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# CONCERNING pH GRADIENTS BETWEEN THE EXTRACELLULAR COMPARTMENT AND FLUIDS BATHING THE BONE MINERAL SURFACE AND THEIR RELATION TO CALCIUM ION DISTRIBUTION *

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In a previous report describing studies of surviving bone samples *in vitro* it was shown that various aspects of bone cell metabolism have an influence on the calcium concentrations achieved in the incubation medium after a steady state is reached (1). Although the Ca and Ca × P concentrations maintained in the media surrounding actively metabolizing bone samples were higher than those maintained by inactivated samples, the underlying mechanisms remained obscure.

Since normal serum is supersaturated with Ca and P with respect to bone mineral and at the same time must be considered to be in diffusion equilibrium with this phase, it has been postulated that the composition of serum differs from the fluid which is in direct contact with the bone mineral (2–5). At low pH, bone mineral solubility is increased. Therefore several workers (2–5) have suggested that, if a pH difference could be shown to exist between the fluid bathing the bone mineral and the circulating fluids, an explanation would be provided for the apparently increased solubility of bone salt *in vivo*. The existence of a pH at the mineral surface below that of serum has been suggested by histochemical studies carried out by Cretin (6), but the relationship of this gradient to cellular metabolism and its significance in the mobilization of Ca and P from the bone has not been directly investigated, partly, at least, because of the inherent difficulty of measuring serially the pH of the microscopic layer of fluid in contact with the mineral in living bone. However, an alternative, albeit indirect, approach to this problem seemed possible using the same type of *in vitro* system employed in previous studies (1).

It was argued that, if bone samples were incubated in media of sufficiently low pH, it was reasonable to presume that any pH gradient sustained by cellular activity in living samples would be abolished. This would be the result, in living samples, of raising the $H^+$ ion concentration in the ambient medium to or above that at the mineral surface, while in heated samples any pH gradient created by cellular activity would be abolished also, but by another mechanism (in this case, cell death). Thus, if the creation and maintenance of a $H^+$ gradient were the mechanism by which cellular activity created the difference in steady state Ca and Ca × P concentration products between living and heat-inactivated samples, this *difference* should disappear as the final pH of the ambient medium decreased, even though the total concentrations of these ions increased.[1] Obviously two factors were critical in such experiments: first, bone cells had to be capable of normal metabolic activity although incubated at low pH; second, rates of ion exchange and solubility of bone mineral must not be altered by the heating procedure. Previous experience (unpublished preliminary experiments) indicating that cells would metabolize normally at pH 6.6, and evidence already published that heating did not change diffusion from bone samples (1), made such an approach seem feasible.

Our experience with such a system is reported below. The results obtained support the view that the maintenance by cellular activity of a $H^+$

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[1] Such an increase in total concentrations after incubation at low pH was anticipated on the basis of preliminary observations in this laboratory and in others (2, 4, 5).

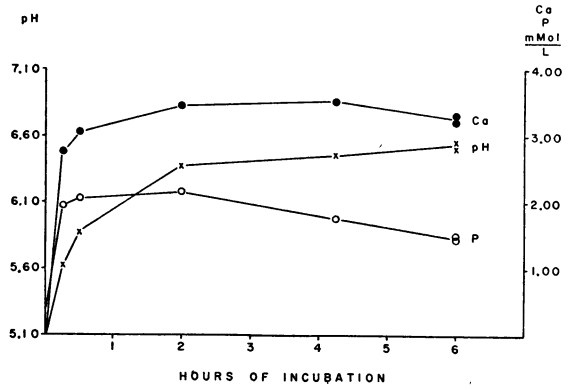


FIG. 1. PLOT OF VALUES OF pH, Ca and P CONCENTRATIONS IN THE MEDIA WITH INCREASING TIME OF INCUBATION, TAKEN FROM A TYPICAL EXPERIMENT. The values at each time represent those found in a single incubation flask, with the exception of the 6-hour point, where two flasks were analyzed. The data for both are given to illustrate the reproducibility of the system.

gradient between the fluid bathing the mineral surface and the ambient medium is a major factor in determining the distribution of Ca and perhaps of P between bone samples and their surrounding fluids *in vitro*. By analogy, the concept that a similar mechanism exists *in vivo* is given added support.

## METHODS

Preparation of bone samples (calvaria from young adult male white Swiss mice), heat-inactivation, analytical techniques for determination of Ca, P, and lactate, and statistical methods were as previously described (1). pH in the media was determined by glass electrode using a Beckman pH meter, model G.

The bone samples were incubated aerobically in a Dubnoff metabolic incubator at 37.8°C for 6 hours in 2 ml of modified Krebs-Ringer medium[2] buffered with Walpole's acetate buffer. Glucose and antibiotics were added to the medium as in previous experiments. The composition of the buffer was varied from one experiment to another in order to obtain the different pH's required. No attempt was made to correct for the small variations in molarity.

Because of the great affinity of bone mineral for H ions, the pH of the medium rose during incubation, despite the presence of buffer. Therefore, in order to obtain a given final pH in the medium, the initial pH had to be considerably lower. In Figure 1 values for pH, together with Ca and P concentrations obtained at different times of incubation of living samples in a typical experiment, are plotted to illustrate this point. As can be seen, during the first period of incubation pH values rose rapidly, and thereafter slowly. At the initial low pH such large amounts of Ca and P were released into the medium that this had become supersaturated with these ions at the pH reached after 2 hours (5). At the end of 6 hours the medium was still supersaturated, and Ca and P concentrations were decreasing as these ions were again deposited on the bone mineral.

These observations indicate two points. First, ions are able to pass from extracellular fluid to bone mineral and vice versa at rates seemingly proportional to the size of the concentration gradient. Second, a steady state distribution of Ca and P between bone sample and medium was not achieved despite 6 hours' incubation because of the continuing slow change of pH. Even when much longer periods of incubation (which might impair the viability of bone cells) were used, a steady state was not achieved for the same reason. However, in contrast to previous experiments, the establishment of steady state conditions was not critical for the purposes of these experiments, and indeed the use of a supersaturated system as the endpoint might be considered desirable, since *in vivo* the circulating fluids are also supersaturated.

In each experiment equal numbers of normal and heat-inactivated bone samples were incubated at the same time. Lactate production was used to determine cell viability and rate of metabolism as outlined previously (1). In the present studies lactate production by the experimental, surviving samples was compared to the amount of lactate produced by normal bones incubated in Krebs-Ringer bicarbonate media (pH 7.4) at the same time. In addition, after the initial incubation, these two groups of samples were transferred to freshly prepared bicarbonate media and incubated once more. Thus, an estimate could be obtained, first, of the depression of metabolism that might occur during incubation at low pH and, second, of the viability of the cells at the end of this incubation.

## RESULTS

*1. Ca and P concentrations in the media: Differences between surviving and heat-inactivated bones at different final pH in the medium.* Final pH and concentrations of Ca and P in the media surrounding both living and heat-inactivated samples are shown in the first two sets of columns in Table I. As was expected from previous reports (2, 4, 5), the highest concentrations of both Ca and P in the media surrounding both types of samples were observed at the lowest final pH. Variations in the absolute values obtained in different experiments were such that a precise inverse relationship between these variables at each pH was not demonstrable.

The important point in relation to the hypothesis under consideration was the *difference* between

[2] The medium contained no Ca; P was present at a concentration of 0.40 mmoles per L.

the concentrations of these ions supported by living and heat-inactivated samples at each pH. These differences are shown in the right-hand columns of Table I. From these data it is apparent that, while the differences in Ca concentrations at a final medium pH of 7.10 were appreciable (0.40 mmoles per L, $p < 0.001$), they gradually disappeared as pH decreased. At pH 6.54 or lower, no reproducible differences in final concentration were found. In experiment N 6, representing an intermediate pH range, the individual values for inactivated samples were 1.81, 1.83, 1.87, and 1.90, while for living samples the values were 2.01, 2.05, 2.08, and 1.79. This last value is considerably below the range of the rest of the group, perhaps because of cell death in this particular sample. Had this value been omitted, the difference would have been statistically significant even at this pH.

No consistent differences in total P concentrations between living and dead samples were found with changes in pH. Therefore, Ca × P concentration products changed with changes in the final Ca concentration. In all experiments Ca × P ion products [3] were in excess of those predicted for the pH value from data on the solubility of dry bone powder (5).

It was important to compare mean final pH values in the medium for living and inactivated samples because the possibility existed that differences in final Ca concentration, where present, might have been the result of a lower pH in the medium surrounding living samples. No consistent difference in final medium pH was found between living and heat-inactivated samples at each pH level. Media surrounding living samples had final pH's which were sometimes above and sometimes below those of inactive samples. These differences were not correlated in any way with differences in final Ca concentration.

*2. Cellular metabolism in surviving bone during and at the end of incubation.* As was pointed out above, it was of critical importance to know whether the disappearance of a difference in Ca concentration in the medium between living and heat-inactivated samples at low pH was due to impairment of cellular metabolism. For this reason the accumulation of lactate in the medium over 6 hours was measured in each incubation flask and compared with that of controls. These data are shown in the first column of Table II.

It is apparent that the accumulation of lactate

[3] Calculated as $[Ca]^3 \times [P]^2$, expressed as milligrams per 100 ml.

TABLE I

*Final pH and concentrations of Ca and P in media surrounding normal and heat-inactivated bone samples*

| Expt. | | Normal | | | Heat-inactivated | | | ΔCa | ΔP |
|---|---|---|---|---|---|---|---|---|---|
| | | Final pH | Ca | P | Final pH | Ca | P | | |
| | | | *mmoles/L* | | | *mmoles/L* | | *mmoles/L* | |
| N 20 | Mean | 7.10 | 2.78 | 0.74 | 7.14 | 2.38 | 0.72 | 0.40 | 0.02 |
| | SD* | 0.14 | 0.06 | 0.03 | 0.09 | 0.03 | 0.01 | | |
| | No.† | 6 | 6 | 6 | 6 | 6 | 6 | | |
| N 5 | Mean | 7.05 | 2.31 | 1.07 | 7.03 | 2.11 | 1.10 | 0.20 | −0.03 |
| | SD | 0.05 | 0.09 | 0.10 | 0.07 | 0.08 | 0.03 | | |
| | No. | 2 | 2 | 2 | 2 | 2 | 2 | | |
| N 6 | Mean | 6.98 | 1.98 | 0.95 | 6.87 | 1.85 | 0.91 | 0.13 | 0.04 |
| | SD | 0.11 | 0.13 | 0.02 | 0.04 | 0.04 | 0.02 | | |
| | No. | 4 | 4 | 4 | 4 | 4 | 4 | | |
| N 7 | Mean | 6.54 | 2.64 | 1.19 | 6.45 | 2.64 | 1.21 | 0.00 | −0.02 |
| | SD | 0.04 | 0.09 | 0.08 | 0.05 | 0.04 | 0.03 | | |
| | No. | 6 | 6 | 6 | 6 | 6 | 6 | | |
| N 2 | Mean | 6.52 | 2.87 | 1.37 | 6.47 | 2.83 | 1.39 | 0.04 | −0.02 |
| | SD | 0.08 | 0.08 | 0.04 | 0.03 | 0.08 | 0.05 | | |
| | No. | 4 | 4 | 4 | 4 | 4 | 4 | | |
| N 8 | Mean | 6.19 | 4.00 | 1.87 | 6.28 | 3.97 | 1.85 | 0.03 | 0.02 |
| | SD | 0.03 | 0.06 | 0.11 | 0.11 | 0.15 | 0.14 | | |
| | No. | 5 | 5 | 5 | 5 | 5 | 5 | | |

* SD = standard deviation of the mean.
† No. = number of incubations.

TABLE II

*Lactate production during and after incubation at different medium pH's*

| | Lactate production* | |
|---|---|---|
| | Initial incubation | Subsequent incubation |
| Controls | | |
| Mean† | 100 | 100 |
| No.‡ | 4 | 4 |
| SD | 10 | 24 |
| pH§ 5.52–7.05 | | |
| Mean | 57 | 52 |
| No. | 4 | 4 |
| SD | 20 | 9 |
| Controls | | |
| Mean | 100 | 100 |
| No. | 4 | 4 |
| SD | 15 | 10 |
| pH 5.33–6.98 | | |
| Mean | 64 | 74 |
| No. | 4 | 3 |
| SD | 17 | 15 |
| Controls | | |
| Mean | 100 | 100 |
| No. | 2 | 2 |
| SD | 4 | 31 |
| pH 5.10–6.54 | | |
| Mean | 81 | 105 |
| No. | 2 | 2 |
| SD | 6 | 8 |
| Controls | | |
| Mean | 100 | 100 |
| No. | 4 | 4 |
| SD | 8 | 7 |
| pH 5.05–6.52 | | |
| Mean | 70 | 54 |
| No. | 4 | 4 |
| SD | 14 | 15 |

* Expressed as per cent of lactate production by controls incubated at pH 7.4 at the same time. All control and subsequent incubations were in bicarbonate buffer. Initial incubation of experimental samples was in acetate buffer.
† Number of determinations.
‡ Standard deviation.
§ Initial and final pH in initial incubation.

in media surrounding surviving bone samples was between 60 and 80 per cent of the "normal" amount (measured in bicarbonate buffer) over the whole pH range studied.[4] The rate of lactate production, if anything, was higher in those samples incubated in the lower range of pH where no differences in Ca concentration were observed. Thus, the presence of a low pH in the medium did not appear to inhibit cellular metabolism, at least as measured by the rate of production of its chief acid end product, lactate.

Although the average lactate production, as shown, was the same over the pH range studied, cellular metabolism and acid production at the end of the incubation period might have been slowing down in the samples in the "lower pH range." To test for this possibility, samples were reincubated in media at pH 7.4 as indicated above. Unfortunately, the lactate values for this group during the subsequent incubation at pH 7.4 showed a considerable spread (see Table II). However, the mean values for the samples in the "lower range" were again higher, indicating that the viability of these samples was at least equal to those in which the final pH had been 7 or above.

## DISCUSSION

The data presented above indicate that the ability of living bone samples to support a Ca concentration in their surrounding media higher than that maintained by heat-inactivated samples is abolished when the pH of the surrounding medium

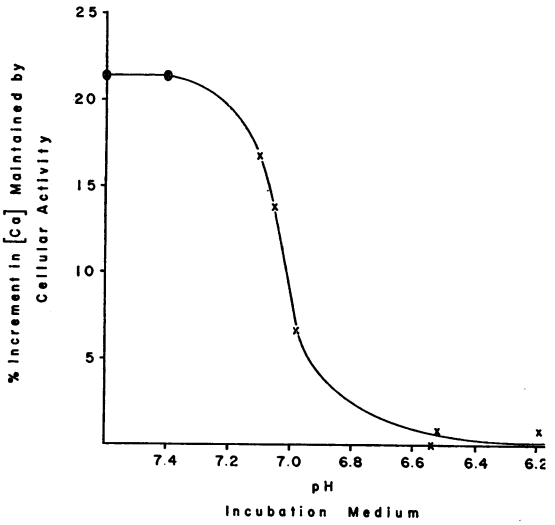


FIG. 2. PERCENTAGE INCREMENTS IN CA CONCENTRATION IN THE MEDIA MAINTAINED BY NORMAL COMPARED TO HEAT-INACTIVATED BONES AT DIFFERENT FINAL MEDIUM pH's. pH values used in this plot were those for incubations utilizing living samples. × = Points calculated from data in Table I; ⊗ = points calculated from data previously published from this laboratory (1).

[4] This degree of reduction in the rate of lactate production was observed in previous experiments (7) when phosphate was substituted for bicarbonate buffer. It seems likely therefore to be related to the nature of the buffer used rather than to the pH per se.

is lowered. This phenomenon occurs despite the fact that the ability of such living samples to produce their chief acid end product, lactate, is not impaired. This observation is consistent with the view that the differences in Ca concentration in the medium between surviving and inactivated samples incubated at medium pH's of 7.1 and above are the result of a pH gradient between the fluid bathing the crystal surface and the medium which is maintained by cellular metabolism.

Nordin has suggested, on the basis of the solubility of bone powder *in vitro,* that were the pH of the fluid in contact with the bone mineral surface between 6.6 and 6.8, the Ca × P ion product in the extracellular fluid would be explained (4). These findings suggest that, in the system used in these and previous experiments, differences in Ca concentration of the medium should approach a maximum at a medium pH of 7.4 or above and a minimum at pH values of 6.6 or below, if the differences are indeed the result of a pH gradient maintained by cell activity. Differences at pH values between 7.4 and 6.6 might be expected to fall along a sigmoid curve with its steepest part centered around a pH of 7.

Figure 2 has been prepared to examine how well data currently available fit this prediction. Differences in medium Ca concentration between living and heat-inactivated samples, expressed as per cent of the inactive sample concentration, have been plotted against final medium pH.[5] The values at pH 7.4 were taken from a previous communication (1), while the others have been calculated from Table I. The data fit the prediction quite well except that the steepest part of the curve is slightly to the left of its predicted position. However, it should be noted that the pH values recorded are those at the end of the incubation period and the mean pH during the incubation was actually lower (Figure 1). It is also of interest that data taken from experiments in which a steady state distribution of Ca was reached (values at medium pH 7.4 to 7.6) fit on a curve described by experiments in which the medium was always supersaturated with Ca and P. This finding suggests that either type of system can be used to examine this phenomenon with equal validity. Finally, a possible explanation for the lack of correlation of Ca concentration observed at steady state with variations in medium pH between 7.6 and 7.4 (1) is supplied, since in that pH range differences in Ca concentration are maximal and small changes in pH should have little measurable effect.

The lack of differences seen in total P concentrations in the medium in this study and in most of the previous experiments (1) is of interest, since these results also may suggest the mechanism for the cellular metabolic effects. As has been mentioned (1), these results might be expected on the basis of MacGregor and Nordin's work (5) if the underlying mechanism were acid production. An explanation for the increased Ca/P ratio in the medium when bone salt is being solubilized by an acid may be that H ions displace Ca ions from the surface, as suggested by Neuman (3). In addition, phosphate and $H^+$ must be removed from the medium if the reaction: $1\ Ca_3(PO_4)_2 + 3\ H^+ + 1\ PO_4^{\equiv} \rightleftarrows 3\ CaHPO_4 \rightleftarrows 3\ Ca^{++} + 3\ HPO_4^{=}$ is to proceed to the right. Thus both phenomena would tend to increase the Ca/P ratios in the medium.

Despite the correlation of these observations with predictions made on Nordin's data, and the concept of a local pH gradient maintained by cellular activity between the fluid at the bone mineral surface and the ambient medium, it must be emphasized that the proof of such a thesis is not yet available. While lactate production was not critically changed at low pH, other aspects of cellular metabolism, not examined so far, could have been greatly modified. That other cellular activities, besides lactate production, are important in the maintenance of higher Ca concentrations by living samples has been indicated in previous work (1). Thus the possibility exists that some aspect (as yet unmeasured) of cellular metabolic activity, critical to Ca mobilization, was abolished by incubation at low pH.

Just how such a $H^+$ gradient between bone mineral and extracellular fluid can be related to the high levels of Ca maintained in the medium by bone samples from parathyroid-treated animals (1) is also not yet clear. The increased passive solubility of the bone mineral, the increased Ca/P ratio, and the increased rates of lactate (1) and citrate (7, 8) release into the medium from living

[5] Expression of values in this manner eliminates differences due to daily experimental variation and the greater solubility of bone mineral at lower pH.

samples taken from animals pretreated with this hormone, certainly fit with the concept that the rate at which bone cells produce acid is involved in this phenomenon. On the other hand, the lack of correlation between levels of lactate (1) and citrate (8) in the medium and levels of Ca and P achieved under steady state conditions, and the similarity in the size of the fraction of total steady state Ca concentration which can be related to cellular activity between bone from normal and parathyroid-treated animals (1) argue against increases in the magnitude of the $H^+$ gradient being the critical factor. The explanation for these apparently conflicting pieces of evidence must await further work.

## SUMMARY

1. Surviving and heat-inactivated bone samples (mouse calvaria) were incubated *in vitro* in media of different pH, and Ca and P concentrations in the media were measured.

2. The ability of actively metabolizing bone samples to maintain higher Ca concentrations in their surrounding media than inactivated samples was abolished when pH in the media was decreased from about 7 to 6.5. This occurred while cellular metabolism continued at the same rate, as judged from lactate production. P concentrations were the same for both groups.

3. These findings are consistent with the view that cellular metabolic effects on Ca concentration in the medium are mediated, at least in part, through pH gradients between the bone fluid compartment and the medium.

4. By analogy, the results support the concept that the existence of a pH gradient between a fluid phase in bone and the circulating fluids is a factor underlying the apparent supersaturation of normal serum with respect to bone mineral.

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