mation. In the present study, while we are transplanting leukemic cells with an unusual chromosome picture, we might also be transplanting a virus which could be the primary causal factor. To date, a number of laboratories have been unable to pass R3149 with use of cell free filtrates(8). No viral particles have as yet been seen after electron microscopic search(12). At present, we consider this leukemia to be a stable mutant and not presently viral associated. It is pertinent to note that this leukemia arose in a female rat(8). The persistence of these leukemic cells as female, even in male rats supports speculation on their origin as a stable mutation and is strong evidence against continuing viral association and multiplication.

The presence of the stable marker chromosome for this leukemia, R3149, should permit further studies of the effects of chemotherapy on this disorder. Dunning and Curtis(9) have reported that this leukemia could be cured by chemotherapy with nitrogen mustard and other related compounds. The effect of such therapy and other agents on the incidence and fate of this marker chromosome will be of interest.

Summary. This report describes a stable marker chromosome for the transplantable Dunning rat leukemia R3149 in Fischer rats. The karyotype of this acute leukemia was female and pseudoeuploid with a giant submetacentric marker chromosome.

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Amorphous-Crystalline Mineral Changes During Endochondral and Periosteal Bone Formation.* (31999)

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The inorganic portion of skeletal tissue has been shown to contain two separate mineral components, amorphous (non-crystalline) calcium phosphate and crystalline apatite(1-4). Bone from young animals, in general, was found to be richer in amorphous mineral than in crystalline apatite, while bone from older animals was always found to contain more calcium phosphate in the crystalline rather than the amorphous state(3,4). These recent quantitative findings are compatible with the earlier electron microscopy studies suggesting that non-crystalline mineral particles may be deposited prior to apatite crystallites during early bone calcification(5-8).

Synthetic, non-crystalline calcium phosphate can be prepared as either a stable(9,10) or a metastable(11,12) salt. The metastable form of amorphous calcium phosphate will spontaneously convert in aqueous media into crystalline apatite *via* dissolution and reprecipitation by means of an autocatalytic, kinetic mechanism(12). Since the water lability be-

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havior of bone mineral *in vitro* indicates that a metastable amorphous calcium phosphate solid is present in skeletal tissue(4), it is possible that this non-crystalline mineral phase may act as a kinetic precursor of crystalline apatite *in vivo* as well as in synthetic systems. On this basis, a series of tissue samples representing progressive stages of endochondral and periosteal bone formation were assayed for both amorphous and apatitic (crystalline) calcium phosphate. This was done in order to determine the histological sequence of mineral deposition throughout the entire calcification process.

Materials. Tissue zones representing progressive stages of endochondral calcification were dissected from the epiphyses, while tissue zones, representative of progressive periosteal calcification were dissected from the diaphyses of foetal calf leg bones. Evaluation of the purity of each tissue zone was based upon histological examination of both the dissected and the remaining undissected tissues at each stage of the dissection process. The tissue zones obtained by this technique were designated as resting cartilage (RC), proliferating cartilage (PC), hypertrophic-proliferating cartilage (HPC), hypertrophic-calcified cartilage (HCC), calcified cartilage (CC), primary spongiosa (PS), cancellous bone (CanB), periosteal bone (PB) and compact bone (ComB). The resting cartilage zone was typical embryonic hyaline cartilage free from either perichondral connective tissue or columnar cartilage. The proliferating cartilage zone consisted of differentiated columnar cartilage, while the HPC, HCC and CC zones represented successive layers of mineralizing columnar cartilage for which no clear cut line of demarcation could be made. The calcified cartilage zone was almost completely free of bone tissue and represents the end point of cartilaginous calcification.

The primary spongiosa sample (PS) was found to be a mixture of calcified cartilage and newly formed trabecular bone. Histological examination of this tissue zone indicated that it consisted of approximately one-third cartilage and two-thirds bone tissue. The cancellous bone sample (CanB) was found to be essentially pure trabecular bone with very little (less than 5%) cartilaginous tissue present. The periosteal bone sample (PB) was scraped from the surface of the foetal calf femoral diaphysis after removal of the periosteum and represents newly formed diaphyseal bone tissue. The harder compact bone sample (ComB) lay beneath the periosteal bone tissue zone and differed from adult haversian compact bone in that it was highly vascular and was composed of dense lamellae. After dissection, each sample was immediately lyophilized and subsequently ground to 200 mesh in a colloid mill (Spex Industries) prior to analysis.

Methods. Percent ash was determined on each lyophilized sample after heating at 600°C for 48 hours. Calcium content was measured by chemical procedures given elsewhere(11), while inorganic phosphate (P_i) was determined by the method of Martin and Doty (13) after treating the tissue with 1 N HCl for 30 minutes at 25°C.

The weight fractions of crystalline apatite and amorphous calcium phosphate contained in each lyophilized tissue zone were measured using an x-ray diffraction technique described elsewhere(2,4). Because of the possible error due to the high background scatter of x-rays by protein constituents, tissue zones with very low ash content (RC, PC and HPC) were refluxed in a Soxhlet apparatus for 6 hours with anhydrous ethylene-diamine to enrich their residual ash contents to values greater than 35%, at which level the error due to background x-ray scatter has been shown to be minimal(4). After refluxing, the enriched samples were vacuum-dried at 100°C and then analyzed as usual by x-ray diffraction to determine their amorphous and crystalline mineral composition. It has been demonstrated previously that this ethylenediamine treatment does not affect the nature of the mineral phase in either synthetically prepared calcium phosphates or bone mineral(2,4,14).

X-ray absorption effects arising from tissue water and organic constituents are taken into account routinely in this quantitative x-ray diffraction analysis of the weight fractions of the amorphous and crystalline phases in bone (2). Absorption of x-ray by inorganic ions other than calcium and phosphate (*e.g.*, sodium, potassium, or chloride) becomes significant for samples with low calcium phosphate

TABLE I. Nature of the Mineral Phase During Progressive Stages of Early Bone Formation in the Foetal Calf.

	Percentage of lyophilized tissue weight					
	Total ash[a]	Total calcium phosphate[b]*	Crystalline apatite[c]	Amorphous calcium phosphate[c]†	β_{002}[d]	Molar Ca/P_i[e]
Resting cartilage	7.6	<0.5	—	—		2.78
Proliferating cartilage	13.4	2.1	1–2	0–1	.650	1.61
Hypertrophic-proliferating cartilage	21.3	11.4	5.7	5.7	.660	1.48
Hypertrophic-calcified cartilage	48.7	43.1	34.5	8.6	.750	1.52
Calcified cartilage‡	69.4	65.9	44.8	21.1	.630	1.56
Primary spongiosa‡	63.2	59.3	38.0	21.3	.665	1.60
Primary spongiosa (calculated as osteoblastic deposition)	60.1	55.9	34.1	21.8	.682	1.61
Cancellous bone‡	65.3	61.3	36.3	25.0	.605	1.62
Periosteal bone	34.7	30.1	17.3	12.8	.540	1.64
Compact bone	64.7	59.7	40.0	19.7	.570	1.63

Relative standard error of reported values: [a] ± 1.0%; [b] ± 1.5%; [c] ± 2.5%; [d] ± 4.0%; [e] ± 1.2%.

* This column represents the sum of each tissue zone's calcium and inorganic phosphate contents.

† These values were calculated on the basis of each tissue zone's total calcium phosphate content.

‡ These tissues were washed free of blood and marrow prior to lyophilization.

contents. Corrections for this effect, utilizing the electrolyte composition of the tissues in question were made for both the high and low ash-content samples, even though these corrections were found to be trivial for samples having high calcium phosphate contents.

For each sample, a function of the average apatite crystal size in the *c*-axis direction was followed by measuring the width at half maximum intensity (in degrees, 2θ) of the apatite 002 reflection, which occurs at approximately 26° 2θ. Suitable corrections were made during this measurement for possible errors due to low ash content(4). This measurement, designated by the term, β_{002}, is inversely proportional to average apatite crystal size(15).

Results. The structural and chemical properties of the mineral phase present in the samples described above are given in Table I. As can be seen, both amorphous calcium phosphate and crystalline apatite were present in all tissue zones containing appreciable mineral deposits. Resting cartilage contained less than 0.5% calcium phosphate, as could be expected since this tissue does not ordinarily calcify. The proliferating cartilage zone contained only 2.1% calcium phosphate and although apatite diffraction peaks appeared in its x-ray diffraction pattern, it was impossible to determine the proportion of its mineral that was amorphous or crystalline because of this very low calcium phosphate content.

The largest incremental increase in the deposition of mineral in cartilage occurred in going from the HPC to the HCC zone. The new mineral formed in the HCC zone was mostly apatite of a very small crystal size, as shown by the large β_{002} value, with little increase in the amorphous calcium phosphate phase occurring at this point. In the CC zone, considerable formation of calcium phosphate also occurred with the deposition of slightly more amorphous than crystalline mineral. In addition, the average size of the apatite crystallites increased from the HCC to the CC zones, as evidenced by the marked decrease in the β_{002} value. The molar Ca/P_i ratios of the newly calcifying cartilage zones ranged from 1.50 to 1.55. Since there appeared to be no direct correlation between amorphous calcium phosphate content and Ca/P_i ratio, it may be possible that the first

TABLE II. Percentages of Crystalline Apatite and Amorphous Calcium Phosphate in Total Mineral Deposited During Early Bone Formation in the Foetal Calf.

	Tissue zone	% Crystalline apatite*	% Amorphous calcium phosphate*
A.	Hypertrophic-proliferating cartilage	50	50
	Hypertrophic-calcified cartilage	80	20
	Calcified cartilage	68	32
B.	Cancellous bone	59	41
C.	Periosteal bone	57	43
	Compact bone	67	33

* Relative standard error of reported values = ± 2.5%.

apatite crystals formed during cartilaginous calcification are calcium deficient since a ratio of 1.67 would be expected for perfect apatite.

The primary spongiosa (PS) zone was a mixture of calcified cartilage and newly formed cancellous bone. Assuming that the calcified cartilage present in the PS zone has a composition similar to that observed for the CC zone and that the Ca/P_i ratio of the newly forming bone is not higher than that in the subsequent CanB zone, it is possible to calculate the proportion of the PS zone that arises from osteoblastic deposition. The tissue proportions calculated were 65-70% new bone and 30-35% calcified cartilage, values which agree quite well with histological examination. Calculations based on these proportions indicate that the newly formed bone tissue present in the PS zone was already well calcified (60% ash) and that its non-crystalline calcium phosphate content was similar to that found in the subsequent CanB zone. However, the average crystal size of the apatite present in the newly formed bone of the PS zone was much smaller than that found for the CanB zone, as judged from β_{002} values.

Table II expresses the amorphous and crystalline calcium phosphate data listed in Table I as percentages of the total calcium phosphate in selected heavily mineralized tissue zones which were representative of the 3 calcification areas examined in this study, *i.e.*, calcification of cartilage (A), deposition of cancellous bone (B) and periosteal deposition of compact bone (C). The osteoblastic deposition within the PS zone was omitted from this Table, since its amorphous-crystalline mineral composition was almost identical with that observed for the CanB zone. The initial mineral deposits in both the calcification of cartilage and the periosteal deposition of compact bone had the largest proportion of amorphous calcium phosphate. This pattern was not exhibited in the deposition of cancellous bone, but this was probably due to the fact that the bone tissue formed in the PS zone was almost as highly calcified as that formed subsequently in the CanB zone. However, the end product in all 3 calcification areas was a mineral with a high percentage of crystalline apatite. It is also interesting to note that the compact bone had a higher percentage of crystalline apatite than did the cancellous bone.

Discussion. The synthesis of crystalline apatite *in vitro* appears to be intermediated by non-crystalline calcium phosphate(11). The first solid precipitated from solution under these conditions is the metastable form of amorphous calcium phosphate and, in the presence of seed apatite crystals, this non-crystalline mineral precipitate rapidly converts into crystalline apatite by an autocatalytic mechanism involving dissolution of the amorphous phase followed by its reprecipitation as small-sized apatite crystals(12). The seed apatite crystal surfaces act as nucleating agents for the formation of new apatite crystals(12) and, if no seed crystals are present originally, there is a lag time in this conversion because seed apatite crystals must be synthesized *de novo*(11). After all of the amorphous precursor is converted to apatite, the crystals increase in size by normal physical-chemical growth mechanisms(11,12).

The mineralization of cartilage can be interpreted on the basis of this *in vitro* system. It has been shown that more than half of the amorphous calcium phosphate contained in

the 3-day-old rat femur, which is principally cartilaginous, can be converted to crystalline apatite by *in vitro* exposure to water(4). Thus, it is probable that a large part of the amorphous calcium phosphate deposited within the HPC, HCC and CC tissue zones is kinetically metastable. Table I shows an increased mineralization in going from the HPC to the HCC zone. The appearance of smaller apatite crystals and the high percentage of crystalline apatite in the HCC zone, as compared to earlier zones, suggest an increased rate of transformation of amorphous to crystalline mineral in this tissue zone. Reasoning from synthetic systems(11,12), it is possible that the apatite crystals in the PC and HPC zones act as seed crystals, which may account for this increase in apatite formation rate. Similarly, the crystal size of the apatite in the CC zone appears to indicate a slower apatite formation rate at the expense of increased crystal growth which might be related in some way to the local mineralization conditions. It then follows that the large increase in amorphous calcium phosphate content in going from the HCC to the CC tissue zone is probably related to a new cycle of heavy mineral deposition with a decreased rate of transformation of the amorphous to the crystalline phase.

Cartilaginous calcification would thus appear to involve the initial precipitation of metastable amorphous calcium phosphate from supersaturated solution followed by a rapid conversion to crystalline apatite mediated by seed apatite crystals. At any given stage of the calcification process, the apatite crystals formed could then grow to larger sizes. Concomitant with the mineral changes, numerous other alterations also take place within cartilaginous tissue during endochondral calcification. Changes in the cell metabolism(16), tissue electrolytes(17) and extracellular matrix composition(17-20) of columnar cartilage occur simultaneously with mineral deposition during endochondral calcification. Therefore, the basic mechanisms of endochondral calcification must encompass all of these factors.

Although the ash and the crystalline apatite content of the newly formed bone tissue in the primary spongiosa was high, its average apatite crystal size was significantly smaller than that observed for the CanB tissue zone. Moreover, the younger periosteal bone PB had a higher weight fraction of amorphous calcium phosphate than did the older compact bone ComB beneath it. This latter evidence is in agreement with the fact that 3-day-old rat calvaria contain less crystalline apatite and more amorphous calcium phosphate than do more heavily mineralized 15-day-old rat calvaria(4). Therefore, it is probable that calcification within bone tissue is also analogous to the *in vitro* system described earlier(11,12). Since amorphous calcium phosphate appears to predominate over crystalline apatite in the early stages of intermembranous bone formation, this non-crystalline mineral phase is most likely the first mineral that is deposited during bone calcification. Since crystalline apatite appears to predominate over amorphous calcium phosphate in the later stages of bone formation, and, since almost two-thirds of the amorphous calcium phosphate content of mature bone mineral can be converted to crystalline bone apatite by *in vitro* exposure to water(4), this non-crystalline bone mineral phase is probably a metastable precursor of crystalline bone apatite. The persistence of labile amorphous calcium phosphate in mature bone tissue(2-4) could be explained by stabilizing influences present within the tissue and/or by a localized absence of aqueous fluid which is necessary for its conversion to crystalline apatite within synthetic systems(11,12).

In addition to a possible role in bone formation, the labile amorphous calcium phosphate of skeletal tissue could be important to mineral metabolism as well. Since metastable amorphous calcium phosphate converts to form crystalline apatite *via* dissolution and reprecipitation, it could just as easily provide mineral ions to metabolism by dissolution without reprecipitation. In terms of physiological, metabolic activity then, the non-crystalline portion of bone mineral might possibly be of significance to calcium homeostasis. Moreover, the chemical and physical properties of amorphous calcium phosphate are different from those of crystalline apatite

1,4,11,12) and interpretations made on data taken from whole bone mineral should take these differences into account.

Summary. Quantitative x-ray diffraction analyses of histological tissue zones representing progressive stages of endochondral and periosteal bone formation reveal that both amorphous and apatitic calcium phosphate salts appear throughout the entire mineralization process. The amorphous-crystalline mineral sequence exhibited by these tissue zones suggests that calcification in both cartilage and bone may entail an initial deposition of amorphous calcium phosphate followed by the conversion of this kinetically metastable, non-crystalline mineral phase to crystalline apatite.

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Hypoxic Decompression and Fat Embolism.* (32000)

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Although known to occur commonly following skeletal fractures, pulmonary fat emboli have also been observed in the absence of overt trauma(1,2,3). Such a situation exists in fatal subatmospheric decompression sickness, in which fat has frequently been found in the pulmonary and systemic vessels (1). The mechanism of fat embolization during decompression, and the source of the fat are not clear. Animal experiments attempting to relate subatmospheric decompression to fat embolization have been few and inconclusive(4,5).

This study was performed to determine if pulmonary fat emboli could be produced by hypoxic subatmospheric decompression. In an attempt to facilitate fat embolization, cholesterol hyperlipemia and ethionine fatty liver were induced prior to decompression. The results indicate that none of the factors tested predisposed to fat embolization in the presence of hypoxic decompression. Nor was decompression alone associated with fat emboli.

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