pressure was constant for the first 2 hours of exposure and began to rise at the same time as the pulse. Loss of peripheral vision as well as subjective signs of oxygen intoxication (dizziness, nausea, etc.) began to appear during the third hour of exposure but no convulsions were produced.

We should like to thank the volunteers who made this study possible as well as our colleagues from the Joint Cardiorespiratory Service of the Royal Victoria Hospital who encouraged us and helped us with the arterial blood sampling.

1. Bean, J. W., Physiol. Rev., 1945, v25, 1.
2. Lambertson, C. J., Kough, R. H., Cooper, D. J., Emmel, G. L., Loeschke, H. H., Schmidt, C. F., J. Appl. Physiol., 1953, v5, 471.
3. Whalen, R. E., Saltzman, H. A., Holloway, D. A., McIntosh, H. D., Sieker, H. O., Brown, I. W., Jr., Am. J. Cardiol., 1965, v15, 638.
4. Duff, J. H., Gundel, W. D., Vignoul, H. G., MacLean, L. D., Canad. Med. Assn. J., 1964, v91, 1051.
5. Saltzman, Herbert A., Circulation, 1965, v31, 454.
6. Behnke, A. R., Forbes, H. S., Motley, E. P., Am. J. Physiol., 1936, v114, 436.

Received February 18, 1966. P.S.E.B.M., 1966, v122.

Effect of Pyrophosphate on Dissolution of Hydroxyapatite and Its Possible Importance in Calcium Homeostasis.* (31123)

HERBERT FLEISCH, J. MAERKI AND R. G. G. RUSSELL
(Introduced by R. V. Talmage)
Laboratory of Experimental Surgery, Schweizerisches Forschungsinstitut, Davos, Switzerland

In previous studies we found that pyrophosphate was present both in plasma(1) and urine(2), in concentrations of 2-3 μM and 10-100 μM respectively. Pyrophosphate inhibited not only calcium phosphate precipitation from metastable solutions *in vitro*(3) but also inhibited calcification in tissue culture (4) and *in vivo*(5). These observations suggested that pyrophosphate might be one of the physiological regulators of calcification (6). During investigations of its possible mechanism of action, pyrophosphate was shown to have a high affinity for hydroxyapatite crystals; apatite crystals remained in equilibrium with physiological concentrations of pyrophosphate (2-3 μM) only after binding large amounts of the compound(7). These studies suggest that at least some of the pyrophosphate known to be present in bone(8,9) is situated on crystal surfaces. The possible effects of pyrophosphate on the behavior of hydroxyapatite crystals seemed therefore to be of potential interest in relation to calcium homeostasis.

In a previous paper we showed that hydroxyapatite treated with pyrophosphate was no longer able to induce precipitation of calcium phosphate even from highly supersaturated solutions(7). Preliminary observations suggested that this treated apatite also showed a somewhat different behavior during dissolution. In the present paper this effect has been investigated in more detail.

Methods. Hydroxyapatite was obtained from the Victor Chemical Co., USA. The crystals were shown to have a molar Ca/P ratio of 1.64. They exhibited an X-ray diffraction pattern characteristic of hydroxyapatite; no other pattern developed after heating at 900°C for 15 hours. Furthermore heating at 500°C for 15 hours, a procedure known to cause pyrophosphate formation from calcium-deficient apatites(10), converted only 0.43% of the P into pyrophosphate. The length of the crystals in electron micrographs was about 1000 Å. These results show that the crystals used were composed essentially of a non-calcium-deficient hydroxyapatite.

* This work has been supported by the Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung; USPHS grant AM 07266-02 of Nat. Inst. of Arthritis and Metab. Dis.; and the Sandoz Stiftung zur Förderung der medizinisch-biologischen Wissenschaften.

Three hundred mg of this apatite were equilibrated for 24 hours at 4°C in 1000 ml of a solution of ionic strength 0.16, containing 0.155 M KCl, 0.01 M diethyl barbituric acid, KOH to adjust the pH to 7.4 and a few crystals of thymol. After this equilibration, radioactive ^{32}P-labeled pyrophosphate (obtained from the Radiochemical Centre, Amersham, England) was added to give a concentration of 2.5 μM, similar to that found in ultrafiltrates of plasma. Since the radioactive pyrophosphate disappeared rapidly from the solution onto the crystals, the compound was again added to the solution and the uptake by the crystals was checked by determining the radioactivity remaining in the solution after removing the crystals by filtration through "Göttingen Membranfilter" MF 150 (mean pore size 1 μ). This procedure was repeated (a total of 24 additions) until the radioactivity due to the pyrophosphate rose to and remained at a level corresponding to the desired concentration of 2-3 μM. At this stage the apatite had then taken up about 4.5% of its phosphorus as pyrophosphate.

The behavior of these crystals, hereafter called "coated apatite," during dissolution was then studied as follows: 4 mg of coated and noncoated apatite were each incubated at 5°C, 20°C and 37°C in 5 ml of a KCl/Barbital solution similar to the one used above, *i.e.*, containing no calcium and no phosphate, but buffered at pH 7.0. Aliquots of the solution were removed at various intervals up to 10 days after the beginning of the incubation. These aliquots were filtered through "Membranfilter" in order to retain the crystals, and the filtrates were analyzed for calcium and phosphorus. Calcium was measured by titrating with EDTA in presence of calcein after oxalate precipitation, and phosphorus was determined spectrophotometrically after reacting with molybdate in the presence of ascorbic acid(11). Hydrolysis of crystal pyrophosphate to orthophosphate was determined by dissolving the crystals at 4°C in a minimum quantity of HCl and extracting the solution with isobutanol/petrol-ether in the presence of molybdic acid(12); this technique separates orthophosphate, which goes into the alcoholic phase, from pyrophosphate, which remains in the aqueous phase. The hydrolysis was calculated by measuring the radioactivity in both layers.

Results. The dissolution curves of the coated and noncoated apatite at 5°C, 20°C and 37°C are shown in Fig. 1-6. It is evident that the coated apatite dissolves to a smaller extent than the noncoated one. This appears to be an effect on the rate of dissolution as well as on the final solubility. The loss of inhibitory action after prolonged incubation, particularly at 37°C, is accompanied by and possibly explained by a considerable spontaneous hydrolysis of the pyrophosphate on the crystals. It is interesting to note that pyrophosphate in solutions of the same pH and composition but without crystals shows nearly no hydrolysis.

Discussion. Previous results had shown that hydroxyapatite in equilibrium with physiological concentrations of pyrophosphate is unable to induce precipitation from metastable solutions of calcium and phosphate(7). The present study indicates that such crystals also differ from normal in that they show a lower solubility during dissolution. Pyrophosphate thus appears to act as a kind of "buffer" around the crystals impeding both their growth and dissolution. It is interesting to note that both effects have also been observed with strontium sulfate crystals treated with polyphosphates(13,14). Although the mechanism is uncertain, it is thought to involve an interaction of the adsorbed polyphosphates with ions in the surface layers of the crystals.

The fact that pyrophosphate, at concentrations normally present in plasma, inhibits dissolution of apatite crystals could be of biological interest. Bone contains considerable quantities of pyrophosphate(8,9), some of which at least is probably situated on crystal surfaces (unpublished results). The amount of pyrophosphate on the surface might influence bone solubility and therefore be involved in calcium homeostasis.

Parathyroid hormone and thyrocalcitonin are both now thought to act on bone, the first increasing(15,16,17), the second decreasing resorption(18). It is tempting to speculate that these actions could be due to changes in the pyrophosphate layer. Although there is

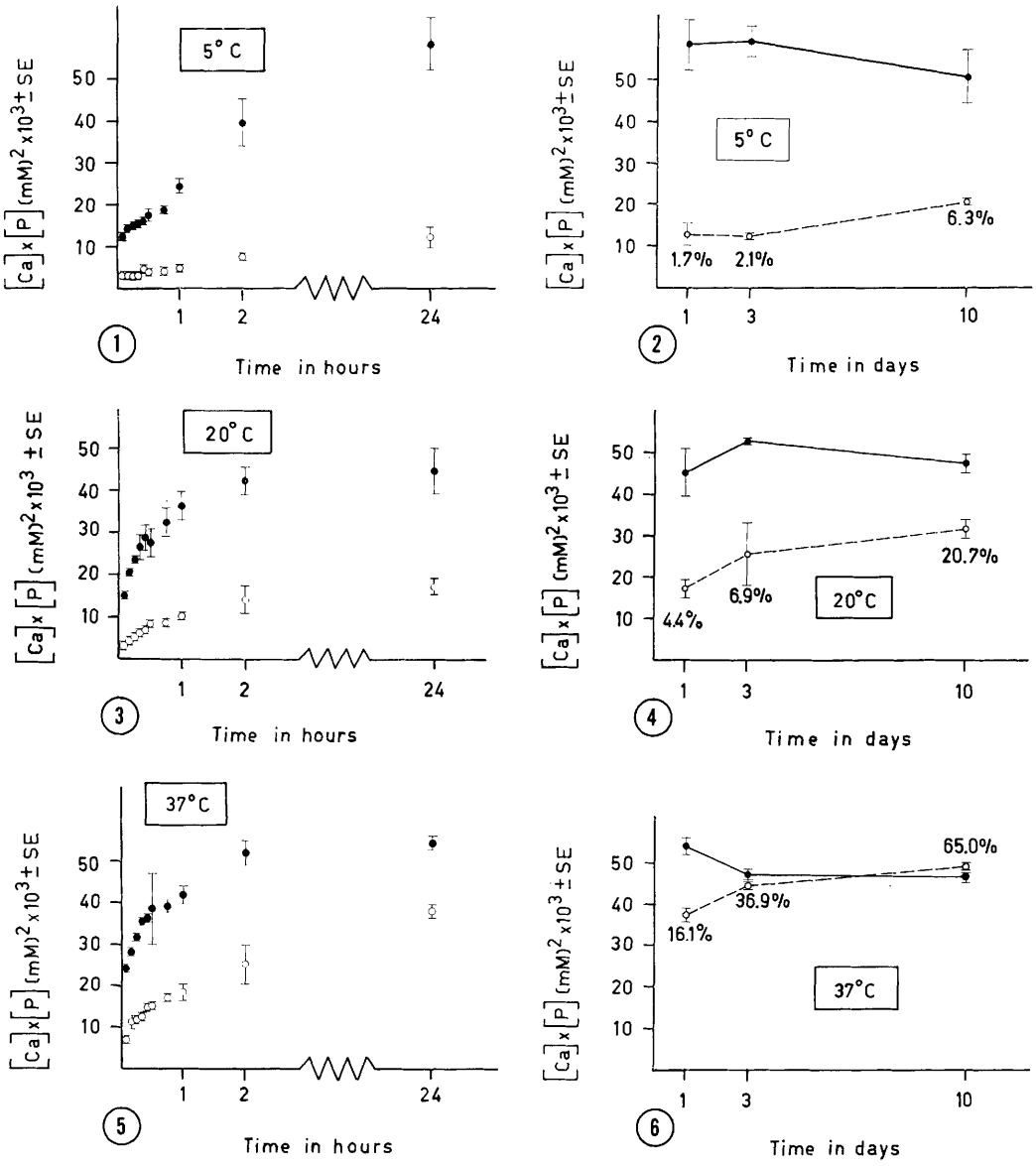

FIG. 1-6. Dissolution curves of hydroxyapatite before (●-●-●) and after treatment with pyrophosphate (○-○-○) at 5°C, 20°C and 37°C. Each point represents the mean of at least 4 experiments ± SE. The extent of the hydrolysis of pyrophosphate in the crystals at 1, 3 and 10 days is shown in %.

at present no direct evidence for this, alterations in pyrophosphate metabolism have been found in several bone diseases; thus urinary excretion of pyrophosphate has been claimed to be several times higher than normal in diseases characterized by increased bone destruction such as hyperparathyroidism, Paget's disease and hyperthyroidism(19), as well as in diseases characterized by defects in mineralization such as hypophosphatasia (20). It is possible that the approximately 2-fold increase in the urinary excretion of pyrophosphate in women at the age of the menopause(21) is related to the onset of osteoporosis at this age and that pyrophosphate is one of the so-called anti-osteoporosis

factors(22). It is an attractive supposition that the regulation of pyrophosphate level in bone might be achieved through alkaline phosphatase, since there is some evidence that this enzyme can also function as a pyrophosphatase(20,23).

Finally, control mechanisms involving pyrophosphate might apply to teeth as well as to bone since this compound is apparently present in saliva in concentrations as high as 100 μM(24), and also in teeth (unpublished results). The effect of pyrophosphate on the dissolution of apatite may constitute part of a physiological mechanism of preventing caries; it might also explain the hitherto unexplained action of condensed phosphates in reducing the incidence of caries in rats(25, 26).

Summary. Hydroxyapatite in equilibrium with physiological concentrations of pyrophosphate showed a markedly reduced solubility during dissolution experiments *in vitro*. Since bone contains pyrophosphate, it is suggested that this phenomenon might be important in calcium homeostasis. It is possible that it is also involved in causation and prevention of caries.

1. Fleisch, H., Bisaz, S., Nature (Lond.), 1962, v195, 911.

2. ———, Am. J. Physiol., 1962, v203, 671.

3. Fleisch, H., Neuman, W. F., ibid., 1961, v200, 1296.

4. Fleisch, H., Straumann, F., Schenk, R., Bisaz, S., Allgöwer, M., ibid., in press.

5. Fleisch, H., Schibler, D., Maerki, J., Frossard, I., Nature (Lond.), 1965, v207, 1300.

6. Fleisch, H., Clin. Orthop., 1964, v32, 170.

7. Fleisch, H., Russell, R. G. G., Straumann, F., Maerki, J., Nature (Lond.), submitted.

8. Perkins, R. H., Walker, P. G., J. Bone Joint Surg., 1958, v408, 333.

9. Cartier, P., Bull. Soc. Chim. Biol., 1959, v41, 573.

10. Dallemagne, M. J., in Handbuch der experimentellen Pharmakologie, Springer-Verlag, Berlin-Göttingen-Heidelberg-New York, 1964, v17, 273.

11. Chen, P. S., Toribara, T. Y., Warner, N., Analyt. Chem., 1956, v28, 1756.

12. Hall, R. J., J. Med. Lab. Technol., 1963, v20, 97.

13. Miura, M., Otani, S., Kodama, M., Shinagawa, K., J. Phys. Chem., 1962, v66, 252.

14. Miura, M., Naono, H., Bull. Chem. Soc. Japan, 1965, v38, 492.

15. Neuman, W. F., Neuman, M. W., The Chemical Dynamics of Bone Mineral, Univ. of Chicago Press, Chicago, 1958.

16. Talmage, R. V., Wimer, L. T., Toft, R. J., Clin. Orthop., 1960, v17, 195.

17. McLean, F. C., Urist, M. R., Bone, Univ. of Chicago Press, Chicago, 1955 and 1961.

18. Milhaud, G., Pérault, A., Moukhtar, M. S., C. R. Acad. Sci. (Paris), 1965, v261, 813.

19. Avioli, L. V., McDonald, J. E., Singer, R. A., J. Clin. Endocrinol., 1965, v25, 912.

20. Russell, R. G. G., Lancet, 1965, vii, 461.

21. Fleisch, H., Bisaz, S., Helv. physiol. pharmacol. Acta, 1963, v21, 88.

22. Urist, M. R., Zaccalini, P. S., MacDonald, N. S., Skoog, W. A., J. Bone Joint Surg., 1962, v44, 464.

23. Cox, R. P., Griffin, M. J., Lancet, 1965, vii, 1010.

24. Sawinski, V. J., Cole, D. F., J. Dent. Res., 1965, v44, 827.

25. König, K. G., Marthaler, T. M., Mühlemann, H. R., Arch. Oral Biol., 1961, v3, 258.

26. Harris, R. S., Das, S. K., Nizel, A. E., J. Dent. Res., 1965, v44, 549.

Received January 3, 1966. P.S.E.B.M., 1966, v122.