Equilibration Studies with Human Bone Powder

II. THE ROLE OF THE CARBONATE ION*

J. MACGREGOR AND B. E. C. NORDIN

From the University Department of Medicine, Gardiner Institute, Western Infirmary, Glasgow W. 1, Scotland

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In the previous paper (1), we demonstrated that when human bone powder was allowed to come into physicochemical equilibrium with bicarbonate-free buffers, it reproduced a constant ion product [Ca++] [PO₄³⁻][CO₃²⁻] over the pH range from 6.6 to 7.6. The free calcium and trivalent phosphate concentrations varied with pH and were reciprocally related to one another, but the concentration of total inorganic phosphate, measured as phosphorus, appeared to be constant irrespective of pH.

Subsequently, in a preliminary communication (2), we showed that the equilibrium phosphate concentration was quite different in a bicarbonate buffer system and that there appeared to be a direct relationship between it and the concentration of bicarbonate ion in the buffer.

The present paper confirms and amplifies these observations.

EXPERIMENTAL PROCEDURE

The equilibration system used has been described before (1). Human bone powder (1 g) was placed in dialysis bags with 10 ml of Tris buffer to which different concentrations (10 to 40 mM) of potassium bicarbonate had previously been added. The majority of the Tris had been added to the bicarbonate, so that the final ionic strength of the buffer was 0.16. In half of the experiments, 4 mM Ca and 1.0 mM phosphate had been added to the buffers to allow equilibration to occur from supersaturation. The sealed bags were placed in polythene bottles with a further 30 ml of buffer solution, and the system was allowed to equilibrate for 72 hours. The pH of the original Tris buffer was approximately 7.2, but each system was left to find its own equilibrium pH value after addition of the bicarbonate, and the final pH values varied from 7.2 to 7.8.

The pH was measured electrometrically at intervals during equilibration. Samples of the buffer fluid outside the bags were analyzed for calcium, total inorganic phosphate, and total CO₂ at 24, 48, and 72 hours from the commencement of the experiments.

Calcium was measured compleximetrically as before (1) in a photocalorimeter by titration with ethylenediaminetetraacetate (disodium salt), with ammonium purpurate used as indicator. Total inorganic phosphate and total CO₂ were measured by published autoanalyzer techniques (Technicon Instruments Limited).

The concentrations of the ion species PO₄³⁻ and CO₃²⁻ were calculated by assuming the dissociation constants 12.4 and 10.25 (pK), respectively. All experiments were performed at room temperature, approximately 20°.

RESULTS

Twenty experiments are reported. Five pairs of experiments were conducted from undersaturation with respect to calcium and phosphate; i.e., the initial buffer was Tris, or Tris plus 10, 20, 30, or 40 mM bicarbonate, with no calcium or phosphate present. In a second series of 10 experiments, identical buffers were used with the addition of calcium (4 mM) and inorganic phosphate (1.9 mM). These were experiments from supersaturation with respect to calcium and inorganic phosphate.

Fig. 1 shows that the calcium concentration varied with pH. At 72 hours, it ranged from 2.08 mM at pH 7.28 to approximately 0.23 mM at pH 7.62.

Fig. 2 shows that the total inorganic phosphate concentration also varied with pH. At 72 hours, it ranged from approximately 0.16 mM at pH 7.28 to approximately 0.81 mM at pH 7.82.

Fig. 3 shows the relation between the ion product pK, [Ca++] [PO₄³⁻][CO₃²⁻] and the pH at 24, 48, and 72 hours in 20 experiments. At 24 hours, it ranged from 27.68 to 25.11, but at 72 hours the range had narrowed to 26.73 to 25.96 and was independent of pH. The arithmetic mean value for this ion product, expressed as a pK, was 26.30.

Fig. 4 shows the relationship between total CO₂ and pH. The initial range of CO₂ concentrations was 0 to 40 mM, and the initial pH, 7.2. It can be seen that the CO₂ concentration at 72 hours ranged from 1.5 mM to 30 mM and that, apart from the four experiments at the lowest pH, there was rather less [CO₂] in the buffers than had been added to the systems originally.

Fig. 5 shows the ion product pKₐ [Ca++] [CO₂⁻] plotted against pH. At 72 hours, it ranged from 8.48 at pH 7.28 to 7.46 at pH 7.80. It does not appear to be a constant or to have a linear relationship with pH. The distribution is similar to that of the total [CO₂] at 72 hours (Fig. 4).

DISCUSSION

It is clear from the results shown in Fig. 3 that bone powder yields the same ion product pKₐ [Ca++] [PO₄³⁻] in buffers whether or not bicarbonate is present. In these 20 experiments, taken from (3).
an ion product has been observed that is independent of pH and is relatively constant whether the systems are initially undersaturated or supersaturated with respect to calcium and phosphate. In comparison with our previous work, it has taken rather longer for this product to be established (72 rather than 24 hours), but the mean value of 26.39 compares with that of 26.39 found previously. This suggests a very reproducible phenomenon. Although the $pK_c$ [Ca$^{++}$][PO$_4^{3-}$] values were identical with those found previously, the absolute values for calcium concentration and particularly inorganic phosphate concentration were quite different. In previous experiments, the total [P] was approximately 0.2 mM over the pH range from 6.6 to 7.8. In the presence of bicarbonate, [P] ranges from approximately 0.2 mM at pH 7.3, with a [CO$_3$] of about 2 mM, to 0.8 mM at pH 7.85, where [CO$_3$] is approximately 20 mM, and the total phosphate concentration appears to be related to pH or carbonate concentration. The correlation between [P] and [CO$_3$] at 72 hours can be seen in Fig. 6 to be very high. Al-
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Fig. 3. The negative logarithm (pKₐ) of the ion product [Ca⁺⁺][HPO₄⁻²] plotted against pH at 24, 48, and 72 hours.

Fig. 4. The relationship between total carbonate concentration and pH after 24, 48, and 72 hours' equilibration with human bone powder.

though the range of pKₐ[Ca⁺⁺][CO₃²⁻] at 72 hours is somewhat narrower than at 24 hours, there is nothing to suggest that a constant product is being established. The distribution of the values suggests a dependence more on the total CO₂ concentration than on anything else.

Nordin (4) has suggested previously that the differences between ion products in experiments from under- and supersaturation reported by other workers might be due to the relatively small amount of solid phase present in the systems employed, and consequently there might be a limit to the amount of inorganic material that can be taken up or given up. This criticism might well be valid in the case of the [Ca⁺⁺][CO₃²⁻] product in these experiments, since the amount of calcium carbonate in the bone employed would be of the order of 40 mg. This possibility has been tested, however, in a further series of five experiments in which the solid to solution ratio in the system was raised by a factor of 10. The products obtained were identical with those in the principal series and ranged from 8.12 at pH 7.55 to 7.82 at pH 7.71. Thus no satisfactory ion product has been established for the carbonate fraction of bone, and
there is no evidence to support the suggestion that this fraction exists as a separate phase of calcium carbonate (5). On the other hand, our present and previously reported work has shown that proportionately much more calcium than phosphate comes out of bone during equilibration, and the associated rise in pH, which always tends to occur, might suggest that calcium carbonate is being preferentially dissolved. Alternatively, one has to postulate that a relatively basic phosphate with high Ca:P ratio is being converted to a less basic form of lower Ca:P ratio, with release of Ca\(^{2+}\) and OH\(^{-}\) ions.

The biological implications of our observations are interesting. In a previous paper (1), we expressed the view that the constancy of the ion product in solutions bathing bone was biologically valid, and we explained the higher absolute phosphate concentrations in tissue fluid by postulating that phosphate from extraskeletal sources, e.g. diet, was continually being mobilized into the tissue fluids to raise the phosphate concentration and lower that of calcium. In the present experiments, the concentration of total phosphate was approximately 0.7 mM when the [CO\(_3\)] was about 23 mM (Fig. 6). Thus, at biological concentrations of bicarbonate, the equilibrium phosphate concentration is approximately 0.7 mM, compared with approximately 1.0 mM in normal human tissue fluids. In fact, Walser (6) has suggested that normal plasma inorganic phosphate is
only about 50% free or unassociated, and if this were so, then addition of bicarbonate to our system yields ionic phosphate concentrations virtually identical with those in extravascular, extracellular tissue fluid.

These results reinforce our previous conclusion that the ion products obtained in vitro are compatible with those found in normal human tissue fluids, provided that one assumes that the environment of the bone salt crystal is at a lower pH than tissue fluids. They also show that further investigation of the nature and role of bone carbonate is urgently required.

SUMMARY

1. Bone powder has been equilibrated with bicarbonate-containing buffers, both from undersaturation and supersaturation with respect to calcium and phosphate.

2. The arithmetic mean product for $pK_e [Ca^{++}][PO_4^{3-}]$ is 26.30, compared with 26.39 in bicarbonate-free buffers.

3. The equilibrium phosphate concentration is dependent on the concentration of bicarbonate in the buffer, although the $pK_e [Ca^{++}][PO_4^{3-}]$ is constant.

4. A constant ion product $pK_e [Ca^{++}][CO_3^{2-}]$ has not been demonstrated, even when the solid solution ratio is raised to 10 g of bone powder per 40 ml of buffer.

5. The results indicate that the equilibrium concentration of inorganic phosphate is similar to that of plasma when physiological concentrations of carbonate are present.

REFERENCES
