THE "CALCIUM-PHOSPHATE PRODUCT" IN AN *IN VITRO* SYSTEM OF SURVIVING BONE[1,2]

By

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INTRODUCTION

It is becoming increasingly apparent that the calcium and phosphate in the tissue fluids are in a state of equilibrium with the mineral of the skeleton (6) and that it is in fact the skeleton which largely sustains the serum calcium under the influence of vitamin D and the parathyroid glands. This being the case, it seemed to us to be of interest to discover whether fresh bone kept alive in a nutrient medium would reproduce, in the medium, concentrations of calcium and phosphate comparable to those seen in the tissue fluid of the living organism. This paper deals primarily with the changes in calcium and phosphate concentrations observed in the nutrient medium of surviving bone preparations *in vitro* during the first 24 to 48 hours after removal from the body.

METHODS

1. *Survival, killing techniques:*

Bone for the experiments came from two sources. Human, cancellous bone was collected between the cortical tables of posterior iliac crest donor sites at the time of graft procurement. Spinous processes, denuded of soft tissues and cartilage, were obtained from young patients undergoing spine fusions for the operative correction of scoliosis. Bone fragments were immediately dropped into sterile flasks containing human

[1] Supported in part by U.S.P.H.S. Grant A-817.

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placental cord serum, chilled to approximately 3° C. The cancellous bone samples were aseptically cut into fragments, and spinous processes were split into mirror halves. Thickness of all cortical-cancellous bone fragments was limited 1 mm. or less. Excess blood and adherent clots were removed by vigorous washing in sterile placental cord serum.

The experimental medium consisted of 90 % Moscona saline (9) or a modification thereof, and 10 % human placental cord serum, to which 50 units of sodium penicillin and 50 gamma of streptomycin sulfate per ml. had been added. Modifications in the Moscona saline were made by adding varying amounts of calcium as $CaCl_2.2H_2O$ and varying the phosphate concentrations by altering the amount of $NaH_2PO_4.H_2O$ added. The concentrations of calcium and phosphate in the final nutrient medium were estimated from the saline formula in each experiment and subsequently determined by chemical analysis.

Approximately one-half gram, wet weight, pieces of bone were deposited in sterile 50 ml. Erlenmeyer flasks and 7 ml. of experimental medium added to each. The flasks were stoppered and placed in an incubator or on an incubated rotary shaker at 37° C. 1 and ½ ml. aliquots were aseptically removed at intervals up to 48 hours for calcium and phosphate determination respectively. In some cases, the pH was measured in a Beckman unit immediately upon removal of the samples.

In a group of experiments on "dead bone", the osseous fragments were slow frozen to —30° C., after the 48 hour nutrient medium samples had been removed. Once frozen the samples were slowly thawed and the process of freezing-thawing repeated. The bone fragments were then returned to the experimental solutions for varying periods.

The viability of cancellous bone was checked by preparing tissue cultures of fragments. Standard Maximow double coverslip cultures were made and carried for 10 days. The survival of cells within the samples was judged upon migration of proliferation of cells from the bony explants. The viability of the largely cortical spinous processes was inferred from pH changes occurring during incubation.

2. *Chemical methods:*

For chemical analysis, ½ or 1 ml. samples of nutrient medium were used. Calcium was estimated by titration with ethylene-diamine-tetra-acetic acid in a Coleman spectrophotometer at 500 millimicrons, using ammonium purpurate as the indicator. Inorganic phosphate was estimated by the method of Fiske and Subbarow (1).

RESULTS

In some preliminary experiments we confined ourselves to observing changes in the calcium concentrations in the medium. The results obtained in two such experiments (4 bottles) are shown in Table 1. In the two bottles in which the concentration of calcium was relatively high, it fell to about 5 mg. % at the end of 5 hours: in the two bottles in which the calcium concentration was low, it had risen to a similar level by the end of 5 hours. In the remainder of the experiments, we have measured both the calcium and the phosphate concentrations in millimoles per liter (m M/L) and have expressed the results as a product with no assigned unitage. No attempt has been made to calculate pK values for the calcium salts because the pH was not measured in many of the early experiments.

TABLE 1*

Experiment	Calcium concentration (mg.%)	
	0 Hours	5 Hours
D 1	3.00	5.00
2	10.00	5.00
G 1	1.76	3.12
2	7.20	4.52

* Experiments involving fresh human cancellous (trabecular) bone.

In 11 experiments involving 24 bottles, fairly consistent results were obtained which are shown in Table 2 and Figs. 1 and 2. The initial concentrations of calcium and phosphate were chosen to yield Ca $\times$ P products above, within and below the physiological range. These initial products extended from 0.03 to 4.85. At the end of 24 hours, the high products had fallen and the low products had risen so that the product range had been reduced to 0.70–2.3.

In all of these experiments in which the initial product was less than 0.6 (R1, T1, T2, BB1, BB2, BB3, SS1, TT1 PP1, WW1, WW3, XX1, BBB1, BBB2) it had risen by the end of 24 hours to fall within the range 0.7 to 1.4. In many of these experiments, a substantial rise occurred within the first 5 hours. In one experiment (BB) the highest product was observed at 5 hours, it had fallen slightly at 24 hours, and was down to 0.3–0.4 at 48 hours. In this experiment the viability test was negative at 24 hours, and we believe that the falling product was due to the death of the preparation. This will be discussed below.

TABLE 2

Experiment	Type of Bone	Time (Hours)	Concentration in medium (mM/L)		Ca × P	Remarks on Viability
			Ca	P		
R 1	Cancellous (Femoral head)	0	0.50	1.93	0.97	Cell growth from 24 hour samples in tissue culture
		24	0.70	1.93	1.35	
2		0	0.50	0.13	0.06	
		24	0.90	1.55	1.39	
3		0	2.00	1.67	3.34	
		24	1.30	1.87	2.42	
T 1	Cancellous (Iliac Crest-intertabular)	0	0.50	0.64	0.32	Cell growth from 24 hour samples in tissue culture
		24	0.90	0.97	0.87	
2		0	0.50	0.97	0.49	
		24	1.00	1.23	1.23	
BB 1	Cancellous (Iliac Crest-intertabular)	0	0.43	0.70	0.30	No growth from 24 hour specimen in tissue culture
		5	1.10	0.89	0.98	
		24	0.90	0.87	0.79	
		48	0.80	0.44	0.35	
2		0	0.43	0.70	0.30	
		5	1.10	0.85	0.94	
		24	0.70	1.08	0.76	
		48	0.90	0.39	0.35	
3		0	0.43	0.70	0.30	
		5	0.90	0.77	0.70	
		24	1.00	0.77	0.77	
		48	0.70	0.42	0.29	
SS 1	Cortical and cancellous (spinous process)	0	0.13	1.43	0.19	
		1	0.25	1.15	0.29	
		3	0.25	1.20	0.30	
		5	0.25	1.10	0.28	
		24	0.75	1.10	0.83	
		48	1.00	1.00	1.00	
		72	1.40	1.00	1.40	
2		0	0.90	1.15	1.03	
		1	1.05	1.25	1.31	
		3	1.00	1.10	1.10	
		5	1.00	1.00	1.00	
		24	1.20	0.85	1.02	
		48	1.10	0.82	0.90	
		72	1.20	1.60	1.92	

TABLE 2 (cont.)

Experiment	Type of Bone	Time (Hours)	Concentration in medium (mM/L)		Ca × P	Remarks on Viability
			Ca	P		
TT 1	Cortical and cancellous (spinous process)	0	0.25	1.43	0.36	Cell growth from 24 hour samples in tissue culture
		2	0.33	1.45	0.48	
		5	0.45	1.08	0.49	
		24	0.90	0.96	0.86	
		48	1.30	1.20	1.56	
		96	1.40	1.00	1.40	
2		0	0.80	1.52	1.22	
		2	0.70	1.62	1.14	
		5	0.89	1.12	0.99	
		24	1.05	0.82	0.86	
		48	1.50	1.00	1.50	
		96	1.75	0.92	1.60	
PP 1	Cortical and cancellous (spinous process)	0	0.50	0.94	0.47	
		1	0.50	0.94	0.47	
		3	0.50	0.94	0.47	
		5	0.50	0.87	0.43	
		24	1.30	0.87	1.12	
WW 1	Cortical and cancellous (spinous process)	0	0.60	0.91	0.55	
		5	0.60	1.12	0.67	
		24	1.00	1.10	1.10	
		48	1.25	0.81	1.02	
2		0	4.30	1.00	4.30	
		5	3.90	0.91	3.55	
		24	2.50	0.75	1.88	
		48	2.50	0.39	0.96	
3		0	0.10	0.25	0.03	
		5	0.40	0.48	0.20	
		24	1.30	0.58	0.80	
		Time pH				
XX 1	Cortical and cancellous (spinous process)	0 7.8	0.45	0.21	0.09	
		24 7.3	1.10	0.75	0.83	
		48 6.9	1.60	0.46	0.73	
2		0 7.8	3.40	0.30	1.02	
		24 7.3	2.60	0.42	1.10	
		48 7.0	2.70	0.28	0.76	

TABLE 2 (cont.)

Experiment	Type of Bone	Time (Hours)	pH	Concentration in medium (mM/L)		Ca × P	Remarks on Viability
				Ca	P		
BBB 1	Cortical and cancellous (spinous process)	0	7.8	1.68	0.15	0.25	Significant O_2 consumption in Warburg apparatus
		24		1.85	0.46	0.85	
		48	6.9	1.98	0.46	0.91	
2		0	7.8	1.08	0.15	0.16	
		24		1.53	0.46	0.70	
		48	7.0	1.65	0.48	0.79	
CCC 1	Cortical and cancellous (spinous process)	0	7.7	2.65	0.93	2.46	
		24	7.3	2.68	0.68	1.82	
2		0	7.7	2.65	0.93	2.46	
		24	7.2	2.60	0.70	1.82	
DDD 1	Cortical and cancellous (spinous process)	0	7.6	2.50	1.87	4.85	
		24		1.89	0.99	1.87	
		48	6.8	2.30	0.90	2.07	
2		0	7.6	2.50	1.87	4.85	
		24		1.77	1.01	1.78	
		48	6.9	2.00	0.89	1.78	

Mean 24 hour Ca × P product = 1.27.
Standard Deviation = .468.
Standard Error = ± .20.

In those experiments in which the initial Ca × P product was about 1–1.3 (R1, SS2, TT2, XX2), very little change in the product occurred during the 24 or 48 hours that they were observed. When the initial Ca × P product was "high" (above 2) it invariably fell to a lower level in 24 hours, though not low enough to meet the products reached in the experiments starting from lower levels. However, in one of these experiments (WW2) which was continued for 48 hours, the product fell to 0.96.

In two experiments (FF and MM) involving 5 bottles discordant results were obtained which are shown in Table 3. In these two experiments, the 24 hour Ca × P product was about 0.2–0.3 which is very close to the product observed with bone powder (10). Tissue cultures of bone fragments removed from FF at the end of 24 hours failed to grow. It is our belief that these bone preparations did not survive, and therefore gave similar "products" to those in experiment BB referred to above. In order to test this hypothesis, some experiments were per-

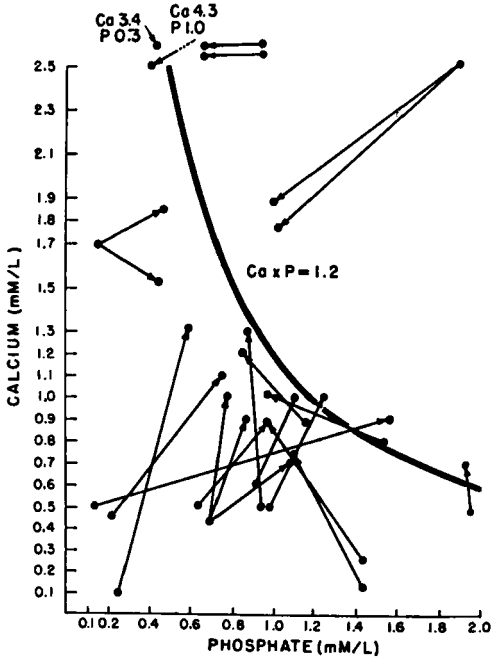

Fig. 1.

Results obtained by 24 hour equilibration of surviving bone preparation in various electrolyte media (Table 2). The black line represents the mean Ca × P product of 1.2 with a standard deviation of ± 0.2. The black dots represent the Ca and P concentrations in millimoles/liter at the beginning of equilibration, and the arrow shows the values at the end of 24 hours. (All experiments except MM and FF in Table 3).

formed with bone preparations which had been "killed" by alternate freezing and thawing. None of the frozen bone samples checked showed any growth of cells in tissue culture. The results obtained in these four experiments with seven bottles are shown in Table 4. The 24 hour product obtained with killed bone ranged from 0.09 to 0.59 in all samples starting from a product of 3.45 or lower. We believe this supports the inference that the discrepancy between the results shown in Table 2 and Table 3 could be attributed to death of the preparations in experiments FF and MM.

In the later experiments listed in Table 2 and Table 4, the pH of the nutrient medium was measured before and after equilibration. A fall in pH was observed during the experiments involving the preparations designed for survival (Table 2), while there was little or no pH change in the media harboring "killed" bone samples (Table 4).

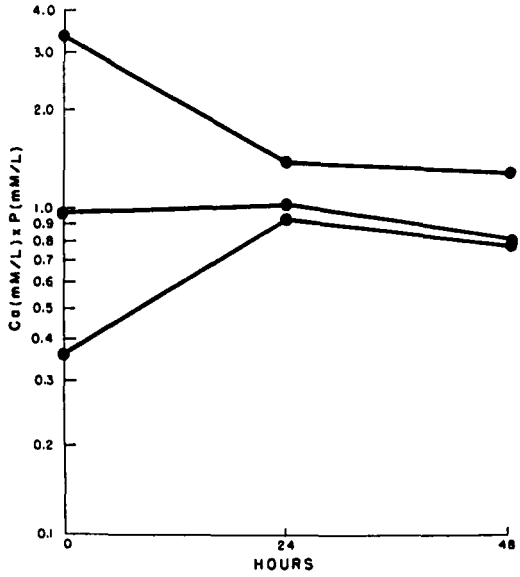

Fig. 2.

Mean Ca (mM/L) × P (mM/L) products at 24 and 48 hours in three groups of experiments—13 in which the starting product ranged from 0.19 to 0.89, 4 from 0.90 to 1.09, and 7 from 1.10 to 4.85. (Data from Table 2).

DISCUSSION

In this study, pH variations must be considered in analysing the results. Since there is a large amount of data in the literature on hydrogen ion concentration changes occurring in tissue culture system, it was felt that is was not necessary to routinely determine pH at the onset of the experiments. However, as it as found that the products in surviving bone systems were higher than the products in dead bone systems, it became evident that pH determinations were required. The range, from 7.8–7.7 at the onset of experiments to 7.3–7.2 after 24 hours is well within expected limits. On the basis of the solubility of bone powder (10), this pH change *per se* could not account for the difference between the Ca × P product obtained with surviving as compared with dead bone.

The three criteria used to judge the viability of cells in surviving bone are adequate when used in combination. Uptake of significant amount of O_2 by bone in the Warburg apparatus is probably the most precise of these criteria. Progressive fall in pH from a level to 7.8 to 6.8 probably was secondary to the elaboration of metabolic end products

TABLE 3

Experiment	Type of Bone	Time (Hours)	Concentration in medium (mM/L)		Ca × P	Viability
			Ca	P		
FF 1	Cancellous (Iliac Crest-intertabular)	0	0	0.80	0	No growth from 24 hour samples in tissue culture
		5	0	0.51	0	
		24	0.50	0.39	0.20	
		48	0.60	0.36	0.22	
2		0	1.00	0.55	0.55	
		5	0.60	0.45	0.24	
		24	0.50	0.52	0.25	
		48	0.90	0.26	0.23	
3		0	3.50	0.80	2.80	
		5	2.30	0.26	0.56	
		24	1.50	0.09	0.14	
		48	1.50	0.13	0.19	
MM 1	Cancellous (Iliac Crest-intertabular)	0	1.90	0.70	1.33	
		5	1.00	0.55	0.55	
		24	0.75	0.60	0.42	
		48	0.50	0.56	0.28	
2		0	1.90	0.70	1.33	
		5	0.80	0.55	0.44	
		24	0.70	0.60	0.42	
		48	0.45	0.56	0.25	

in a closed system and can be considered an index of cell viability. The emigration or proliferation of cells from bone explants is indicative of the presence of live cells when it occurs. However, when a zone of outgrowth fails to appear about a bony explant, one cannot be sure of the status of cell viability, since cells are often ensnared by matrix and prevented physically from emigrating.

The system utilized to promote cell survival was based upon standard tissue culture tecnique. Media were made up as recommended by Hanks (3). Thickness of all specimens was 1 mm. or less, since penetration of nutrients by diffusion into most soft tissues is probably limited to a depth of 1 mm. from the surface. In bony matrix, diffusion is probably limited to 0.2 mm., this dimension representing the largest diameter of viable osteons and the thickest plates of cancellous bone that do not carry a self-contained blood supply (2). The thoracic spinous process from a young adult is largely cancellous bone, but does present a thin,

TABLE 4*

Experiment	Type of Bone	Time (Hours)	Concentration in medium (mM/L)		Ca × P
			Ca	P	
PP 2	Cortical cancellous (spinous process)	0	0.90	0.75	0.68
		2	0.80	0.90	0.72
		5	0.73	0.67	0.49
		24	0.65	0.87	0.57
		48	0.50	0.85	0.43
SS 1	Cortical cancellous (spinous process)	0	0.13	0.56	0.19
		5	0.50	0.56	0.28
		24	0.30	0.78	0.23
2		0	3.00	1.15	3.45
		5	2.50	0.25	0.63
		24	1.90	0.16	0.31
TT 1	Cortical cancellous (spinous process)	0	0.25	1.43	0.36
		5	0.60	0.80	0.48
		24	0.40	0.22	0.09
2		0	3.00	1.52	4.56
		5	2.60	0.31	0.80
		24	1.90	0.31	0.59
		Time pH			
DDD 1	Cortical cancellous (spinous process)	0 7.6	2.45	1.80	4.40
		24	1.93	0.97	1.87
		48 7.4	1.63	0.85	1.39
2		0 7.6	2.45	1.80	4.40
		24	1.90	0.99	1.88
		48 7.4	1.95	0.85	1.65

* When observations involving viable samples PP 2, SS 1, TT 1, TT 2, DDD 1, and DDD 2 (listed in Table 2) were completed, bone was subjected to repeated freezing and thawing before the experiments listed in this table were performed. No growth on tissue culture of any frozen sample.

outer cortical shell measuring approximately 0.5 mm. wide. Therefore, under the conditions of the viable bone experiments, a certain percentage of cells probably failed to receive effective diffusional nutrition and perished.

Repeated slow freezing and thawing was used to "kill" the cells. Ray (13) has reported that frozen bone may harbor viable cells. However, he is not specific on technical points of tissue culture. Most *in vitro* systems use unfiltered chick embryo juice and plasma, both of which may con-

tain sufficient quantities of living cells to establish a cell population. There are numerous reports attesting to the survival of cells during freezing under optimal circumstances (8, 12). Freezing conditions compatible with cell life range from a partial initial dehydration, with an agent such as glycerol, and a "slow freeze" at —78° C to partial or no dehydration and a "fast freeze" at —120° C to —196° C. However, repeated slow freezing at —30° C, without initial exposure to glycerol, coupled with slow thawing can hardly be considered conducive to maintaining a live cell population. Consistently negative viability indices were obtained with frozen material.

The results of calcium and phosphorus determinations are slightly less uniform than those obtained with bone powder by Nordin (10). The data indicate, however, that surviving bone preparations will consistently take up mineral from a nutrient medium if the Ca × P product is above 2.0, or give up mineral to the medium if the product is below 0.6. The "equilibration point" appears to be about 1.27, only small changes occurring when this was the initial product. There is probably a limit both to the amount of mineral which can be taken up or given up by a given quantity of bone, and to the speed at which these processes can take place, but these limits cannot be estimated from the data. It is possible that the rather high products observed after 24 hours equilibration with high calcium and phosphate solutions could have been due to failure to reach equilibrium in this time. It is also possible that there exits a real, if small difference between the Ca × P product when approached from above and from below. It has frequently been suggested that the "solubility product" at which bone salt forms is higher than the "solubility product" at which it will dissolve (7). It is believed at the present time that the first step in the formation of bone salt is the precipitation of $CaHPO_4$ and that this changes spontaneously into hydroxyapatite with a lower solubility than the original precipitate (7).

The 24 hour mean "product" of 1.27 is slightly lower than the product of calcium and phosphate in the tissue fluids. Taking the normal tissue fluid (i.e. ultrafiltrable) calcium to be 6.5 mg. per 100 ml. (5) (1.6 mM/L) and the phosphate 3.5 mg. per 100 ml. (1.1 mM/l) then the normal product is about 1.8. However, this product is presumably maintained by the parathyroid glands. In hypoparathyroidism the tissue fluid calcium might be 4 mg. % (1 mM/L) and the phosphate 4.5 mg. % (1.5 mM/L) making a product nearer to the one we have observed. The residual discrepancy might be explained in one of two ways. First,

Vitamin D was absent from our system. We believe that both vitamin D and parathyroid hormone may increase the "solubility" of bone and that the physiological Ca × P product may be in part the outcome of their combined action (11). However, in some later experiments, the addition of Vitamin D, parathyroid hormone or frozen parathyroid glands to both the systems or surviving and killed bone has as yet failed to alter the "equilibration point". Another explanation for the discrepancy between our mean product of 1.27 and the physiological products of 1.5 to 1.8 might lie with the percentage of cells that failed to receive adequate nutrition in the surviving bone samples. The results of our experiments indicate that living bone has a higher equilibration Ca × P product with nutrient medium than does dead bone. Since all of our surviving preparations probably contained a varying percentage of dead bone, the resultant product should therefore be lower than it would, had 100 % of the cells survived.

The Ca × P product was consistently lower in the "killed" bone preparations than in comparable surviving samples. Only two 24 hour products from the "dead" bone study were higher (Table 4, DDD 1 and 2) than the lowest 24 hour level obtained with "living" bone. These two anomalous results occurred in samples where the initial Ca × P product was very high (4.40). It may be argued that the lower products obtained in the "killed" bone experiments are not solely caused by killing cells of the bone samples. Certainly, many physical changes are brought about by freezing (4). Such changes might account for an alteration in the calcium and phosphorus binding capacity of bone. However, the close correlation of the "killed" bone results and those obtained with bone powder prepared in quite a different way (10) speak against an obscure physical change as an explanation for the lower products.

CONCLUSIONS

1. Surviving human bone *in vitro* will take up mineral from a nutrient solution if the product of Ca × P (concentrations expressed in millimoles/liter) is above 2.0 or give up mineral to the medium if the product is below 0.6.

2. The "equilibration point" for the Ca × P product in equilibrium with viable bone appears to be about 1.27. The concentrations of calcium and phosphorus in the nutrient media after equilibration are slightly lower than those seen in the tissue fluids of the living organism. The discrepancy between the *in vitro* product of 1.27 and the higher

products seen *in vivo* may in part be explained by the death of certain portions of the bone fragments during the survival experiments.

3. The results suggest that dead bone reaches equilibrium with a somewhat lower Ca × P product than does viable bone.

RESUME

1. Un os humain survivant *in vitro* absorbera la matière minérale d'une solution nutritive si le produit de Ca × P (concentrations exprimées en millimoles/litres) est environ 2.0 ou rendra la matière minérale si la moyenne du produit est inférieure à 0.6.

2. Le « point d'équilibration » de Ca × P en équilibre avec l'os viable paraît s'établir autour de 1,27. Les concentrations de calcium et de phosphore de l'agent nutritif après équilibration sont légèrement moins élevées que celles constatées dans les fluides des tissus de l'organisme vivant. La différence entre le produit *in vitro* de 1,27 et les produits plus élevés constatés *in vivo* peuvent en partie s'expliquer par la mort de certaines parties des fragments de l'os durant les expériences de survivance.

3. Ces résultats paraissent indiquer que l'os mort atteint son équilibre avec un produit Ca × P un peu moins élevé que l'os viable.

ZUSAMMENFASSUNG

1. *In vitro* überlebender menschlicher Knochen nimmt Mineralien von der ernährenden Lösung auf, wenn das Produkt von Ca × P (Konzentrationen in Millimol/Liter ausgedrückt) grösser als 2,0 ist oder gibt Mineral an das Medium ab wenn das Produkt unter 0,6 ist.

2. Der „Gleichgewichtspunkt" für Ca × P im Gleichgewicht mit lebenstüchtigen Knochen scheint ungefähr 1,27 zu sein. Die Konzentrationen von Calcium und Phosphor im ernährenden Medium nach Ausgleichung sind etwas niedriger als jene, die in den Gewebsflüsigkeiten des lebenden Organismus gesehen werden. Die Verschiedenheit zwischen dem *in vitro* Produkt von 1,27 und den höheren Produkten, welche *in vivo* gesehen werden, können teilweise durch das Absterben von gewissen Teilen der Knochenfragmente während der Überlebungsversuche erklärt werden.

3. Die Ergebnisse machen es wahrscheinlich, dass abgestorbener Knochen das Gleichgewicht mit einem etwas niedrigeren Ca × P Produkt ereicht, als lebender Knochen.

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