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Bone Growth in Organ Culture: Effects of Phosphate and Other Nutrients on Bone and Cartilage*

Patricia J. Bingham** and Lawrence G. Raisz

Departments of Pharmacology and Toxicology and of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, N.Y.

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A method for studying the growth of fetal rat long bones in a chemically defined medium in organ culture is described. Cartilage ends and bone shafts were analyzed separately for growth and mineralization by measuring the collagen, calcium, and phosphate content, dry weight, and incorporation of labeled proline into hydroxyproline. Growth and mineralization of the bone shaft were slower in a chemically defined medium than *in vivo*. Growth could be enhanced by supplementation of the medium with non-essential amino acids, albumin or serum. Cartilage ends showed a greater increase in weight and collagen content than the shafts, and medium supplements had less effect on their growth. Bone shaft growth and mineralization were enhanced by increasing medium phosphate concentration over a range of 1.5 to 4.5 mM whether or not the medium was supplemented with serum or albumin. At a low medium calcium concentration (0.5 mM) bone shaft growth and mineralization were impaired. At a low magnesium concentration (0.5 mM) mineralization was enhanced, but growth was impaired.

Key words: Bone — Cartilage — Calcification — Collagen — Phosphate.

Une méthode pour l'étude de la croissance des os longs de foetus de rat, en culture d'organe, dans un milieu chimiquement défini, a été mise au point. Les extrémités cartilagineuses et les parties centrales de l'os sont analysées séparément pour leur croissance et minéralisation en étudiant leur contenu en collagène, calcium et phosphate, poids sec, et incorporation de proline marquée en hydroxyproline. La croissance et la minéralisation des parties centrales osseuses sont plus lentes dans un milieu chimiquement défini qu'*in vivo*. La croissance peut être accélérée en ajoutant au milieu des acides aminés non essentiels, de l'albumine ou du sérum. Les extrémités cartilagineuses présentent une augmentation plus importante en poids et contenu en collagène que les parties centrales et l'adjonction de diverses substances a moins d'effet sur la croissance. La croissance et la minéralisation des parties centrales sont augmentées en élevant la concentration du milieu en phosphate de 1.5 à 4.5 mM, avec ou sans adjonction de sérum ou d'albumine. A une concentration faible de calcium (0.5 mM), la croissance et la minéralisation des parties centrales sont arrêtées. A une concentration faible en magnésium (0.5 mM), la minéralisation est augmentée, mais la croissance est arrêtée.

For reprints: Dr. Lawrence G. Raisz, Department of Pharmacology and Toxicology) University of Rochester Medical Center, 260 Crittenden Boulevard, Rochester, New York 14642, U.S.A.

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** Research Fellow, Monroe County Chapter of the Arthritis Foundation 1969–70, and Pharmacology-Morphology Fellow, Pharmaceutical Manufacturers Association Foundantion, 1970–72. Present address: Department of Medicine, Royal Melbourne Hospital, Victoria 3050, Australia.

Es wird eine Methode beschrieben, mit welcher das Wachstum der Röhrenknochen von Rattenembryos in einem chemisch bestimmten Medium in Organkultur untersucht werden kann. Die Knorpelenden und Knochenschäfte wurden gesondert auf Wachstum und Mineralisation geprüft, indem Collagen-, Calcium- und Phosphatgehalt, das Trockengewicht und der Einbau von markiertem Prolin in Hydroxyprolin gemessen wurden. Wachstum und Mineralisation des Knochenschaftes waren langsamer in einem chemisch bestimmten Medium als in vivo. Das Wachstum konnte beschleunigt werden, indem dem Medium nicht-essentielle Aminosäuren, Albumin oder Serum beigegeben wurden. Die Knorpelenden zeigten eine viel stärkere Zunahme an Gewicht und Collagengehalt als die Schäfte, und Anreicherung des Mediums hatte weniger Wirkung auf ihr Wachstum. Das Wachstum und die Mineralisation der Knochenschäfte nahmen zu, wenn die Phosphatkonzentration im Medium zwischen 1,5 und 4,5 mM erhöht wurde, und zwar unabhängig davon, ob dem Medium Serum oder Albumin beigegeben wurde oder nicht. Bei niederer Calciumkonzentration (0,5 mM) im Medium wurden Wachstum und Mineralisation der Knochenschäfte beeinträchtigt. Bei niedriger Magnesiumkonzentration (0,5 mM) wurden die Mineralisation erhöht, das Wachstum hingegen gehemmt.

Introduction

The use of organ culture to study bone growth was pioneered by Fell over 30 years age [7], but the subsequent studies have suffered from two major shortcomings: 1) Most systems have required supplementation of the medium with embryo extract, plasma or serum [10, 12, 19, 28, 32]. Such chemically undefined media are difficult to reproduce and contain unknown concentrations of hormones, vitamins and other nutrients. 2) No distinction is made between cartilage and bone. In some instances cartilage rudiments with little calcified matrix are used [2, 11, 12, 29]. In others, calcified bone is not separated from cartilage [7, 31]. Any investigation of skeletal growth must differentiate between bone and cartilage since these have chemically different matrices [15, 16] and can responds differently to changes in the environment [23]. The present studies were therefore undertaken to find methods for studying bone growth in organ culture using a chemically defined medium in which factors regulating mineralization and matrix formation of bone and cartilage could be analyzed separately.

Methods

Culture Technique

The techniques were modified from those previously used to study bone resorption [4, 21, 25]. Fetuses were obtained from 18-day pregnant rats. The entire radius and ulna were separated and dissected, but a small amount of muscle was left adhering to the surface of the bone to protect the perichondrium or periosteum. The radius and ulna were washed with Eagle's medium and transferred to a culture dish. In some experiments the two bones were cultured at the surface of the medium on a Millipore filter held by a metal screen in a watch glass containing 0.5 ml of medium. These are referred as "sessile cultures." In other experiments two or four bones were cultured in a 35 mm plastic petri dish (Falcon) containing 0.5 or 1.0 ml of medium. The dishes were kept in a humidified chamber on a rocking platform (Bellco) moving at 7 cycles/min. These are referred to as "rocking cultures". The cultures were maintained at 37° and gassed with 5% CO_2 in air. The medium was changed every two or three days. Whenever possible, bones from the same litter or similar sized bones from two litters were used for one experiment.

The chemically defined medium used, BGJ (Gibco), was the same as BGJb as originally described by Biggers *et al.* (1961) except as follows: The glucose concentration was 2 g/l,

Table 1. Summary of culture conditions

Experiment	Data presented in		Culture system	Basal medium	Supplements		Days in culture
	Table	Figure			Non-essential amino acids[a]	Proteins[b]	
Growth in a chemically defined medium	2	1, 2	Sessile	BGJ	None	None	4
Effect of amino-acid supplements	3		Rocking	BGJ	High AA or low AA	None	4
Effect of adding albumin or serum	6	3, 5	Seesile	BGJ	None	2,5 or 10 mg/ml BSA or 20% FRS or 20% ARS	3
Effect of phosphate							
In a chemically defined medium	5		Sessile	BGJ	High AA	None	3
Graded levels of PO_4	6	4	Rocking	BGJ	None	1 mg/ml BSA	4
In media supplemented with FRS or BSA	4	3	Sessile	BGJ	Low AA	1 mg/ml BSA or 10% FRS	7
Comparison of hydroxyproline content of bones and medium	7		Sessile	BGJ	High AA	10% FRS	3
Effect of lowering calcium or magnesium content	8		Rocking	BGJ	None	1 mg/ml BSA	4

[a] "High AA" supplement consisted of L-alanine (2.8 mM), L-aspartic acid (1.1 mM), glycine (10.7 mM), L-proline (3.5 mM), and L-serine (1.9 mM). The same amino acids at 1/20th of these concentrations were used for the "low AA" supplement.
[b] BSA = bovine serum albumin; FRS = fetal rat serum; ARS = thyroparathyroidectomized adult rat serum.

and ascorbic acid and sodium acetate were added at 50 to 100 mg/l. The concentrations of calcium, magnesium, and phosphate were varied, but the standard medium contained 1 mM Ca and Mg and 3 mM PO_4. The medium was made up fresh for each experiment from concentrated solutions of salts and amino acids (which were kept at 4°) and vitamins and cofactors (which were kept at −20°). Immediately before use, concentrated sodium bicarbonate solution was added under an atmosphere of 5% CO, to bring the medium pH to 7.3. Ascorbic acid was also added at this time since it is labile in unbuffered medium.

The different culture conditions used are summarized in Table 1. The medium was supplemented with non-essential amino acids, albumin or serum. The albumin used was bovine Fraction V (Pentex). "Fetal rat serum" was obtained from 18- or 19-day rat fetuses which were washed in saline and decapitated, and the outflow from the neck was collected and centrifuged. The supernatant presumably included tissue fluid as well as serum. The concentration of calcium was 1.5 to 2 mM; phosphate, 3 to 5 mM; and protein, 1–2 g/100 ml. Adult rat serum was obtained from thyroparathyroidectomized rats. The concentration of calcium was about 1.5 mM; phosphate, 3.5 mM; and protein, 5 g/100 ml. This serum was heat inactivated at 60° for 30 min.

Experimental Design

The radius and ulna were treated as a unit. The two bones responded similarly in culture and the shafts were similar in size. The ulnar cartilage was larger than that of the radius in 18-day explants; they showed similar growth in culture, so that the percentage increase from control was less for the ulnar than for the radial cartilage. Cultures were paired for quantitative analysis of growth and mineralization in different ways: 1) The radius and ulna from one limb were taken for analysis at the time of explantation and the growth response of the bones from the other limb was measured after a period of culture. 2) Bones from opposite limbs of the same fetus were subjected to different treatments in culture and then compared. 3) Bones from two embryos were arranged so that the radius of one was combined with the ulna of the other to give four sets that could be cultured under different conditions and compared with each other. Controls obtained at explantation are referred to as "18 day explants." In some experiments the bones were precultured and then transferred to test media. Control bones were then obtained at the end of preculture and are called "0-time controls". Data were analyzed by Student's "t" test for paired or nonpaired samples as appropriate.

Analytic Methods

Eighteen-day explants, 0-time controls and cultured bones were rinsed with saline, and in most experiments the cartilage ends were cut from the bone shaft under a dissecting microscope for separate analysis. When viewed by transillumination, the calcified shaft was a well defined, dark cylinder which could be separated precisely from the translucent cartilage ends. This was true for 18-day explants as well as for cultured bones. The bones were extracted with cold 5% TCA overnight and the extracts analyzed for calcium by atomic absorption spectrophotometry and for inorganic phosphorus [5].

The TCA-extracted shafts and ends were defatted with acetone and ether, dried, weighed on a Cahn electrobalance, and hydrolyzed in sealed ampoules with 4 N HCl for 22 h at 110°. Matrix formation was evaluated by analysis of the labeled hydroxyproline formed from ^{3}H-proline (10 μCi/ml of L-proline-^{3}H(T)-Amersham Searle—266 mCi/mM) [13] or by measurement of stable hydroxyproline content using a micromodification of the method of Cheng (1969). By using small initial volumes for hydrolysis and a 5 cm cuvette for reading absorption, the sensitivity of the stable hydroxyproline analysis was increased so that 1 μg of hydroxyproline gave an absorbance of 0.16 O.D. The standard curve was linear over a range of 0.6 to 4 μg; duplicate standards varied by less than 3%. The collagen content was calculated by multiplying the hydroxyproline content by 7.46. Recovery of hydroxyproline from acid-extracted, lyophilized skin collagen was 95–102%.

The calcium and phosphate content of the medium was analyzed in all experiments and in some experiments the labeled hydroxyproline content was also determined.

Histology

For general histology, bones were fixed in formol-acetic-ethanol or 10% neutral buffered formalin and decalcified with 0.1 M disodium ethylene-diamine-tetracetic acid overnight. Five micrometer paraffin sections were stained with toluidine blue, Lillie's modified trichrome, or Van Gieson's picro-fuchsin. To localize areas of mineralization, bones were fixed in 80% ethanol and embedded without decalcification. Five micrometer sections were stained by the method of Von Kossa and counterstained with safranin; alternate sections were stained with trichrome. Although silver deposits in the Von Kossa stain localized phosphates or carbonates rather than calcium, recent electron microprobe studies [9] indicate that the stain corresponds closely with sites of deposition of insoluble calcium salts in bone and cartilage.

For autoradiography bones which had been cultured with ^{3}H-proline were fixed with 10% neutral buffered formalin and decalcified. Five micrometer paraffin sections were covered with Kodak AR 10 stripping film and exposed for four days. After development the sections were stained with methyl green-pyronin.

Table 2. Growth of rat long-bone rudiments in a chemically-defined medium (BGJ)

	Shafts			Cartilage ends		
	Extracted dry wt (μg)	Calcium (μg)	^{3}H-Hydroxy-proline (dpm/xg dry wt)	Extracted dry wt (μg)	Calcium (μg)	^{3}H-Hydroxy-proline (dpm/μg dry wt)
18-day explants	24.4 ± 1.0	2.3 ± 0.1	—	104 ± 7	0.44 ± 0.05	—
Cultured bones						
1 day	23.9 ± 1.0	3.6 ± 0.2	2404 ± 103	102 ± 8	0.33 ± 0.02	1963 ± 83
2 days	24.5 ± 4.5	4.5 ± 0.2	3051 ± 206	126 ± 8	0.44 ± 0.04	2162 ± 46
3 days	23.1 ± 1.7	4.8 ± 0.2	3522 ± 71	128 ± 4	0.55 ± 0.04	2326 ± 138
4 days	31.0 ± 3.0	6.2 ± 0.2	2898 ± 452	150 ± 9	0.53 ± 0.06	1744 ± 188

Values are mean ± S.E. for 4 sets (radius plus ulna) of bones. ^{3}H-proline (10 μc/ml) was present for final 24 h of each culture period.

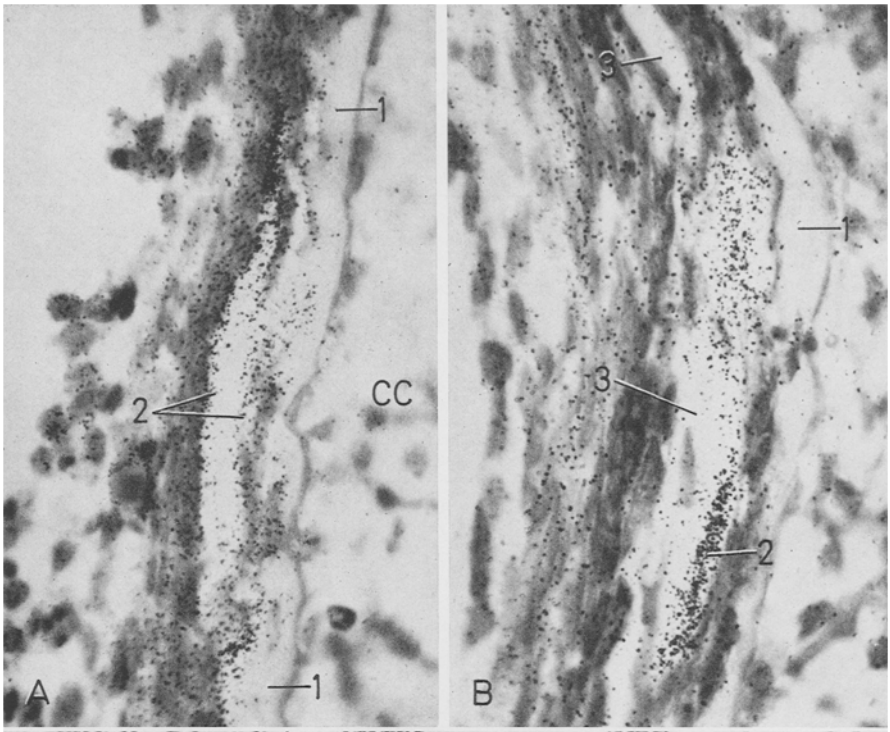
Results

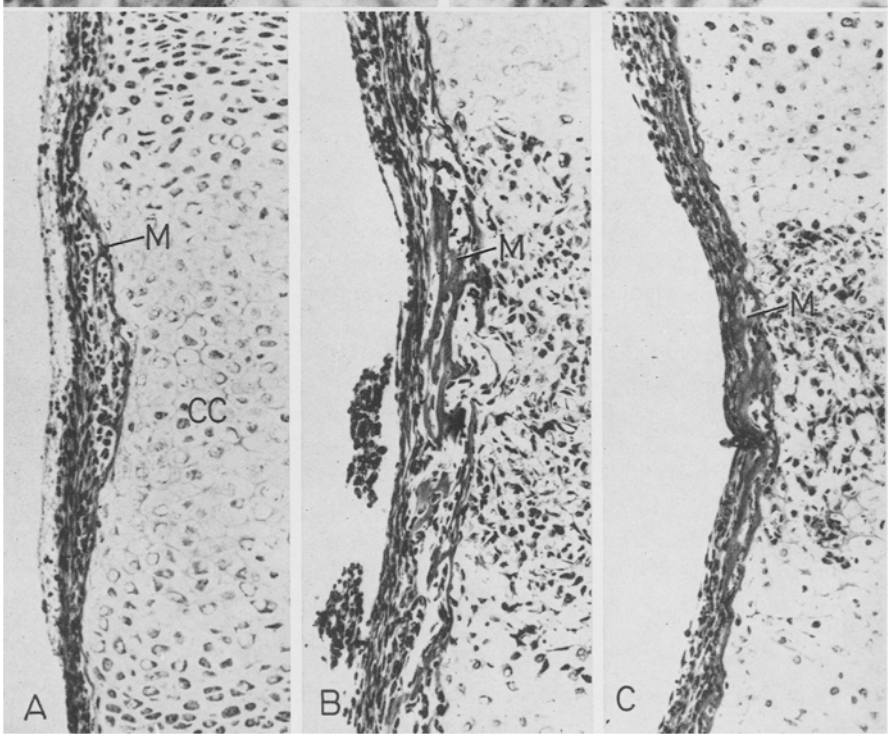
Fetal rat long bone rudiments were explanted on the 18th day of pregnancy; at this time the rudiment consists largely of proliferating cartilage, but a thin cylinder of periosteal woven bone is beginning to form around a core of lightly calcified cartilage. During the next few days *in vivo*, the shaft rapidly enlarges and the cartilage core calcifies and then is resorbed. The epiphyseal cartilage grows less rapidly.

In contrast, after four days of organ culture in a chemically defined medium the bones increased in length by 40 to 60%, but this was due primarily to an increase in the size of the cartilage ends (Table 2). The length of the shaft was increased by periosteal apposition and both the periosteal bone and the cartilage core became more heavily calcified. There was only a small, late increase in demineralized dry weight, which represents the net result of additions by formation and losses by resorption.

The 24-h incorporation of ^{3}H-proline into labeled hydroxyproline was maintained throughout the 4-day culture period in both the cartilage ends and the shafts. Autoradiographic studies showed that the incorporation of ^{3}H-proline in the bone shaft was due mainly to new matrix synthesis by osteoblasts (Fig. 1), and there was little label in the cartilage core. When the bones were incubated with unlabeled medium for a further 42 h, the heavily labeled matrix was covered by new, less labeled matrix, mainly on the periosteal side.

During the first few days in culture new trabeculae of woven bone were found similar in appearance to bone matrix formed *in vivo* (Fig. 2). However, after longer periods in culture, particularly where growth was stimulated by addition of serum (Fig. 3) the new matrix often consisted of coarse bundles of collagen containing many fibroblast-like cells. In many cultures both types of matrix were present with a gradual transition from one to the other (Fig. 4).





Figs. 1 and 2

The bone shafts always showed an increase in calcium content when cultured in standard medium. The calcified periosteal bone matrix increased in both width and length, and cartilage calcification became more intense and extended further into the hypertrophic cartilage (Figs. 3 and 4). In cultures with high $Ca \times PO_4$ products in the medium, small silver-staining deposits were occasionally seen in the connective tissue of the periosteum and perichondrium (not shown in the Figures). These may have represented precipitates from the medium or ectopic calcification. There was no mineral deposition in the proliferative or resting cartilage, or in the coarse fibroblastic matrix which was formed late in culture.

Histologically, explants cultured for 4 days were qualitatively similar to 19 day fetal long bones, but quantitatively there was less new bone and less cartilage resorption. Bones grown in "sessile" cultures often showed distortion of the cartilage and decreased intensity of metachromatic staining associated with acid mucopolysaccharides. Moreover, in sessile cultures periosteal growth tended to be greater on the surface immersed in the medium than on the upper surface exposed to the gas phase. In the "rocking" cultures the bones were alternately immersed in medium and exposed to the gas phase. There was no increase in total matrix synthesis but distortion was reduced and mineralization was somewhat greater. Responses to phosphate, albumin and serum were similar in the two culture systems.

Effect of Amino Acid Supplements

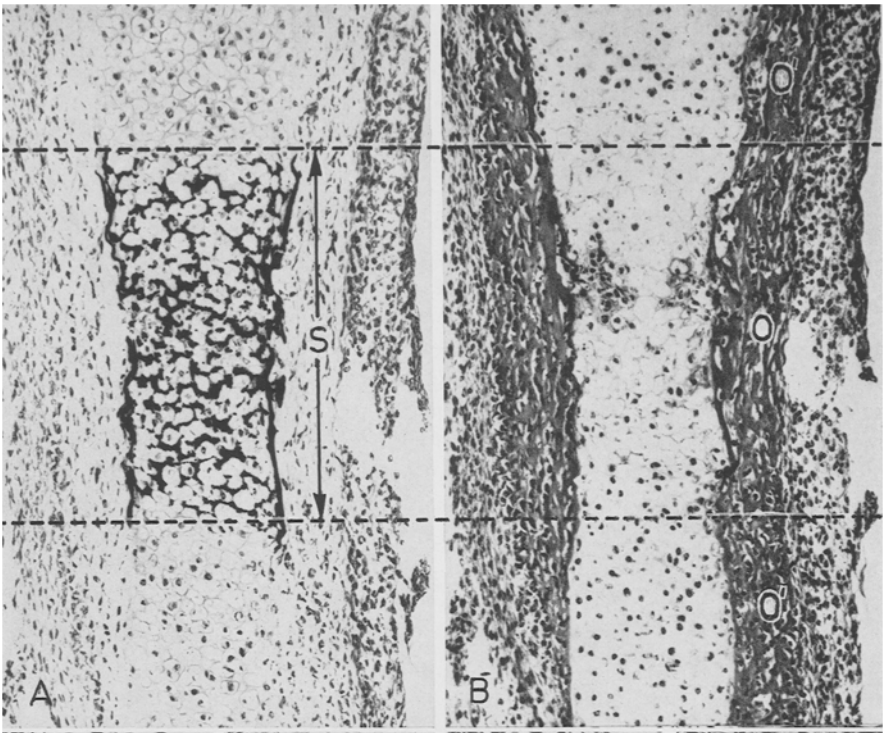
The addition of the non-essential amino acids, alanine, aspartic acid, glycine, proline, and serine, at high concentrations did not increase growth or mineralization of the shaft, and produced only a small increase in cartilage weight (Table 3). But when these amino acids were added at a lower concentration which approximated that in rat serum [30], matrix formation was significantly enhanced. Neither level of amino-acid supplementation affected calcification.

Effect of Adding Albumin or Serum

The addition of either bovine serum albumin or rat serum increased the dry weight of whole cultured bones (Fig. 5). Histologically these bones showed less cell

Fig. 1 A and B. Synthesis of periosteal bone matrix by shafts of fetal rat long-bones *in vitro*. (A) Autoradiograph of shaft after six hours in culture with ^{3}H-proline (10 μCi/ml), showing (*1*) original unlabeled bone spicules present before exposure to ^{3}H-proline, and (*2*) labeled matrix, newly synthesized in culture, being laid down on their surfaces. Note that there is little label over the calcified cartilage core (*CC*). (B) Paired bone to A, which, after the 6 h pulse with ^{3}H-proline, was washed, transferred to fresh medium containing excess unlabeled proline and cultured for a further 42 h. Autoradiograph shows (*1*) original unlabeled bone spicules, (*2*) labeled matrix laid down during and shortly after the 6 h pulse with ^{3}H-proline and (*3*) newer matrix which is lightly labeled, formed during the subsequent 42-h culture in unlabeled medium. Periosteal surface to the left; methyl green-pyronin, × 720

Fig. 2 A—C. Comparison of shaft growth *in vivo* and *in vitro*. (A) Shaft of 18-day fetal rat radius with a cartilage core (*CC*) and a thin layer of periosteal bone matrix (*M*). (B) Shaft of 19 day fetal rat radius. In 24 h, *in vivo*, there is growth of the shaft and resorption of the cartilage core. (C) Shaft of fetal rat radius explanted at 18 days and cultured for 4 days in unsupplemented BGJ medium. Note that the same type of growth has occurred *in vitro* as *in vivo*, but that the extent of cartilage resorption and periosteal bone formation is less than in the 19 day bone. Periosteal surface to the left, trichrome stain, × 120



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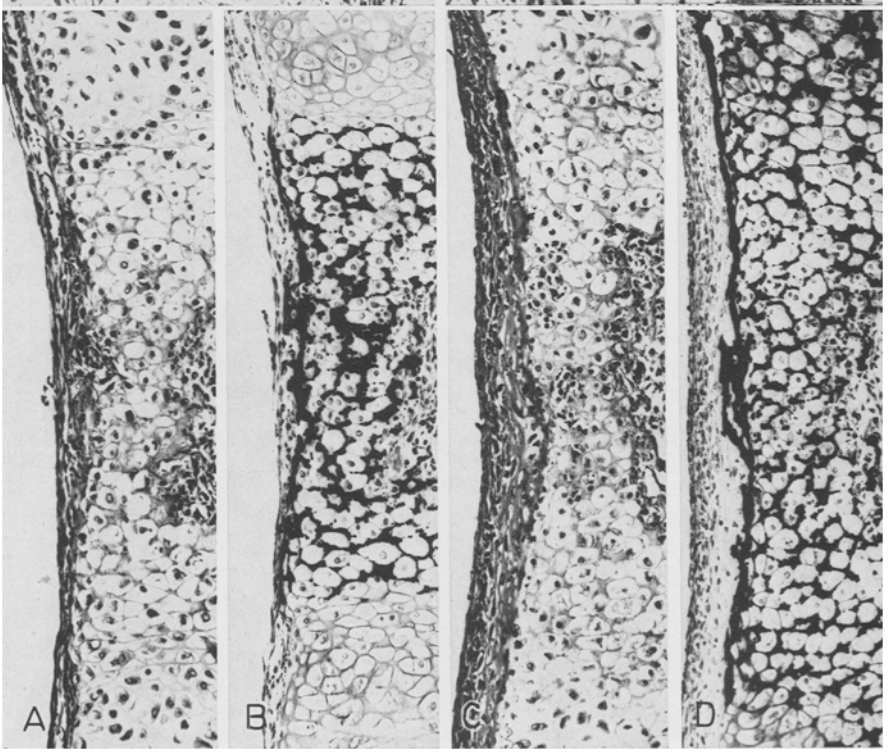

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Figs. 3 and 4

Table 3. Growth of rat long-bone rudiments *in vitro*-Effect of adding amino acid supplements (AA) to the medium

	Shafts			Cartilage ends		
	Extracted dry wt (μg)	Calcium (μg)	Collagen (μg)	Extracted dry wt (μg)	Calcium (μg)	Collagen (μg)
18-day explants	27.5 ± 0.1	3.0 ± 0.5	3.4 ± 0.2	88 ± 2	0.6 ± 0.2	8.8 ± 0.2
Cultured bones						
Unsupplemented BGJ	28.0 ± 2.7	10.2 ± 0.5	5.0 ± 0.2	205 ± 2	0.7 ± 0.1	27.3 ± 1.5
BGJ+low AA	33.7 ± 2.1	9.6 ± 0.5	6.9 ± 0.3[a]	271 ± 12[a]	0.8 ± 0.1	36.1 ± 1.9[a]
BGJ+high AA	24.2 ± 1.6	9.8 ± 0.6	4.0 ± 0.3	245 ± 15[a]	0.7 ± 0.1	30.4 ± 2.6

Values are mean ± S.E. for 4 sets of bones. For composition of the amino acid supplements, see Table 1.

[a] Significantly different from cultures in unsupplemented BGJ ($P<0.05$).

death and more matrix in both the shaft and ends. Addition of serum did not enhance calcification. At high concentrations of albumin the calcium content of the shaft actually decreased, and histologically there was greater resorption and remodelling of the calcified cartilage core. Addition of fetal rat serum markedly increased collagen synthesis compared to cultures with albumin (Table 4) or BGJ alone (data not shown), but this was due largely to an overgrowth of uncalcified fibroblastic tissue both perichondrially and periosteally (Fig. 3).

Effect of Phosphate

Over a range of 1.5 to 4.5 mM, increasing the medium phosphate concentration resulted in increased calcification, increased collagen content, and increased synthesis of labeled hydroxyproline in the bone shafts. These changes occurred whether the bones were cultured in unsupplemented or supplemented medium

Fig. 3A and B. Overgrowth of fibrous osteoid in bone rudiments cultured for 7 days in BGJ supplemented with 10% fetal rat serum. (A) Section showing mineralized area of the bone shaft (*S*), von Kossa stain. (B) Adjacent section showing fibrous osteoid (*O*) in the shaft and similar matrix (*O'*) extending beyond the calcified shaft and surrounding hypertrophic zone of the cartilage ends. This matrix did not calcify and does not have the appearance of typical bone matrix. Trichrome stain, × 120

Fig. 4A—D. Effect of phosphate on growth of the shaft of rat long-bone rudiments *in vitro*. (A) Shaft of bone cultured for 4 days in BGJ containing 2.0 mM P and supplemented with 1 mg/ml bovine serum albumin. There is little periosteal matrix, trichrome stain. (B) Adjacent section showing calcification, von Kossa stain. (C) Shaft of paired bone cultured under the same conditions except that medium phosphate concentration was 3.5 mM. There is an increase both in typical bone matrix and in periosteal fibroblastic matrix. trichrome stain. (D) Adjacent section showing corresponding area of calcification. The shaft is longer, and new areas of periosteal matrix and hypertrophic cartilage are calcified, but the new periosteal fibroblast matrix has no calcified von Kossa stain, × 120. In all views, the periosteal surface is to the left

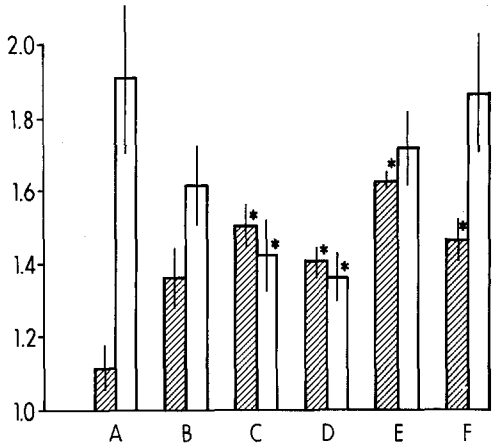

Fig. 5. Ordinate: Ratio (± SE) of 4 or 5 sets of cultured whole bones to paired 18 day explants. ▨ extracted dry weight; □ calcium. Growth of rat long bone rudiments *in vitro*: Effect of adding albumin or serum to the culture medium. Sets of bones (radius + ulna) were cultured for 3 days in (A) unsupplemented BGJ; (B) BGJ + 2 mg/ml bovine serum albumin (BSA); (C) BGJ + 5 mg/ml BSA; (D) BGJ + 10 mg/ml BSA; (E) BGJ + 20% fetal rat serum; (F) BGJ + 20% thyroparathyroidectomized adult rat serum. The cultured bones were compared to 18-day explants from the opposite limbs of the same embryos. Asterisks indicate significant ($p<0.05$) differences from group (A) in dry weight or calcium ccntent

(Table 4–7). At phosphate concentrations of 2 mM or less the shafts often lost weight, although their collagen content was maintained or increased. Since bone resorption may be increased in low phosphate media [25] it was possible that changes in collagen-labeled hydroxyproline content were due to changes in breakdown. However, in cultures labeled continuously with ^{3}H-proline there was no difference in the proportion of labeled hydroxyproline released into the medium at high or low phosphate concentrations (Table 7). At higher phosphate concentrations the shaft showed more calcification both periosteally and in cartilage. At 4.5 mM phosphate there was mineral in the perichondrium and periosteum and a small increase in calcium content of the cartilage ends (Table 6). With increasing phosphate, the calcium-phosphate molar ratio decreased. In 18-day explants the ratio was 1.7 to 1.95. In bones cultured at 2 mM phosphate the ratio was 1.79, while it fell to 1.46 at 4.5 mM phosphate.

In the presence of 10% fetal rat serum high phosphate increased collagen content and labeled hydroxyproline incorporation in both bone shafts and cartilage ends, but there was no increase in calcification (Table 4). The increased collagen synthesis probably was due to the fibroblastic overgrowth noted above (Fig. 3).

Effect of Calcium and Magnesium

When the medium calcium concentration was decreased from 1.0 to 0.5 mM, the growth and calcification of the shaft were significantly reduced (Table 8).

Table 4. Effect of phosphate on growth of rat long bone rudiments *in vitro*-In media supplemented with 10% fetal rat serum (FRS) or 1 mg/ml bovine serum albumin (BSA)

	Shafts				Cartilage ends			
	Extracted dry wt (μg)	Calcium (μg)	Collagen (μg)	^{3}H-Hydroxy-proline (dpm/μg collagen)	Extracted Dry wt (μg)	Calcium (μg)	Collagen (μg)	^{3}H-Hydroxy-proline (dpm/μg collagen)
18-day explants	17.1 ± 1.3	1.7 ± 0.2	2.6 ± 0.2	—	87 ± 4	0.26 ± 0.01	8.5 ± 0.2	—
Cultured bones								
FRS+1.5 mM P	20.1 ± 2.2^b	2.6 ± 0.1	5.4 ± 0.4^b	585 ± 26^b	287 ± 9	0.32 ± 0.02	36.5 ± 1.9	1668 ± 150
FRS+3.0 mM P	25.2 ± 2.4^b	$3.6 \pm 0.2^{a,b}$	$8.1 \pm 0.2^{a,b}$	$1203 \pm 217^{a,b}$	$320 \pm 8^{a,b}$	0.31 ± 0.04	$45.5 \pm 1.4^{a,b}$	$2679 \pm 112^{a,b}$
BSA+1.5 mM P	10.6 ± 1.8	2.1 ± 0.2	2.7 ± 0.1	204 ± 9	225 ± 7	0.34 ± 0.03	33.2 ± 1.8	1181 ± 175
BSA+3.0 mM P	17.8 ± 1.2^b	4.0 ± 0.3^b	4.4 ± 0.7^b	260 ± 12^b	266 ± 14	0.34 ± 0.04	34.3 ± 2.1	1238 ± 185

Values are mean ±S.E. for 3 sets of 18-day explants, or 6 sets of cultured bones. ^{3}H-proline (10 μc/ml) was added to the medium for the first 24 h of culture.

[a] Significantly different from cultures in FRS+1.5 mM P ($P<0.05$).
[b] Significantly different from cultures in BSA+1.5 mM P ($P<0.05$).

Table 5. Effect of phosphate on growth of rat long bone rudiments *in vitro* in a chemically-defined medium

	Shafts			Cartilage ends		
	Extracted dry wt (μg)	Calcium (μg)	^{3}H-Hydroxyproline (dpm × 10^{-2}/culture)	Extracted dry wt (μg)	Calcium (μg)	^{3}H-Hydroxyproline (dpm × 10^{-2}/culture)
0-time control	30.3 ± 1.7	3.4 ± 0.1	—	95 ± 5	0.5 ± 0.1	—
1.0 mM P	28.3 ± 2.8	3.5 ± 0.3	63 ± 6	149 ± 8	0.3 ± 0.04	484 ± 47
Change from control	−2.0 ± 3.7	+0.1 ± 0.04	—	+54 ± 7	−0.2 ± 0.1	—
0-time control	27.4 ± 2.9	2.5 ± 0.3	—	96 ± 3	0.3 ± 0.04	—
3.0 mM P	34.4 ± 1.3	4.5 ± 0.3	90 ± 4[a]	137 ± 8	0.4 ± 0.1	479 ± 44
Change from control	+7.0 ± 4.0	+2.0 ± 0.4[a]	—	+41 ± 7	+0.1 ± 0.1	—

Values are mean ± S.E. for four sets of bones. All bones were precultured for 24 h in BGJ containing 3.0 mM P; half the bones were then taken as 0-time controls and the rest were cultured for 48 hours in medium containing ^{3}H-proline (10 μc/ml) and either 1.0 mM or 3.0 mM P.

[a] Significantly different from cultures in 1.0 mM P ($P<0.05$).

Table 6. Effect of phosphate on growth of rat long bone rudiments *in vitro*. Graded levels of phosphate

	Shafts				Cartilage ends		
	Extracted dry wt (μg)	Calcium (μg)	Phosphorus (μg)	Collagen (μg)	Extracted dry wt (μg)	Calcium (μg)	Collagen (μg)
18-day explants	23.8 ± 1.2 (9)	2.7 ± 0.2 (9)	1.1 ± 0.1 (9)	2.7 ± 0.1 (7)	82 ± 3 (9)	0.39 ± 0.2 (9)	8.2 ± 0.5 (7)
Cultured bones							
2.0 mM P	g19.0 ± 1.9 (6)	5.5 ± 0.2 (6)	2.3 ± 0.1 (6)	4.1 ± 0.7 (2)	166 ± 5 (6)	0.53 ± 0.04 (6)	20.9 ± 0.4 (2)
2.5 mM P	23.6 ± 1.6 (6)	8.3 ± 0.3 (6)[a]	3.6 ± 0.1 (6)[a]	4.3 ± 0.2 (4)	181 ± 7 (6)	0.61 ± 0.03 (6)	22.0 ± 1.8 (2)
3.0 mM P	27.1 ± 1.0 (12)[a]	9.7 ± 0.4 (12)[a]	4.6 ± 0.2 (12)[a]	5.3 ± 0.3 (8)	158 ± 5 (12)	0.55 ± 0.03 (12)	19.9 ± 1.0 (9)
3.5 mM P	32.8 ± 1.8 (12)[a]	11.5 ± 0.4 (12)[a]	5.6 ± 0.3 (12)[a]	6.3 ± 0.2 (9)[a]	154 ± 3 (12)	0.56 ± 0.03 (12)	19.8 ± 0.7 (6)
4.5 mM P	36.5 ± 1.2 (6)[a]	12.7 ± 0.3 (6)[a]	6.5 ± 0.3 (6)[a]	6.9 ± 0.3 (6)[a]	139 ± 6 (6)[a]	0.72 ± 0.02 (6)[a]	17.2 ± 1.0 (5)[a]

Values are mean ± S.E. for (n) sets on bones.

[a] Significantly different from cultures in 2.0 mM PO_4 ($P<0.05$).

Table 7. Effect of phosphate on growth of rat long bone rudiments *in vitro*. Comparison of hydroxyproline content of bones and medium

	^{3}H-Hydroxyproline (cpm $\times 10^{-2}$/culture		
	Shafts	Cartilage ends	Medium
2.0 mM P	48 ± 2	548 ± 6	289 ± 34
4.0 mM P	178 ± 67[a]	600 ± 29	360 ± 58

Values are mean ± S.E. for 4 sets of bones cultured in BGJ with 10% FRS for 3 days. ^{3}H-Proline (10 μCi/ml) was present throughout.

[a] Significantly different from cultures in 2.0 mM P ($P<0.05$).

Table 8. Growth of rat long bone rudiments *in vitro*. Effect of lowering medium calcium or magnesium concentration

	Shafts			Cartilage ends		
	Extracted dry wt (μg)	Calcium (μg)	Collagen (μg)	Extracted dry wt (μg)	Calcium (μg)	Collagen (μg)
18-day explants	22.3 ± 1.7	2.0 ± 0.1	2.8 ± 0.1	77 ± 1	0.6 ± 0.02	6.2 ± 0.04
Cultured bones						
Standard BGJ	21.8 ± 1.4	6.3 ± 0.4	3.9 ± 0.2	148 ± 4	0.8 ± 0.02	16.9 ± 0.9
Low calcium BGJ	17.0 ± 0.4[a]	3.7 ± 0.3[a]	3.0 ± 0.2[a]	134 ± 6	0.6 ± 0.01	15.4 ± 0.8
Low magnesium BGJ	23.8 ± 1.6	12.7 ± 0.9[a]	4.1 ± 0.2	121 ± 6	0.7 ± 0.02	13.6 ± 0.8[a]

Values are mean ± S.E. for 4 sets of 18-day explants or 6 sets of cultured bones. Standard BGJ contained 1 mM Ca, 3 mM P, 1 mM Mg; in low calcium BGJ, Ca was 0.5 mM; in low magnesium BGJ, Mg was 0.5 mM.

[a] Significantly different from cultures in regular BGJ ($P<0.05$).

Cartilage growth was slightly but not significantly decreased. It was not possible to study higher calcium concentration with 3 mM phosphate concentration because of precipitation of calcium and phosphate from the medium during incubation. Reduction of magnesium concentration from 1.0 to 0.5 mM resulted in increased calcification in the shaft. Cartilage dry weight and collagen content were significantly decreased.

Discussion

An ideal organ culture system for the study of skeletal growth would be one in which it was possible to test substances for their differential effects on growth *vs* resorption, mineralization *vs* matrix formation, and cartilage *vs* bone. The responses should resemble qualitatively those that occur *in vivo*, although

substantial quantitative differences might be expected in organ cultures that lack the vascular proliferation and muscular stress so important for skeletal development. The culture system described in this report fulfills some, but certainly not all, of these requirements.

In cultures of long bone rudiments explanted from 18-day rat fetuses it was possible to separate the cartilage ends from the bone shafts and study their mineralization and matrix deposition separately. Growth of the cartilage ends was well sustained in modified BGJ, our standard chemically defined medium. Cartilage growth was increased by supplementation with non-essential amino acids, albumin or serum, and decreased at low concentrations of magnesium. Since the estimates of collagen content and synthesis were based on hydroxyproline, the accumulation of partially hydroxylated collagen would not have been detected. However, hydroxylation of collagen was well maintained in this culture system, as long as ascorbic acid was added (Chen and Raisz, unpublished observations).

Compared with the cartilage ends, the changes in the bone shaft were complex and difficult to analyze. Bone formation occurred largely on the periosteal surface while endosteally both bone and calcified cartilage were being resorbed. Calcification occured in both bone and cartilage. It was not possible to analyze cartilage and bone mineral separately, but from histological appearances variables which affected mineralization appeared to affect both processes similarly.

Collagen synthesis was well sustained in the bone shaft in a chemically defined medium. Autoradiographic results indicated that most of the incorporation of proline was in periosteal cells which were synthesizing bone matrix. There was relatively little labeling of the calcifying cartilage core. Unlike the cartilage ends, the bone shaft showed large alterations in the amount and labeling of collagen under different culture conditions. Supplementation with the non-essential amino acids at a low concentration increased the amount of collagen in bone. In acute experiments with rat calvaria [8], uptake and incorporation of proline were maximal at a medium concentration of 0.15 mM (our low amino-acid supplement gave a proline concentration of 0.17 mM), and it was suggested that proline supply might be rate limiting for collagen synthesis.

The effects of albumin and serum on bone growth were complex. Others have found that bone collagen synthesis could occur without protein in the medium in organ culture [14, 17], but it was reported that mineralization of new matrix did not occur in the absence of serum [10]. In the present study the addition of albumin, adult rat serum or fetal rat serum did not enhance mineralization, but all produced an increase in the collagen content and hydroxyproline labeling of the bone rudiments. Much of the increase, however, could not be ascribed to better synthesis of normal cartilage or bone matrix, but to fibroblastic proliferation and formation of a connective tissue matrix that resembled tendon or subcutaneous tissue more than bone. Extensive fibroblastic outgrowth has been observed when bones are cultured on plasma clots in the presence of embryo extract [7].

Striking effects on bone matrix formation were obtained by changing the medium phosphate concentration. The phosphate concentration in fetal rat serum is usually 3 mM or greater. A standard phosphate concentration of 3 mM was selected for our chemically defined medium partly because it usually did not cause

precipitation of calcium-phosphate salts from the medium in the presence of 1 mM calcium at pH 7.3. Precipitation did occur at higher pH, Ca, or PO_4 concentrations. Addition of serum could prevent this precipitation.

Over the range of 1.5 to 4.5 mM increases in phosphate were associated with increased collagen content and labeled hydroxyproline incorporation in the bone shaft, as well as with increased mineralization. It seems likely that phosphate directly enhanced the synthesis of bone collagen. Alternative explanations include:

1) an increase in labeled proline uptake or a decrease in the nonlabeled proline precursor pool, which seems unlikely since phosphate enhanced collagen growth at tracer, physiological and high concentrations of proline;

2) inhibition of resorption, which would not readily explain the changes observed above 2 mM, where the phosphate effect on resorption tends to level out [4, 25] and would not explain the increase in medium hydroxyproline with high phosphate; 3) a technical artefact which resulted because an increased proportion of cartilage collagen was dissected with the bone shaft as its mineralization increased, which could only explain those experimental results in which an increase in shaft collagen was accompanied by a decrease in cartilage collagen. In most experiments collagen content and labeled hydroxyproline in the cartilage ends remained constant or increased.

The phosphate effect is specific to the extent that cartilage was less affected than bone. When there was increased collagen in cartilage, this could be ascribed to increased connective tissue on the surface of the cartilage rather than any increase in the cartilage matrix itself. The collagen of bone and connective tissue differs from cartilage collagen in chemical composition [15–17]: bone collagen molecules contain two α_1 chains and one α_2 chain, whereas cartilage matrix contains a molecule made up of three α_1 chains of a different type. We have recently observed Raisz, McGoodwin and Martin, unpublished observations) that the ratio of α_2 to α_1 chains synthesized in culture is much higher in the bone shafts than in the cartilage ends, and that increasing the phosphate concentration further increases the proportion of the chains.

The mechanism of the phosphate effect is unknown. Since phosphate is used in so many steps in cell metabolism, it seems unlikely that only one process is affected in bone. Noncollagenous protein synthesis may be enhanced since there is a small increase in tryptophan incorporation in high phosphate medium (Bingham and Raisz, unpublished observation), and there is no tryptophan in collagen. Since the phosphate concentration in fetal rats is at the upper end of the range we have studied *in vitro*, the changes could represent the effects of phosphate depletion. There is striking impairment of bone growth during severe phosphate depletion *in vivo* [1]. Mineralization was also impaired, and it is possible that decreased matrix synthesis was secondary to decreased mineral deposition. This seems an unlikely explanation for our experiments with fetal rat serum, in which high phosphate concentrations resulted in greater accumulation of unmineralized fibroblastic connective tissue. Phosphate may be important in physiologic regulation of growth. There is a general correlation between body growth and phosphate concentration in mammals [26, 36]. Growth hormone

increases phosphate concentration and this could mediate its effects on bone. High phosphate concentrations also occur in azotemic renal osteodystrophy in which osteosclerosis and increased bone formation may occur [22].

In the present study, no attempt was made to quantitate cartilage and bone resorption. Bone resorption can be affected by albumin, serum and phosphate [4, 25, 33, 34]. Resorption of bone and cartilage is accomplished by the same type of cell which is called an osteoclast or chondroclast depending on its location. In cartilage there is another type of breakdown which involves specific loss of protein polysaccharide complexes and may be important in mineralization [18]. This could explain the decrease in extracted dry weight accompanied by increased collagen content and calcification in our cultures. The role of phosphate in this process is unknown.

Most effects on mineralization in the present study can probably by explained by changes in the calcium-phosphate activity product or formation constant [35]. In organ culture it is difficult to distinguish between physiologic and abnormal mineralization. In chick bone [27] calcification was increased when the bone was split or killed, and it was postulated that the cells normally limit calcification. However, the increase in mineralization with increasing phosphate in our cultures was associated with better cell preservation. The decrease in mineralization at a high concentration of albumin could have been due to decreased calcium ion activity or to inhibition of crystal nucleation of calcium-phosphate salts. The increase in calcification at low magnesium concentration could have been due to a lowering of the formation constant for calcium phosphate salts. Although we have not determined the type of mineral formed, the low calcium-phosphate ratio in the bones is more consistent with deposition of amorphous calcium phosphate than of hydroxyapatite [20].

The culture system described here, while not ideal, should enable us to study hormonal and other factors influencing bone development [24]. The results support the view that the synthesis of cartilage and bone matrix and mineralization are all regulated separately and should be quantitated separately.

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References

1. Baylink, D., Wergedal, J. S., Stauffer, M.: Formation, mineralization, and resorption of bone in hypophosphatemic rats. J. clin. Invest. **50**, 2519–2530 (1971)
2. Biggers, J. D.: Cells and tissues in culture, ed. E. N. Willmer, vol. 3, p. 197. New York: Academic Press 1965
3. Biggers, J. D., Gwatkin, R. B. L., Heyner, S.: Growth of embryonic avian and mammalian tibiae on a relatively simple chemically defined medium. Exp. Cell Res. **25**, 41–58 (1961)
4. Brand, J. S., Raisz, L. G.: Effects of thyrocalcitonin and phosphate ion on the parathyroid hormone stimulated resorption of bone. Endocrinology **90**, 479–487 (1972)
5. Chen, P. S., Jr., Toribara, T. Y., Warner, H.: Microdetermination of phosphorus. Analyt. Chem. **28**, 1756–1758 (1956)

6. Cheng, P. H.: An improved method for the assay of hydroxyproline in rat skin. J. invest. Derm. **53**, 112–115 (1969)
7. Fell, H. B.: Biochemistry and physiology of bone, ed. G. H. Bourne, p. 402. New York: Academic Press 1956
8. Finerman, G. A. M., Downing, S., Rosenberg, L. E.: Amino acid transport in bone. II. Regulation of collagen synthesis by perturbation of proline transport. Biochim. biophys. Acta (Amst.) **135**, 1008–1015 (1967)
9. Gardner, D. T., Hall, T. A.: Electron-microprobe analysis of sites of silver deposition in avian bone stained by the v. Kóssa technique. J. Path. **98**, 105–109 (1969)
10. Goldhaber, P.: Remodeling of bone in tissue culture. J. dent. Res. **45**, 490–499 (1969)
11. Ito, Y., Endo, H., Enomoto, H., Wakabayashi, K., Takamura, K.: Ossification in tissue culture: II. Chemical development of the femur of chick embryo in various liquid media. Exp. Cell Res. **31**, 119–127 (1963)
12. Jeffrey, J. J., Martin, G. R.: The role of ascorbic acid in the biosynthesis of collagen: I. Ascorbic acid requirement by embryonic chick tibia in tissue culture. Biochim. biophys. Acta (Amst.) **121**, 269–280 (1966)
13. Juva, K., Prockop, D. J.: Modified procedure for the assay of H^3- or C^{14}-labeled hydroxyproline. Analyt. Biochem. **15**, 77–83 (1966)
14. Melcher, A. H., Hodges, G. M.: *In vitro* culture of an organ containing mixed epithelial and connective tissue on a chemically defined medium. Nature (Lond.) **219**, 301–302 (1968)
15. Miller, E. J.: Isolation and characterization of the cyanogen bromide peptides from the $\alpha 1$ (II) chain of chick cartilage collagen. Biochemistry **10**, 3030–3035 (1971)
16. Miller, E. J., Matukas, V. J.: Chick cartilage collagen of a new type of $\alpha 1$ chain not present in bone or skin of the species. Proc. nat. Acad. Sci. (Wash.) **64**, 1264–1268 (1969)
17. Past, W. L.: The uptake of FE^{59} by osseous tissue arising *in vitro*. Amer. J. Path. **45**, 873–881 (1964)
18. Pita, J. C., Cuervo, L. A., Madruga, J. E., Muller, F. J., Howell, D. S.: Evidence for a role of proteinpolysaccharides in regulation of mineral phase separation in calcifying cartilage. J. clin. Invest. **49**, 2188–2197 (1970)
19. Porter, K.: Production of bone matrix in vitro: I. Method and histologic observations. J. dent. Res. **50**, 31–40 (1971)
20. Posner, A. S.: Crystal chemistry of bone mineral. Physiol. Rev. **49**, 760–792 (1969)
21. Raisz, L. G.: Bone resorption in tissue culture: factors influencing the response to parathyroid hormone. J. clin. Invest. **44**, 103–116 (1965)
22. Raisz, L. G.: Bone formation and resorption in tissue culture; effect of agents implicated in renal osteodystrophy. Arch. intern. Med. **126**, 887–889 (1970)
23. Raisz, L. G.: The pharmacology of bone: introduction. Fed. Proc. **29**, 1176–1178 (1970)
24. Raisz, L. G., Bingham, P. B.: Effect of hormone on bone development. Ann. Rev. Pharmacol. **12**, 337–352 (1972)
25. Raisz, L. G., Niemann, I.: Effect of phosphate, calcium, and magnesium on bone resorption and hormonal response in tissue culture. Endocrinology **85**, 446–452 (1969)
26. Raisz, L. G., O'Brien, J. E., Au, W. Y. W.: Parathyroid size and uptake of alpha-aminoisobutyric acid in intact and hypophysectomized rats. Proc. Soc. exp. Biol. (N.Y.). **119**, 1048–1053 (1965)
27. Ramp, W. K., Neuman, W. F.: Some factors affecting mineralization of bone tissue in culture. Amer. J. Physiol. **220**, 270–274 (1971)
28. Ray, R. D., Mosiman, R., Schmidt, J.: Tissue-culture studies of bone. J. Bone Jt Surg. A**36**, 1147–1165 (1954)
29. Reynolds, J. J.: The effect of ascorbic acid on the growth of chick bone rudiments in chemically defined medium. Exp. Cell Res. **42**, 178–188 (1966)
30. Scharff, R., Wool, I. G.: Concentration of amino acids in rat muscle and plasma. Nature (Lond.) **202**, 603–605 (1964)
31. Schwartz, P. L., Wettenhall, R. E. H., Bornstein, J.: The growth of newborn rat tibiae in a continuous-flow organ culture system. J. exp. Zool. **168**, 517–529 (1968)

32. Shaw, J. L., Bassett, C. A. L.: An improved method for evaluating osteogenesis *in vitro*. Anat. Rec. **149**, 57–63 (1964)
33. Stern, P. H.: Albumin-induced resorption of fetal rat bone in vitro. Calcif. Tiss. Res. **7**, 67–75 (1971)
34. Stenr, P. H., Raisz, L. G.: An analysis of the role of serum in parathyroid hormone-induced bone resorption in tissue culture. Exp. Cell Res. **46**, 106–119 (1967)
35. Taves, D. R., Neuman, W. F.: Factors controlling calcification *in vitro*: fluoride and magnesium. Arch. Biochem. Biophys. **108**, 390–397 (1964)
36. Widdowson, E. M., McCance, R. A.: The effect of food and growth on the metabolism of phosphorus in the newly born. Acta paediat. (Uppsala) **48**, 383–387 (1959)