# COMMUNICATIONS TO THE EDITOR

## ON THE ROLE OF PHOSPHATASE IN THE NUCLEATION OF CALCIUM PHOSPHATE BY COLLAGEN[1]

*Sir:*

A clarification of the solubility behavior of the aqueous calcium-phosphate system in general[2] and of bone mineral in particular[1,2,3,4] has led several laboratories to the view[2,5,6] that the mineralization process may involve a special property of collagen (or some molecule or grouping closely associated with the collagen) of "inducing" crystal nucleation, whereupon the body fluids contain more than sufficient quantities of calcium and phosphate to carry crystal formation to completion. At least, there are numerous reports[2,5,6,7,8] of collagen's ability to cause crystals of calcium phosphate to form from stable or metastable solutions. The process has been shown to be very specific[5,7] and morphologically resembles calcification *in vivo*.[5,9] Finally, collagen has served successfully as a nucleator in the physiological range of ion concentrations.[10,11]

A disturbing aspect of the nucleation concept has been the lack of a function to be provided by the enzyme, alkaline phosphatase. Usually, when an enzyme is found to occur in nature, the biochemist, by habit and on teleological grounds, assumes that the function of the enzyme is to provide the *products* of the reaction which it catalyzes. This assumption was made by Robison[12] when he found the enzyme phosphatase to be closely associated with the process of biological calcification. In fact, he elaborated a theory of the calcification in which phosphate ions were purported to be supplied at the exact locus of calcification by the enzyme acting on some unidentified substrate(s), ester phosphate(s).[12] However, there is precious little ester phosphate in the circulating body fluids, about $10^{-4}$ $M$[13] and calcification, after all, does occur extracellularly.

In 1950, an alternative suggestion was made, *i.e.*, the enzyme might function not to supply products but rather to destroy a substance harmful or inhibitory to crystallization.[14] There are, in fact, many detoxicating enzymes, *d*-aminoöxidase for example, which destroy harmful substances.

TABLE I

THE PRESENCE IN SERUM OF A NUCLEATION-INHIBITOR AND ITS DESTRUCTION BY ALKALINE PHOSPHATASE

Conditions were: $\mu = 0.16$; $t = 37°$; $p$H 7.4; time of equilibration, 3 days. To prevent bacterial contamination neomycin was added to the ultrafiltrate containing flasks at a level of 10 mg. %. Ultrafiltration was carried out by the Toribara technique.[15] Serum when incubated with phosphatase contained 1 mg. purified alkaline intestinal phosphatase (Mann) per ml.

| | | | Precipitation point Ca × Pi in (mM).² | |
|---|---|---|---|---|
| | Sample | Expts. | Mean | Std. error of mean |
| I | Control solutions | 11 | 1.61 | 0.11 |
| II | Diluted ultrafiltrate of dog serum | 5 | 2.76 | 0.33 |
| III | Dilute ultrafiltrate (preincubated with phosphatase) | 5 | 1.83 | 0.21 |
| | Paired differences (II − III) | 5 | 0.93 | 0.21 |

To test whether such an inhibitory substance may occur *in vivo*, ultrafiltrates were prepared from dog, beef and human sera. Portions of these ultrafiltrates were added in the volume ratio 1 ml. of ultrafiltrate to 11.5 ml. of incubating fluid. The ion product Ca × $P_{inorganic}$ in (mM.)² required to precipitate calcium phosphate in these solutions, nucleated by an active collagen, was determined by techniques published elsewhere (Fig. 1, ref. 2f). An elevation of the required product was taken as evidence of interference or inhibition of the nucleation process. Even at the 1:11.5 dilution, the ultrafiltrates of the sera of all three species were markedly inhibitory. Especially interesting was the observation that incubation of dog sera with alkaline phosphatase removed nearly all of the inhibitory action of the ultrafiltrates. These latter data are given in Table I.

These results prompted a survey of a number of phosphate esters to determine how much of an inhibitory substance is needed to interfere with the nucleation process. These various substances were ineffective at concentrations of $10^{-4}$ $M$ or less: phosphoethanolamine, glycerophosphate, phosphoserine, phosphocholine, creatine phosphate, disphosphopyridine nucleotide, triphosphopyridine nucleotide, and adenosine monophosphate (heparin and chondroitin sulfate were also ineffective). These were inhibitory at $10^{-4}$ $M$ but relatively ineffective at $10^{-5}$ $M$: adenosine diphosphate, adenosine triphosphate and trimetaphosphate. Pyrophosphate was inhibitory at $10^{-5}$ $M$ but not at $10^{-6}$ $M$. Thiamine pyrophosphate and tripolyphosphate were inhibitory at $10^{-6}$ $M$ but not at $10^{-7}$ $M$. Hexametaphosphate and two polyphosphates averaging 11 and 27 P's in chain length

(1) This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, N. Y.

(2) (a) W. F. Neuman and M. Neuman, "The Chemical Dynamics of Bone Mineral," The University of Chicago Press, Chicago, Ill., 1958; (b) B. S. Strates, W. F. Neuman and G. J. Levinskas, *J. Phys. Chem.*, **61**, 279 (1957).

(3) B. E. C. Nordin, *J. Biol. Chem.*, **227**, 551 (1957).

(4) B. E. C. Nordin, *ibid.*, **235**, 1215 (1960).

(5) M. J. Glimcher, *Rev. Modern Phys.*, **31**, 359 (1959).

(6) A. E. Sobel, P. A. Laurence and M. Burger, *Trans. N. Y. Acad. Sci.*, **22**, 233 (1960).

(7) M. J. Glimcher, A. J. Hodge and F. O. Schmitt, *Proc. Natl. Acad. Sci. U. S.*, **43**, 860 (1957).

(8) B. N. Bachra, A. E. Sobel and J. W. Stanford, *Arch. Biochem. Biophys.*, **84**, 79 (1959).

(9) S. Fitton Jackson and J. T. Randall, "Bone Structure and Metabolism," Ciba Foundation Symposium, ed. G. E. W. Wolstenholme and C. U. O'Connor, London, 1956.

(10) C. C. Solomons and W. F. Neuman, *J. Biol. Chem.*, in press.

(11) H. Fleisch and W. F. Neuman, THIS JOURNAL, **82**, 996 (1960).

(12) R. Robison, "The Significance of Phosphoric Esters in Metabolism," New York University Press, New York, N. Y., 1932.

(13) Documenta Geigy Scientific Tables, 1956, p. 326.

(14) W. F. Neuman in "Metab. Interrelations," Josiah Macy, Jr., Foundation, 1950, p. 187; *cf.* also *ibid.*, 1951, p. 205.

(15) T. Y. Toribara, *Anal. Chem.*, **25**, 1286 (1953).

were sufficiently inhibitory even at $10^{-7}$ $M$ to raise significantly the concentrations of calcium and phosphate required to "seed" collagen with crystals.

It may prove difficult, indeed, to identify the substance or substances present in serum which are inhibitory to the seeding of calcium phosphate crystals by collagen. In any case, the ester phosphate fraction of serum, while too small to serve as a normal substrate, in the conventional sense, is sufficiently large to contain one or several highly inhibitory polyphosphates, hydrolyzable by bone phosphatase. Thus, the present experiments provide an important new function for phosphatase in biological calcification, but do not preclude other functions yet to be established by further investigations.

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## THE CYCLOÖCTATETRAENYL DIANION

*Sir:*

We wish to report experimental substantiation in a simple unsubstituted carbocyclic system of the theoretical prediction of molecular orbital theory[1] that a high degree of resonance stabilization is associated with a closed shell of $(4n + 2)$ $\pi$ electrons for $n = 2$.

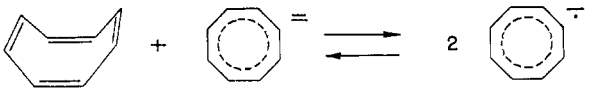
The demonstration that cycloöctatetraene is non-planar[2] has suggested that the strain imposed on the underlying molecular framework might prohibit the preparations of such a system.[2,3,4]

Extensive evidence is available which suggests that cycloöctatetraene has a large affinity for electrons and forms a stable dianion. The reaction of cycloöctatetraene in ether or liquid ammonia solution with alkali metals to form di-alkali derivatives, which on hydrolysis yield cycloöctatrienes and on carbonation yield diacids[7]; the reaction of cyclooctatetraene with sodium triphenylmethyl to form a mixture of hexaphenylethane and a di-alkali derivative which yields cycloöctatrienes on hydrolysis and a *bis*-diol on treatment with benzophenone[8]; the electrolytic reduction of cycloöctatetraene in aqueous ethanolic solution to 1,3,6-cycloöctatriene and the polarographic data which indicate the reaction to be a reversible (the Heyrovsky-Ilkovič equation is obeyed) two electron reduction[9]—all these reactions, well-known for aromatic compounds, but uncharacteristic of olefins, imply that the cycloöctatetraenyl dianion possesses unusual stability.[10]

In tetrahydrofuran solution cycloöctatetraene undergoes a very ready reaction with two moles of potassium metal, and on cooling large, almost colorless, but pale yellow and probably solvated crystals of dipotassium cycloöctatetraenide precipitate. These crystals are difficult to isolate, for on drying and exposure to air they explode. Solutions however, are stable.

The n.m.r. spectrum[12] of dipotassium and dilithium cycloöctatetraenide—a single sharp peak of expected intensity insignificantly displaced from the resonance of cycloöctatetraene itself—is not in accord with the covalent 1,4 structure which usually appears in the literature,[13] nor with rapid exchange averaging among such covalent structures, for in either case the spectrum should be shifted to higher fields than characterize the spectrum of cycloöctatetraene itself. Increased proton shielding would be expected on addition of electrons to the molecule, and in this case must be compensated, if the ring flattens, or aromatizes, by the displacement to low fields characteristics of aromatic molecules, due, at least in part, to the diamagnetic ring current induced in the applied magnetic field.[14] It is this latter representation of a flattened eight-membered ring carrying two negative charges which alone appears in accord with the spectrum.[15]

That the n.m.r. spectrum of the solvent, tetrahydrofuran, is clearly resolved when less than two moles of potassium is allowed to react with cyclooctatetraene proves that large concentrations ($> 10^{-3}$ $M$) of paramagnetic ions, which would



(1)

(1) E. Hückel, "Grundzüge der Theorie ungesättigter und aromatischer Verbindungen," Verlag Chemie, Berlin, 1938.

(2) The chemistry of cycloöctatetraene has recently been reviewed: R. A. Raphael, "Non-Benzenoid Aromatic Compounds," D. Ginsburg editor, Interscience Publishers, Inc., New York, N. Y., 1959.

(3) Wilson Baker, "Perspectives in Organic Chemistry," A. Todd editor, Interscience Publishers, Inc., New York, N. Y., 1956, p. 28 ff.; K. Mislow, *J. Chem. Phys.*, **20**, 1489 (1952).

(4) Indirect solutions to related problems have been successfully devised by Sondheimer[5] and also by Allinger.[6]

(5) F. Sondheimer and R. Wolovsky, *Tetrahedron Letters*, No. **3**, 3 (1959); F. Sondheimer, R. Wolovsky and Y. Gaoni, THIS JOURNAL, **82**, 755 (1960); F. Sondheimer and R. Wolovsky, *ibid.*, **81**, 4755 (1959).

(6) N. L. Allinger and G. A. Youngdale, *Tetrahedron Letters*, No. **9**, 10 (1959).

(7) (a) W. Reppe, O. Schlichting, K. Klager and T. Toepel, *Ann.*, **560**, 1 (1948); (b) A. C. Cope and F. A. Hochstein, THIS JOURNAL, **72**, 2515 (1950).

(8) G. Wittig and D. Wittenberg, *Ann.*, **606**, 1 (1957).

(9) R. M. Elofson, *Anal. Chem.*, **21**, 917 (1949); J. H. Glover and H. W. Hodgson, *Analyst*, **77**, 473 (1952); L. E. Craig, R. M. Elofson and I. J. Ressa, THIS JOURNAL, **75**, 480 (1953).

(10) The ready equilibria between metallic derivatives of cyclooctatrienes and cycloöctatetraenes[11] are also reminiscent of hydroquinone-quinone systems. The facile base-catalyzed isomerization of 1,3,6-cycloöctatriene[7b] might also have a common origin with the above reactions.

(11) A. C. Cope and M. R. Kinter, *ibid.*, **73**, 3424( 1951); A. C. Cope and H. O. van Orden, *ibid.*, **74**, 175 (1952).

(12) Accompanying communication, T. J. Katz, *ibid.*, **82**, 3785 (1960).

(13) W. Reppe, O. Schlichting, K. Klager and T. Toepel, *Ann.*, **560**, 1 (1948); R. A. Raphael, "Chemistry of Carbon Compounds," E. H. Rodd, editor, Elsevier Publishing Company, New York, N. Y., 1953, Vol. IIA, p. 261; E. E. Royals, "Advanced Organic Chemistry," Prentice-Hall, Inc., New York, N. Y., 1954, p. 545.

(14) J. A. Pople, W. G. Schneider and H. J. Bernstein, "High Resolution Nuclear Magnetic Resonance," McGraw-Hill Book Company, Inc., New York, N. Y., 1959, pp. 180–183, 247 ff.

(15) In this connection, the known spectra of the cyclopentadienyl anion,[16] the tropylium ion,[16] and allylmagnesium bromide[17] offer pertinent orientational references.

(16) J. R. Leto, F. A. Cotton and J. S. Waugh, *Nature*, **180**, 978 (1957).

(17) J. E. Nordlander and J. D. Roberts, THIS JOURNAL, **81**, 1769 (1959).