concentrations of cyclic AMP prevented osmotic lysis of lysosomes (*10*). Although the bidirectional effect of cyclic AMP on the IgE-dependent antigen-induced release of chemical mediators from human lung tissue has been related to the secretory nature of that reaction, the findings that the inhibitory action of cyclic AMP is preceded by several biochemical events in that reaction (*2*) and that the inhibition is manifest against cytolytic reactions as noted herein suggest that an increase in endogenous cyclic AMP affects IgE-dependent antigen-induced and cytolytic mediator release by an action either at different sites or at a common site involving both cellular and granular membranes.

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References and Notes

1. L. M. Lichtenstein and S. Margolis, *Science* **161**, 902 (1968); R. P. Orange, M. Kaliner, P. J. LaRaia, K. F. Austen, *Fed. Proc.* **30**, 1725 (1971); M. Kaliner, S. I. Wasserman, K. F. Austen, *N. Engl. J. Med.* **289**, 277 (1973).
2. M. Kaliner and K. F. Austen, *J. Exp. Med.* **138**, 1077 (1973).
3. ———, *J. Immunol.*, in press.
4. M. D. Valentine, K. J. Block, K. F. Austen, *ibid.* **99**, 98 (1967).
5. K. F. Austen, K. J. Bloch, A. R. Baker, B. G. Arnason, *Proc. Soc. Exp. Biol. Med.* **120**, 542 (1965).
6. M. K. Bach, K. J. Bloch, K. F. Austen, *J. Exp. Med.* **133**, 521 (1971).
7. K. F. Austen and W. E. Brocklehurst, *ibid.* **113**, 521 (1961).
8. Highly purified human C6 was donated by P. Ward, University of Connecticut School of Medicine, Farmington.
9. M. M. Mayer, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2954 (1972).
10. L. J. Ignarro and C. Colombo, *Science* **180**, 1181 (1973).
11. M. Kaliner, R. P. Orange, K. F. Austen, *J. Exp. Med.* **136**, 556 (1972); A. G. Gilman, *Proc. Natl. Acad. Sci. U.S.A.* **67**, 305 (1970).
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Parathyroid Hormone Effects in Rats Treated with Diphosphonate

Abstract. *The ability of disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP; 40 milligrams per kilogram of body weight per day) to reduce the hypercalcemic effect of parathyroid hormone in thyroparathyroidectomized rats was confirmed. However, treatment with this large dose of EHDP enhanced the hypophosphatemic effect of a low dose of parathyroid hormone (10 international units per 100 grams of body weight), apparently by promoting the renal excretion of phosphate. The data suggest that EHDP may have a direct effect on the renal action of parathyroid hormone and, in this way, may also affect vitamin D metabolism by the kidney.*

Diphosphonates have been reported to inhibit bone resorption (*1*, *2*) and to prevent soft tissue calcification (*3*). At least part of the skeletal effect has been attributed to the binding of the pyrophosphate analog on the surface of apatite crystals (*4*). In rats, disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP), at a daily dose of 40 mg per kilogram of body weight for 3 days, reduced the hypocalcemic response to calcitonin but did not affect the hypophosphatemic action of the hormone (*5*). A similar reduction in the hypercalcemic response of EHDP-treated rats to parathyroid hormone (PTH) also has been reported (*6*). The studies reported here suggest that the renal response of the rat to PTH is not inhibited by pretreatment with this dose of EHDP, but in fact actually may be enhanced.

Male rats weighing between 140 to 200 g (Zivic-Miller strain) were injected subcutaneously with EHDP, 40 mg kg^{-1} day^{-1} (I. Y. Rosenblum, Procter & Gamble Co.), between 9 and 10 a.m. for five successive days. This dose level may have been toxic since it reduced the gain in weight of these growing rats by 25 percent. On the afternoon of the fifth day, all rats were thyroparathyroidectomized (TPTX), and food and water were continuously supplied. The morning of the sixth day, a single subcutaneous injection of PTH, either 10 or 20 international units (I.U.) per 100 g of body weight (Wilson Laboratories, 250 units per milligram of trichloroacetic acid powder), was administered. Blood samples (from tail vein) were obtained prior to hormone injection and at 1 and 3 hours after injection. In one experiment the rats were bilaterally nephrectomized 2 hours prior to injection of PTH. Plasma calcium (Ca) and inorganic phosphate (P_i) were determined by standard methods (*7*, *8*). Statistical comparisons were made by Student's *t*-test.

In preliminary experiments the following reported effects of EHDP treatment in rats were confirmed: (i) Ca and P_i fluxes into bone were markedly reduced, as were the rates of removal of previously deposited radionuclides; (ii) after 5 days of treatment, the concentration of plasma Ca was slightly elevated, while the concentration of plasma P_i was significantly lowered; and (iii) following TPTX or parathyroidectomy, the rate of fall in plasma Ca and the rate of rise in plasma P_i were significantly reduced by treatment with EHDP. The inhibition of the ability of PTH (10 to 20 I.U. per 100 g of body weight) to raise plasma Ca concentrations in TPTX rats was also confirmed; however, these experiments suggested that the hypophosphatemic action of PTH was enhanced by 5 days of treatment with EHDP, despite the fact that plasma P_i concentrations at the time of PTH injection were between 1.0 and 1.5 mg/100 ml lower than in controls. The experiments described below confirmed this finding.

Using the standardized procedures (18 hours after TPTX; animals either fasted or fed), we made a comparison of the immediate effects of PTH on plasma Ca and P_i in control rats and in those treated with EHDP. The data, summarized in Fig. 1, demonstrate that in EHDP-treated rats the hypercalcemic action of PTH was reduced while its hypophosphatemic effect was augmented. The increased hypophosphatemia in EHDP-treated animals after injection of PTH was more apparent at the lower dose of the hormone and at 1 hour after its injection than at the higher dose or at 3 hours after injection of PTH. A similar sequence of changes was observed in plasma ^{45}Ca and ^{32}P activities when the radionuclides were injected 3 to 6 days prior to PTH administration.

Preliminary data on the effect of EHDP on renal excretion of Ca and P_i have been obtained. Based on the values of both P_i and ^{32}P in urine, the increase in P_i excretion following administration of PTH was greater in EHDP-treated rats than in nontreated animals. Changes in the concentration of Ca in urine followed a different pattern. In EHDP-treated TPTX rats, urinary Ca was increased almost ten-

fold over controls not treated with EHDP. Following injection of PTH, this value dropped to approximately that of the controls. Quantitative urinary measurements to verify these changes have not yet been reported.

In order to study further the role of the kidney in this increased sensitivity of EHDP-treated rats to PTH, 20 I.U. of this hormone (per 100 g of animal body weight) was injected into rats, previously treated for 3 days with EHDP, 2 hours after nephrectomy. The hypophosphatemic response to PTH was negated by nephrectomy in the EHDP-treated TPTX rats as well as in those not treated with the diphosphonate. Nephrectomy did not abolish the relative hypercalcemic effects of PTH on plasma calcium in the two groups of rats (Table 1).

The time course of development of the effects of this dosage of EHDP on the action of PTH was examined by repeating the experimental procedure except that the rats were TPTX 24 or 48 hours after the first injection of EHDP. While inhibition of the hypercalcemic effect of PTH was apparent by 24 hours after the initial administration of the drug, the hypophosphatemic effect of PTH was unaffected for 3 days of treatment, indicating that the renal effect of EHDP is slower in developing. The first significant decrease in plasma P_i also occurred between the fourth and fifth day of drug treatment.

Table 1. Plasma phosphate and calcium values after nephrectomy. The initial bleeding was performed on all rats 2 hours after nephrectomy and 18 hours after TPTX. Concentration of PTH injections was 20 I.U. per 100 g of body weight. Values are given as means ± the standard error of the mean.

Treatment	Animals (No.)	Before PTH	After PTH	
			1 hour	3 hours
		Phosphate		
TPTX (no PTH)	4	10.6 ± 0.3	11.5 ± 0.5	12.5 ± 0.5
TPTX plus PTH	5	10.1 ± 0.5	10.8 ± 0.6	11.5 ± 0.6
EHDP-TPTX (no PTH)	5	9.6 ± 0.2	10.1 ± 0.2	11.2 ± 0.3
EHDP-TPTX plus PTH	5	10.0 ± 0.2	10.6 ± 0.3	11.8 ± 0.4
		Calcium		
TPTX (no PTH)	4	8.3 ± 0.6	8.5 ± 0.8	7.5 ± 1.2
TPTX plus PTH	5	8.9 ± 0.6	9.5 ± 0.6	10.3 ± 0.9
EHDP-TPTX (no PTH)	5	10.0 ± 0.1	10.6 ± 0.3	10.5 ± 0.2
EHDP-TPTX plus PTH	5	9.8 ± 0.4	10.2 ± 0.4	10.7 ± 0.4

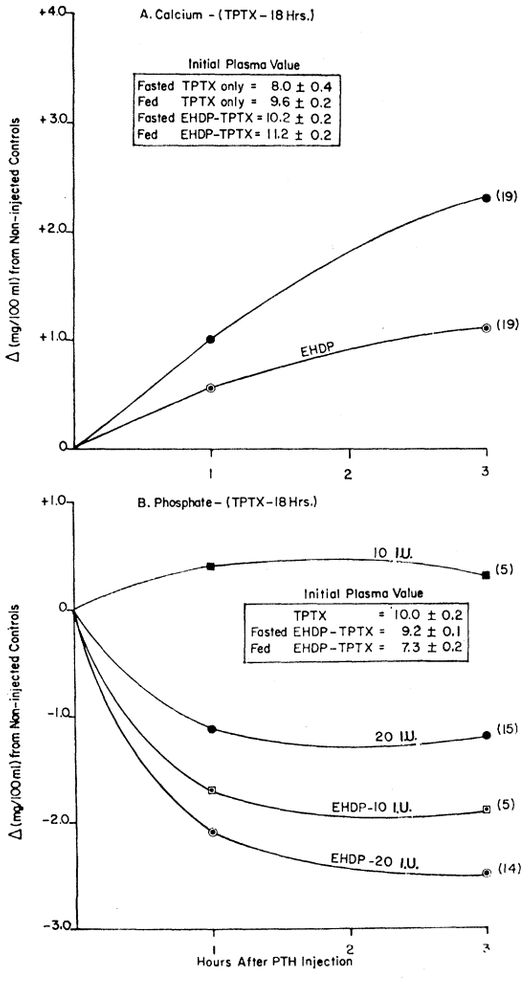

Fig. 1. Plasma calcium and phosphate changes (Δ) in milligrams per 100 ml of plasma after PTH injection in TPTX rats. All data from EHDP-treated rats were statistically different ($P < .005$) from respective controls. Rats administered PTH were injected with either 10 or 20 I.U. per 100 g of body weight. In (B) changes in values of plasma calcium from rats receiving two levels of PTH have been combined. Numbers in parentheses indicate the number of animals. Closed symbols refer to mean values of control rats; open symbols, to mean values of EHDP-treated rats.

The contrasting effect of this drug in man and rat is difficult to explain. Administration of EHDP lowers concentrations of plasma P_i in the rodent while raising them in humans (*9*). In addition, Recker *et al.* (*9*) have reported no significant change in the phosphaturic effect of the administration of PTH in humans who were maintained on oral doses of EHDP. However, they concluded that the EHDP-induced rise in plasma phosphate values was the result of a renal effect of the drug. Plasma P_i concentrations in man are normally low (3 mg/100 ml) whereas those in the young rat are high (7 to 9 mg/100 ml); but in rats maintained on a rachitogenic diet, which lowers concentrations of both plasma Ca and P_i, EHDP raises rather than lowers the P_i (*10*). It is possible, therefore, that the opposite net effects on plasma P_i concentrations might be due to the difference in the starting values of plasma P_i rather than to different effects of the drug on renal metabolism.

It has recently been reported that adult human subjects treated for long periods of time with EHDP develop widened osteoid seams and signs of osteomalacia (*2, 11*). Rats administered large doses of EHDP absorb less calcium from the gut (*12*) and, even though receiving adequate or excess vitamin D, may develop rickets (*13*). Whereas most of these bone changes can be attributed to a direct action of EHDP on bone, an effect of the drug on renal hydroxylation of 25-hydroxycholecalciferol also has been reported (*10, 14*). Whether this is a direct effect of EHDP on renal 1-hydroxylase or an indirect result of changes in endogenous hormone levels is open to question. However, based on the results of the experiments described here, we conclude that the administration of EHDP in sufficiently large doses may

have a direct effect on the renal action of PTH, an agent which by itself has been implicated in the control of vitamin D metabolite hydroxylation (*15*).

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References and Notes

1. R. Smith, R. G. G. Russell, M. Bishop, *Lancet* **1971-I**, 945 (1971); H. Fleisch, J. P. Bonjour, D. B. Morgan, J. J. Reynolds, R. Schenk, R. Smith, R. G. G. Russell, in *Endocrinology 1971*, S. Taylor, Ed. (Heinemann, London, 1972), p. 430.
2. J. Jowsey, B. Riggs, P. J. Kelly, D. L. Hoffman, P. Bordier, *J. Lab. Clin. Med.* **78**, 574 (1971).
3. C. A. L. Bassett, A. Donath, F. Macagno, R. Preisig, H. Fleisch, M. D. Francis, *Lancet* **1969-II**, 845 (1969); R. L. Cram, R. Barmada, W. B. Geho, R. D. Day, *N. Engl. J. Med.* **285**, 1012 (1971).
4. M. D. Francis, *Calcif. Tissue Res.* **3**, 151 (1969).
5. R. V. Talmage, J. J. B. Anderson, J. W. Kennedy, III, *Endocrinology*, in press.
6. H. Fleisch, R. G. G. Russell, M. D. Francis, *Science* **165**, 1262 (1969); R. G. G. Russell, R. C. Mühlbauer, S. Bisaz, D. A. Williams, H. Fleisch, *Calcif. Tissue Res.* **6**, 183 (1970).
7. J. B. Hill, *Clin. Chem.* **11**, 127 (1965); G. Kessler and M. Wolfman, *ibid.* **10**, 686 (1964).
8. P. S. Chen, Jr., T. Y. Toribara, H. Warner, *Anal. Chem.* **28**, 1756 (1965); method of Hycel, Inc., Houston, Texas.
9. R. R. Recker, G. S. Hassing, J. R. Lau, P. D. Saville, *J. Lab. Clin. Med.* **81**, 258 (1973).
10. L. F. Hill, G. A. Lumb, E. B. Mawer, S. W. Stanbury, *Clin. Sci.* **44**, 335 (1973).
11. R. Smith and R. G. G. Russell, *Semin. Drug Treat.* **2**, 77 (1972); S. W. Stanbury, *Clin. Endocrinol. Metab.* **2**, 239 (1972).
12. D. B. Morgan, J. P. Bonjour, A. B. Gasser, K. O'Brien, H. A. Fleisch, *Isr. J. Med. Sci.* **7**, 384 (1971); A. B. Gasser, D. B. Morgan, H. A. Fleisch, L. J. Richelle, *Clin. Sci.* **43**, 31 (1972); J. P. Bonjour, H. F. DeLuca, H. Fleisch, V. Trechsel, L. A. Matejowec, J. L. Omdahl, *Eur. J. Clin. Invest.* **3**, 44 (1973); H. W. Sampson, E. L. Krawitt, A. S. Kunin, J. L. Matthews, *Fed. Proc.* **32**, 424 (abstr.) (1973).
13. J. Jowsey, K. E. Holley, J. W. Linman, *J. Lab. Clin. Med.* **76**, 126 (1970); W. R. King, M. D. Francis, W. R. Michael, *Clin. Orthopaed.* **78**, 251 (1971).
14. H. F. DeLuca, *Biochem. J.*, in press.
15. M. Garabedian, M. F. Holick, H. F. DeLuca, I. T. Boyle, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1673 (1972).
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Failure to Confirm Cyclic AMP as Second Messenger for Norepinephrine in Rat Cerebellum

Abstract. *Microiontophoretic applications of adenosine 3′,5′-monophosphate (cyclic AMP) to spontaneously active, electrophysiologically identified Purkinje cells of the rat cerebellum failed to mimic the strong depressant action of norepinephrine on the same cells. These findings, in combination with a reevaluation of other studies, cast doubt on the hypothesis that cyclic AMP mediates the depressant actions of norepinephrine in the cerebellum.*

Bloom and colleagues have proposed that adenosine 3′,5′-monophosphate (cyclic AMP) mediates the inhibitory action of norepinephrine (NE) on cerebellar Purkinje neurons (*1–3*). However, the failure of others to replicate their findings in the cerebellum (*4*) and the lack of evidence for the mediation by cyclic AMP of the depressant effects of NE on neurons of the cerebral cortex (*5*) suggest that the evidence for the role of cyclic AMP in the actions of NE needs further scrutiny.

One type of so-called "interlocking" (*2, 3*) evidence has been derived from studies with pharmacological agents such as methylxanthines, prostaglandins, and nicotinate, with the implicit assumption that the results obtained arise because of direct and specific manipulations of cyclic AMP metabolism (*2, 3*). This assumption is untenable in view of the multiple and diverse actions of the pharmacological agents that could interact with NE through mechanisms unrelated to cyclic AMP (*5*). In fact, in the brainstem, where cyclic AMP depressed many of the neurons also depressed by NE (*6*), these pharmacological tools failed to provide any decisive evidence that the similar effects of cyclic AMP and NE were causally related rather than merely coincidental.

Because the data from studies with methylxanthines, prostaglandins, and nicotinate are not unequivocal and are at best only suggestive as to the involvement of cyclic AMP in the mediation of NE's effects on neurons, the evidence crucial for this hypothesis is the demonstration that applications of cyclic AMP mimic the effects of NE. Accordingly, we have attempted to replicate the crucial experiments of Bloom and colleagues (*1–3*), who reported strong depressant effects of NE and cyclic AMP on rat cerebellar Purkinje neurons, and to give considerable attention to the precise repetition of their experimental paradigms including species, anesthesia, techniques for applying drugs, and identification of neurons.

Experiments were performed on 12 male hooded rats (250 to 500 g), 6 of which were anesthetized with halothane and N_2O and 6 with methoxyflurane and N_2O. Spontaneously firing Purkinje cells were identified in the cerebellar vermis by antidromic activation from the deep cerebellar nuclei or by their characteristic climbing fiber bursts (Fig. 1). Extracellular action potentials were recorded by the central barrel (2*M* NaCl) of seven-barreled micropipettes whose outer barrels were filled by centrifugation immediately before use with the following drugs: L-norepinephrine bitartrate, 0.2*M*, *p*H 5.0 (Sigma); adenosine 3′,5′-monophosphate, 0.5*M*, *p*H 6.0 to 7.5 (Calbiochem); aminophylline, 0.2*M* (Sigma); and 2*M* NaCl. Drugs were applied into the vicinity of neurons by microiontophoresis, and their effects were assessed from rate meter records of extracellular action potentials and from oscilloscope displays of the potentials, which were often photographed.

We have overcome the objections (*2, 3*) to the work of Godfraind and Pumain (*4*) by (i) using spontaneously firing, electrophysiologically identified Purkinje neurons of gas (nonbarbiturate) anesthetized rats and by (ii) employing a continuous automatic balancing current (*7*) in addition to conventional current controls with each neuron. Iontophoretic applications of aminophylline prior to tests of cyclic AMP were utilized to minimize enzymatic degradation of cyclic AMP by phosphodiesterase. These doses of aminophylline produced profound depression of Purkinje cell firing. In order to eliminate the possibility of additive effects (*5*), the cell was allowed the

Table 1. Actions of norepinephrine and cyclic AMP on Purkinje cells.

Anesthetic	Number of cells					
	Norepinephrine			Cyclic AMP		
	Excited	Depressed	No effect	Excited	Depressed	No effect
Halothane	0	21	3	5	3	12
Methoxyflurane	2	10	1	5	3	3
Combined data	2	31	4	10	6	15