action (6–8).

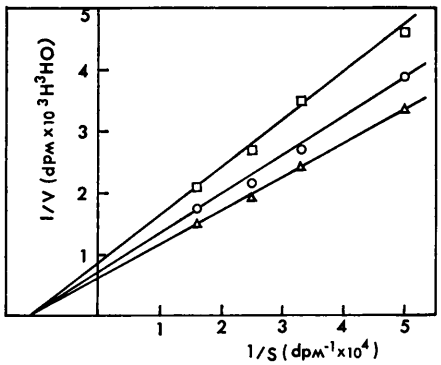

FIG. 3. Plot of the reciprocal of the initial velocity (V) versus the reciprocal of tritiated protocollagen concentration (S) in the presence of various concentrations of Dilantin. (○—) 4 and (□—) 8 μmoles/tube. The control tubes (△—) were incubated in the absence of Dilantin.

Dilantin-induced gingival hyperplasia appears to involve increased connective tissue and the increase in collagen reflects a larger cell population rather than a specific stimulation of collagen synthesis (4). Dilantin also stimulates the proliferation, *in culture*, of fibroblastic cells derived from many different tissues (11–14). The inhibition of PPH by Dilantin suggests that the drug may not be directly involved in the increased synthesis of collagen. Dilantin undergoes metabolic alteration *in vivo* and the possibility that a metabolite of Dilantin may be involved in the stimulation of gingival connective tissue cannot be ruled out.

Summary. Dilantin (sodium 5,5′-diphenylhydantoinate) induces hyperplastic changes involving increased collagen, in the gingivae. Our experiments indicate that Dilantin inhibits protocollagen proline hydroxylase (PPH) in a noncompetitive manner and the inhibition is reversed by adding more Fe^{2+}. Our observations suggest that Dilantin may inhibit PPH by complexing Fe^{2+} and it may not be directly involved in increased collagen synthesis.

1. Merritt, H. H., and Putnam, T. J., J. Amer. Med. Ass. **111**, 1068 (1938).

2. Panuska, H. J., J. Periodontol. **32**, 15 (1961).

3. Babcock, J. R., J. Amer. Dent. Ass. **71**, 1447 (1965).

4. Aas, E., "Hyperplasia Gingivae Diphenylhydantoinea." Scandinavian Univ. Books, Universitetsforlaget, Oslo, (1963).

5. Ebadi, M. S., and Scott, P. M., Clin. Toxicol. **4**, 39 (1971).

6. Bhatnagar, R. S., Rapaka, S. S. R., Liu, T. Z., and Wolfe, S. M., Biochim. Biophys. Acta **271**, 125 (1972).

7. Krebs, H. S., Biochim. Biophys. Acta **4**, 249 (1950).

8. Halme, J., Kivirikko, K. I., and Simons, K., Biochim. Biophys. Acta **198**, 460 (1970).

9. Scocca, J. J., Panny, S. R., and Bessman, M. J., J. Biol. Chem. **244**, 3698 (1969).

10. Chvapil, M., Hurych, J., Ehrlichova, E., and Jichy, M., Eur. J. Biochem. **2**, 229 (1967).

11. Shafer, W. G., Proc. Soc. Exp. Biol. Med. **104**, 198 (1960).

12. Shafer, W. G., J. Dent. Res. **40**, 680 (1961).

13. Nease, W. J., J. Periodontol. **36**, 22 (1965).

14. Buchanan, R. A., Kinkel, A. W., Goulet, J. R., and Smith, T. C., Neurology **22**, 1809 (1972).

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