thyronine probably act as 'competitive' inhibitors[7-9]. Studies with FMN are complicated by the fact that the iodinated forms of tyrosine and thyronine are deiodinated upon illumination in the presence of this sensitizer[10-13]. If photochemical deiodination occurred rapidly in the present experiments, some of the protective compounds present could change significantly during the period of illumination. This might account for the very similar results obtained with the different protective agents when FMN is used as the sensitizer.

The protective efficiencies of the different compounds varied widely with eosin Y as sensitizer as shown in Figure 2; for example, DIT was a good protector while potassium iodide showed relatively little protective effect. Eosin sensitizes the photochemical deiodination of T_4[14], although its efficiency in this respect as compared to FMN is not known. The sensitivities of the other protective compounds in our series to photochemical deiodination with eosin have not been determined.

Finally, the results with methylene blue as sensitizer are described in Figure 3. As may be seen, the protective efficiencies of the different compounds vary enormously. In contrast to FMN and eosin, methylene blue does not sensitize the photochemical deiodination of T_4[14]. The methylene blue system is interesting because the degree of inhibition demonstrated by these protective compounds is similar, in general to their known biological metabolic activity[15]. This system, then, may merit further study as a potentially simple photochemical system for the in vitro assay of thyroid compounds and congeners in terms of their likely pharmacological activity[16].

Zusammenfassung. Es wurde Tyrosin- und Thyroninwirkung auf die farbensensibilisierende Photoinaktivierung des Trypsins untersucht. Sensibilisatoren waren Methylenblau, Flavinmononukleotid und Eosin Y. Die Analoge von Tyrosin und Thyronin haben sehr ähnliche konzentrationsabhängige Schirmwirkung bei Verwendung von Flavinmononukleotid als Photosensibilisator. Die Abschirmungseffekte der Sensibilisatoren werden besprochen.

B. W. Glad, J. D. Spikes and L. F. Kumagai

Departments of Anatomy, Medicine, Molecular and Genetic Biology, University of Utah, and Veterans Administration Hospital, Salt Lake City (Utah 84112, USA),
15 May 1968.

[7] L. Weil, W. C. Gordon and A. R. Buchert, Archs Biochem. Biophys. *33*, 90 (1951).
[8] S. Isaka and J. Kato, J. Coll. Arts Sci. Chiba Univ. *1*, 44 (1962).
[9] L. A. Ae. Sluyterman, Biochim. Biophys. Acta *60*, 557 (1962).
[10] M. Suzuki, I. Ishikawa, S. Shimizu and K. Yamamoto, Biochim. Biophys. Acta *51*, 403 (1961).
[11] S. Lissitzky, M. Benevent and M. Roques, Biochim. Biophys. Acta *51*, 407 (1961).
[12] G. Morreale de Escobar, P. L. Rodriquez, T. Jolin and F. Escobar del Rey, J. biol. Chem. *238*, 3508 (1963).
[13] D. Reinwein and J. E. Rall, J. biol. Chem. *241*, 1636 (1966).
[14] I. Ishikawa, Gunma J. med. Sci. *11*, 243 (1962).
[15] R. Pitt-Rivers and W. R. Trotter, *The Thyroid Gland* (Butterworth Inc., Washington, D.C. 1964).
[16] We thank Miss Carol F. Hodgson, Mr. D. H. Tolboe and Mr. J. J. Kaiser for their technical assistance. This work was supported under the following grants: U.S. Atomic Energy Commission Contracts Nos. AT (11-1)-119 and AT (11-1)-875, National Institutes of Health Contract No. CY-5225, NSF Postdoctoral Fellowship No. 44173, American Cancer Society Institutional Research Grant No. IN30-F, and Veterans Administration Research Project No. M7-66.

Formation of Hydroxyapatite Nuclei Induced by Dehydration of Calcifying Solutions

Of fundamental importance to our understanding of the process of biological calcification is knowledge of the mechanism(s) which induce the formation of nuclei of crystallization of hydroxyapatite from calcifying media. Nuclei formation is a phase transition and may be described as the emergence from a metastable solution of the first microaggregates (nuclei) of a new solid phase. Based on theoretical studies of energy requirements[1], nuclei formation is a distinct process and should be considered separately from the subsequent step of growth of these nuclei into larger aggregates of crystals of hydroxyapatite. Although our knowledge of the kinetics of nuclei formation is meager, empirical data[2] would indicate that nucleation of calcium phosphate salts is favored by an elevation of the activity product of calcium and phosphate ions. In a biological calcifying system in dynamic equilibrium, an increase in the activity product of calcium and phosphate ions could be simply accomplished by the addition of a quantity of one or both ions. Alternatively, removal of water from the system would result in a relative increase in calcium and phosphorus ion product without necessitating an absolute increase. It is known that the first case is true[3], but the alternative, although appearing obvious is not fully documented by direct experimentation. Thus in the foregoing experiment, we examined this premise that nuclei of crystallization of hydroxyapatite could be induced to form in a nuclei-free metastable calcifying solution by the simple technique of partially removing water from the system.

Materials and methods. The barbiturate-buffered calcifying solution of Fleisch and Neuman[4] was used in this experiment. In our test system, we utilized the simple expedient of slow freezing to remove water from the calcifying solutions. 100 ml of barbiturate-buffered calcifying solutions (nuclei-free), pH 7.4 $\pm$ 0.05, with Ca $\times$ P products ranging from 25–60 were placed in 125 ml glass bottles. The lower third of the bottles were immersed in an ice-salt bath at $-$ 10 °C. After an interval of approximately $^1/_2$ h, ice crystal began to form in the bottom of the bottles and rapidly spread toward the top. When the ice crystals occupied approximately 50% of the total volume of calcifying solution, the bottles were rapidly withdrawn from the ice bath and the concentrated liquid supernatant phase quickly decanted and saved. Controls were of 2 types: (a) untreated calcifying solutions and (b) calcifying solutions which were partially frozen as above and then

[1] R. Ginell, J. chem. Phys. *34*, 992 (1961).
[2] A. E. Sobel, in *Tooth Enamel* (Ed. M. V. Stack and R. W. Fearnhead; John Wright and Sons, Ltd., Bristol 1965), p. 1.
[3] B. S. Sherman and A. E. Sobel, Archs oral Biol. *10*, 323 (1965).
[4] H. Fleisch and W. F. Neuman, Am. J. Physiol. *200*, 1296 (1961).

Nucleation in calcifying solutions induced by removal of water by partial freezing

(A) Untreated control calcifying solutions			(B) Calcifying solutions after freezing and thawing			(C) Supernatant phase of calcifying solution after partial removal of water by freezing		
Ca (mg%)	P (mg%)	Ca × P	Ca (mg%)	P (mg%)	Ca × P	Ca (mg%)	P (mg%)	Ca × P
5.0	5.0	25	5.0	5.0	25	6.3	6.3	40
6.0	5.0	30	6.0	5.0	30	7.5	6.3	47
7.0	5.0	35	7.0	5.0	35	9.4	6.7	63[a]
8.0	5.0	40	8.0	5.0	40	10.5	6.5	68[a]
9.0	5.0	45	9.0	5.0	45	12.0	6.7	80[a]
10.0	5.0	50	10.0	5.0	50	13.0	6.5	85[a]
11.0	5.0	55[a]	11.0	5.0	55[a]	14.2	6.6	94[a]
12.0	5.0	60[a]	12.0	5.0	60[a]	15.5	6.5	101[a]

[a] Nuclei formation occurred at these concentrations as evidenced by precipitation after incubation the solutions at 37 °C for 24 h (see text).

allowed to thaw. This latter control served to check the possibility that ice crystals themselves might act as nuclei-forming agents. Calcium[5] and phosphorus[6] analyses were performed on aliquots of the concentrated supernatant phase and on the control solutions.

Since it is extremely difficult to chemically analyze directly for the presence of nuclei in these solutions (1×10^9 nuclei of hydroxyapatite of $25 \times 50 \times 100$ Å size weigh less than 4×10^{-4} µg), we employed an indirect test[3] based on the observation that nuclei-free metastable calcifying solutions do not produce a precipitate upon incubation at 37 °C for 24 h. However, if nuclei are present in the calcifying solutions, a precipitate of hydroxyapatite representing crystal growth of the nuclei will appear in the solution upon subsequent incubation.

Results and discussion. As seen in the Table, the control calcifying solutions (A) do not spontaneously form nuclei as long as the Ca × P product is less than 55. Ice crystals per se in these solutions do not act as nuclei-forming agents, since freezing, thawing and incubating these solutions at 37 °C give the same results as the untreated controls (section B in the Table). However, in those calcifying solutions which were partially frozen (C), the liquid supernatant phase shows a relative increase in calcium, phosphorus and Ca × P products at all levels of initial concentration. In these 'dehydrated' calcifying solutions, nuclei were detected in those instances where the Ca × P had been elevated to 63 or higher.

Although the present experiment was performed using a simplified chemical system, the results have value for future investigations of biological calcifying systems. Organic macromolecules such as collagen, elastin and bovine submaxillary mucin, among other substances, have been shown to possess nucleating properties[3,7–11]. The nucleating ability of organic 'catalysts' has been variously explained by such mechanisms as 'cluster capture' of calcium phosphate, epitaxy, reduction of the minimum Ca × P product required for initiating nucleation, and conversely, concentration of calcium and/or phosphate ions[2]. It may be that all these mechanisms are valid and that different biological mineralizing systems may use any or all of these mechanisms according to variations in the nature of the organic matrix and/or the intracellular and extracellular milieu. Based on this concept of multiple mechanisms of calcification and the results of the present experiment, we should like to offer the proposal that the removal of water, if employed by a biological catalyst or system could accomplish at least one of the postulated mechanisms, i.e. the concentration of calcium and phosphate ions, thus inducing nucleation with subsequent calcification. In considering the formation of a solid hydroxyapatite, an important step might be the transformation of hydrated ions in solution to dehydrated ions in the solid. The role of nucleating catalysts and other factors in this model system is under investigation[12].

Zusammenfassung. Es wird gezeigt, dass bei Entfernung eines Teiles der wasserlöslichen Phase einer meta-stabilen, verkalkenden Lösung, Kalk- und Phosphorionen so angereichert werden, dass «kristallisierte Kerne» von Hydroxyapatit auftreten.

B. S. SHERMAN, S. LEIBOWITZ and A. E. SOBEL (deceased)

Department of Anatomy, State University of New York, Downstate Medical Center, Brooklyn (N.Y. 11203) and Graduate Hospital, University of Pennsylvania, Philadelphia (Penna., USA), 1 April 1968.

[5] B. N. BACHRA, A. DAUER and A. E. SOBEL, Clin. Chem. *4*, 107 (1958).
[6] C. H. FISKE and Y. SUBBAROW, J. biol. Chem. *66*, 375 (1925).
[7] A. E. SOBEL, S. LEIBOWITZ, R. G. EILBERG and F. LAMY, Nature *211*, 45 (1966).
[8] B. S. STRATES, W. F. NEUMAN and G. J. LEVINSKAS, J. phys. Chem. *61*, 279 (1957).
[9] W. F. NEUMAN and M. W. NEUMAN, *The Chemical Dynamics of Bone Mineral* (Univ. of Chicago Press, Chicago 1958).
[10] M. J. GLIMCHER, A. J. HODGE and F. O. SCHMIDT, Proc. natn. Acad. Sci. USA *43*, 860 (1957).
[11] M. J. GLIMCHER, in *Calcification in Biological Systems* (Am. Ass. Advance. Sci., Washington, D.C. 1960), p. 421.
[12] This work was supported in part by grant No. DE-02456-03 from the National Institute of Dental Research, National Institutes of Health and by contract No. 4234 (00) with the Office of Naval Research, Department of the Navy.