Partition of Calcium, Phosphate, and Protein in the Fluid Phase Aspirated at Calcifying Sites in Epiphyseal Cartilage

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ABSTRACT A reproducible method, adapted from renal micropuncture techniques, was developed for sampling 10–40 mμl of a clear fluid from epiphyseal cartilage of normal or rachitic rats in vivo, either from the hypertrophic cell zone (C_{fl}) or surface resting cell cartilage (L_{fl}).

Characterization of this fluid depended upon quantitation of protein, total inorganic phosphate (P_{i_t}), total calcium (Ca_t), nucleotide, and hemoglobin in volumes of 20 mμl. Established methods for macroscale measurements of each of these parameters have been modified to permit direct spectrophotometric readings on samples of 10^{-10}–10^{-11} g.

The fluid from hypertrophic and peripheral resting cell cartilage was of an extracellular nature as evidenced by a high chloride and sodium, as well as low potassium, protein, and nucleotide content.

The pH of fluid isolated from endochrondral plates in vivo was measured under oil as a function of P_{CO_2} and the computed bicarbonate was elevated above concurrent serum levels.

After ultracentrifugation of C_{fl} of normal, rachitic, and healing rachitic animals, non–protein-bound calcium (Ca_f) and phosphate (P_{i_f}) were determined on supernatant fluids. The hypertrophic cell cartilage fluid of rachitic rats was distinguished by a high ratio C_{fl}/serum of P_{i_f}. This ratio returned to normal during treatment of rickets. The upper limit for ionic activity A[1] $Ca^{++} \times A\ HPO_4^{=}$ was too low to initiate precipitation of brushite or dicalcium phosphate but was in a range of supersaturation in respect to crystalline apatites. Thus these data are consistent with initiation of calcification by heterogeneous nucleation of mineral in the septal matrix but can be reconciled alternately with a precipitation mechanism only if the site of initial mineral phase separation is outside the septal matrix.

INTRODUCTION

Theories on mechanisms of normal endochondral calcification and dystrophic calcification of cartilage in man have been developed from studies of a variety of biological as well as physiochemical systems. Considerable information has become available concerning possible pathways whereby calcium and phosphate in the fluid phase of cartilage might be initially translocated into a solid phase. The initiating event in such calcification has been postulated by various investigators to result from increased concentration of the relevant ions (1), removal of shielding compounds from nucleation sites (2), destruction of circulating inhibitors (3), or synthesis of new nucleation sites (4). Combinations of one or more of these processes are also possible (5). Direct proof for any of these postulates has been

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[1] The symbol A has been used throughout this paper to represent ionic activity which is frequently denoted by a.

difficult, partly because in the assessment of hard tissue metabolism both in vivo and in vitro parameters measured by most classic techniques must reflect the average function of a large population of cells with diverse biological roles; among these cells only a portion is concerned with the process of calcification.

In the current report it is demonstrated that a distinctive fluid with extracellular characteristics can be aspirated in the amount of 10–40 mμl from the hypertrophic zone of the upper tibial epiphyseal cartilage within or near a calcifying site or normally growing, rachitic, as well as healing rachitic rats in vivo. The latter preparations permitted testing the fluid phase during conversion of an essentially uncalcified into a calcified matrix. Evidence is presented that this fluid phase is altered in certain respects favorable to calcification.

METHODS

Animal preparations. For the normal control group, weanling rats of the Holtzman strain were placed, at the age of 3 wk, on normal rat food for 3 wk. Other animals designated for control and treated rachitic groups received USP rachitogenic diet No. 12[2] for 21–26 days. All animals were sacrificed at age 42–47 days. The Ca/P ratio was analyzed in duplicate for each batch of rachitogenic feed and averaged 5.88 with 1.2% calcium content. The normal feed, Purina laboratory chow, contained 1.3% calcium with a Ca/P ratio of 1.38 and 5.3 U of vitamin D per g dry weight. Precautions as to darkness and separation of animals were taken as described by Bills (6). Rachitic animals for which experimental data are presented were considered to have severe vitamin D deficiency as displayed by: (*a*) steady slow gain of total body weight during induction of rickets; (*b*) low serum inorganic phosphate; and (*c*) absence of gross mineral aggregates as detected by von Kossa stains in the epiphyseal plate cartilage or proximal metaphysis (Table III).

Rachitic animals received various regimens in an attempt to demonstrate a precipitation mechanism for mineral phase separation in the cartilage septa during healing of rickets. Rapid focal healing was induced by intraperitoneal administration of sodium phosphate (7). Rapid focal healing was registered in animals (Table III) who received 0.1 mmole of disodium phosphate in 1 ml of saline intraperitoneally 24 hr before being sacrificed (7). Rapid diffuse healing was induced by a program of either starvation (7, 8) or administration of one dose of Vitamin D_2, 1500 U, together with a normal diet during the 24 or 48 hr period before being sacrificed. Slow or incomplete healing was induced by a daily dose of 0.1 U of vitamin D_2 and continuation of the rachitogenic diet (6).

[2] General Biochemicals, Chagrin Falls, Ohio.

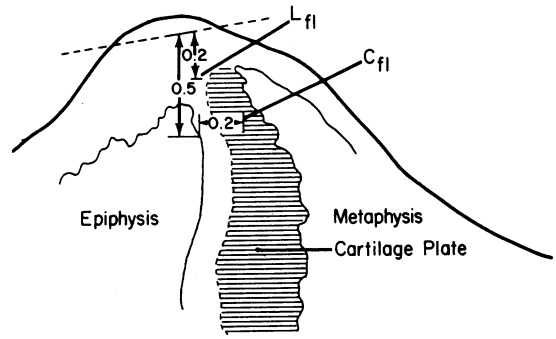

FIGURE 1 Diagram of sagittal section of upper tibial epiphyseal plate, showing sites of collection for the hypertrophic cell fluid (C_{fl}) and lymph fluid (L_{fl}).

For all administrations mentioned, vitamin D_2 was emulsified in distilled water and the specified dose combined in 0.2 ml was administered by an oral tube.

Sampling of cartilage fluid. Rats were prepared and anesthetized with Nembutal as conventionally employed for renal micropuncture. The rats were placed supine in a plastic chamber and the left knee clamped in 30° flexion and bathed in oil kept at 37°C. For some experiments, the oil was equilibrated with 5% CO_2 and 95% nitrogen. After a small skin incision adjacent to the infrapatellar ligament, the perichondrium was displaced by blunt dissection and the epiphyseal cartilage plate exposed with almost no bleeding. Marrow, metaphysis, epiphyseal plate, and epiphysis were clearly identified under a magnification of × 50 with a dissecting microscope. Pyrex capillary tubing, 0.8 mm O.D. and 0.4 mm I.D., was drawn into micropipettes with 10–15 μ tips. After sharpening these micropipettes we mounted them on a micromanipulator[3] and filled them with dust-free Paraffin White Oil, USP (Saybolt 340–355). An aspirator with fine control, developed in this laboratory, was employed although other conventional aspiratory systems could be used. Micropipettes, coated with silicone, were calibrated by: (*a*) placement of a small plastic mark on the external surface; (*b*) aspiration of a solution of 30% *p*-nitrophenol to the mark followed by dilution to 20 μl in 0.10 M NaOH; (*c*) measurement of absorbance at 400 mμ in the ultramicrocuvette (see below); and (*d*) calculation of volume from a standard curve (9). The micropipette was carefully placed for collection of fluid from the distal hypertrophic cell cartilage septa (C_{fl}) in the juxtametaphyseal margin of the cartilage plate identified clearly as a silver-grey structure with the tip 0.5 mm below the tissue surface and 2.0 mm lateral to the medial border of the tibial plateau (Fig. 1). Negative pressures of approximately 100–300 mm Hg were applied for 10–30 min; thereby a sample of clear fluid, 10–40 mμl, was routinely obtained and delivered under oil to a micro concavity slide.[4] With the micromanipulator and holder in the same position used for aspiration, a micropipette containing 7% trichloroacetic acid, 10% Car-

[3] Laboratory Associates, Inc., Belmont, Mass.

[4] Clay Adams A-1475.

bowax, and 5% ferric chloride in water was replaced to approximately the same position in the cartilage. About 50 mμl of this mixture was injected as a marker. Serial histological sections were then made through the cartilage with cryostat microtome sections cut at 10 μ thickness. These sections were subjected to a Prussian blue reaction for iron and counterstained with phloxine (Fig. 2).

After each micropuncture aspiration, a blood sample was taken from the aorta and the animal was sacrificed by a blow on the head. Part of each contralateral knee for all groups of animals was fixed in formalin, embedded in paraffin, sectioned at 10 μ thickness, and stained with 0.1% toluidine blue and by the von Kossa method, as previously described (10). Degree of calcification of cartilage upper tibial epiphyses (Table III) was assessed according to Bills on the von Kossa stained sections (6).

Biochemical methods. Total calcium (Ca_t) present in serum, as well as calcifying solutions, was measured by the method of Clark and Collip (11) and total (acid-soluble) inorganic phosphate (P_{i_t}) in the same samples was measured by the method of Kuttner and Cohen (12) as modified by Polley (13). Chloride was measured by the electrometric method of Ramsay, Brown, and Croghan on serum, as well as cartilage fluid (14). Other methods, described below, depended upon the use of an ultramicrocuvette with a total capacity of 2–8 μl (9). This cuvette was oriented precisely in the Zeiss PMQ-2 spectrophotometer by a simple positioner and holder. The optical properties of this cuvette in the ultramicrocalcium method have been reported previously by the authors (9).

The Ca_t in diluted microscopic fluid samples was determined by precipitating it as the oxalate from a solution of tetrabutylammonium oxalate. The calcium oxalate was then reacted with chlorophosphonazo III and the resulting colored compound was analyzed spectrophotometrically (9, 15).

For measurement of P_{i_t} in cartilage fluid, a 15–20 mμl sample was diluted to 0.5 μl with CO_2-free demineralized water. Further dilution with 15% trichloroacetic acid to 2 μl, centrifugation at 10,000 *g* for 10 min at 5°C, yielded the total acid-soluble phosphate; 9.5 μl of 0.25% ammonium molybdate was added in a chamber of 20 μl capacity. Finally, 0.5 μl of 0.12% aqueous stannous chloride was added, mixed, and after 5 min of color development the readings were made at 660 mμ in the ultramicrocuvette, with Corex glass end-windows.[5,6] The same cuvette was employed in testing blank, standard, and sample with exact orientation in the positioner. Nucleotides were determined from the ratio of absorbance at 260 and 280 mμ with quartz glass end-windows in the ultramicrocuvette (16).[4] After a 6 μl dilution of the initial sample, 2 μl was used for the total protein determination by the method of Lowry, Rosebrough, Farr, and Randall (17).[4] Range of recoveries of protein, chloride, calcium, and phosphate added to reconstituted serum (Clinical Chemistry Control Serum #450–30)[7] was 95–103% (Table I). Hemoglobin was quantitated in C_{fl} by a standard method (18).[3] Samples were diluted with 10% Na_2CO_3 and read at 560 mμ. Measurements were made in the ultramicrocuvette; Beer's law was obeyed with the use of standards in the range of 50–600 mg/100 ml of hemoglobin in 0.9% saline.

TABLE I

Recovery Values for Ultramicro Methods Applied to Reconstituted Serum Standards

Determination	Range of recovery values	Concentration in starting samples	n*
P_{i_t}‡	98.4–101	1, 3, 6, 9, and 12 *mg/100 ml*	20
Chloride	97.6–101	85, 100, 110, and 120 *mEq/liter*	16
Protein	95.0–103	4, 6, and 8 *g/100 ml*	12
Ca_t‡	96.1–102	3, 6, 9, and 12 *mg/liter*	18

* No. of complete determinations on 20 mμl starting samples.

‡ P_{i_t}, total inorganic phosphate; Ca_t, total calcium.

We measured pH in ultramicro samples of heparinized blood and C_{fl}, using Corning 015 pH glass capillary tubing drawn into ultramicropipettes. These were sealed to form exploring electrodes with tip diameter of 15 μ (19). Pyrex glass capillaries were similarly prepared for reference electrodes. Voltage differences were read in a Cary vibrating reed electrometer (Applied Physics Corporation, Monrovia, Calif.) provided with a bucking potentiometer. Only electrodes with performance $\rightleftharpoons$ 50 mv/pH U were used. Electrodes were tested in standard buffers at 37°C, $\mu = 0.16$. The buffers, which contained 3% hide powder collagen and 2% protein chondroitin sulfate, registered on a model 76 Beckman pH meter, with an expanded scale, the following pH levels: 6.40, 6.71, 7.10, 7.75, 8.20, and 8.50. The standard deviation from the same mean values measured with the microelectrodes ranged from ±0.005 to ±0.010. After collection and transfer under a layer of oil, samples were equilibrated with CO_2 and nitrogen in a constant temperature bath at 37°. The gas mixture was adjusted to a constant CO_2 tension by a Godart mixing pump. The actual CO_2 content delivered was monitored by an infrared analyzer. pH readings on microscopic samples were made at time 0, 10, 20, 30, and 40 min. Current data represent the mean of the last two or three readings which were approximately constant. Values for observed P_{CO_2} and pH were fitted into the Henderson-Hasselbalch equation, using the CO_2 conversion factor of 0.0314 mmole/liter of H_2CO_3 per mm Hg (Table V).

Calcium and phosphate not bound to proteins or other macromolecules separable by ultracentrifugation (Ca_f and Pi_f) were measured by the method of Loken, Havel, Gordan, and Whittington (20) for human sera modified to an ultramicroscale. For the ultracentrifugations, 25–40 mμl of cartilage fluid samples were collected under CO_2-equilibrated oil and after aspiration of a bubble of 5% CO_2 and nitrogen into the tip, the end was thermally sealed. Centrifugation of the capillaries was performed in a Spinco Model L ultracentrifuge, with a special holder for the

[5] These methods were modified only slightly to accommodate ultramicro quantities of starting sample.

[6] Adenosine triphosphate and guanosine monophosphate (4×10^{-3} mole/liter) incubated at 37°C for 0.5 hr carried through this procedure registered 0–0.1 mg/100 ml of P_{i_t}.

[7] Hyland Laboratories, Los Angeles, Calif.

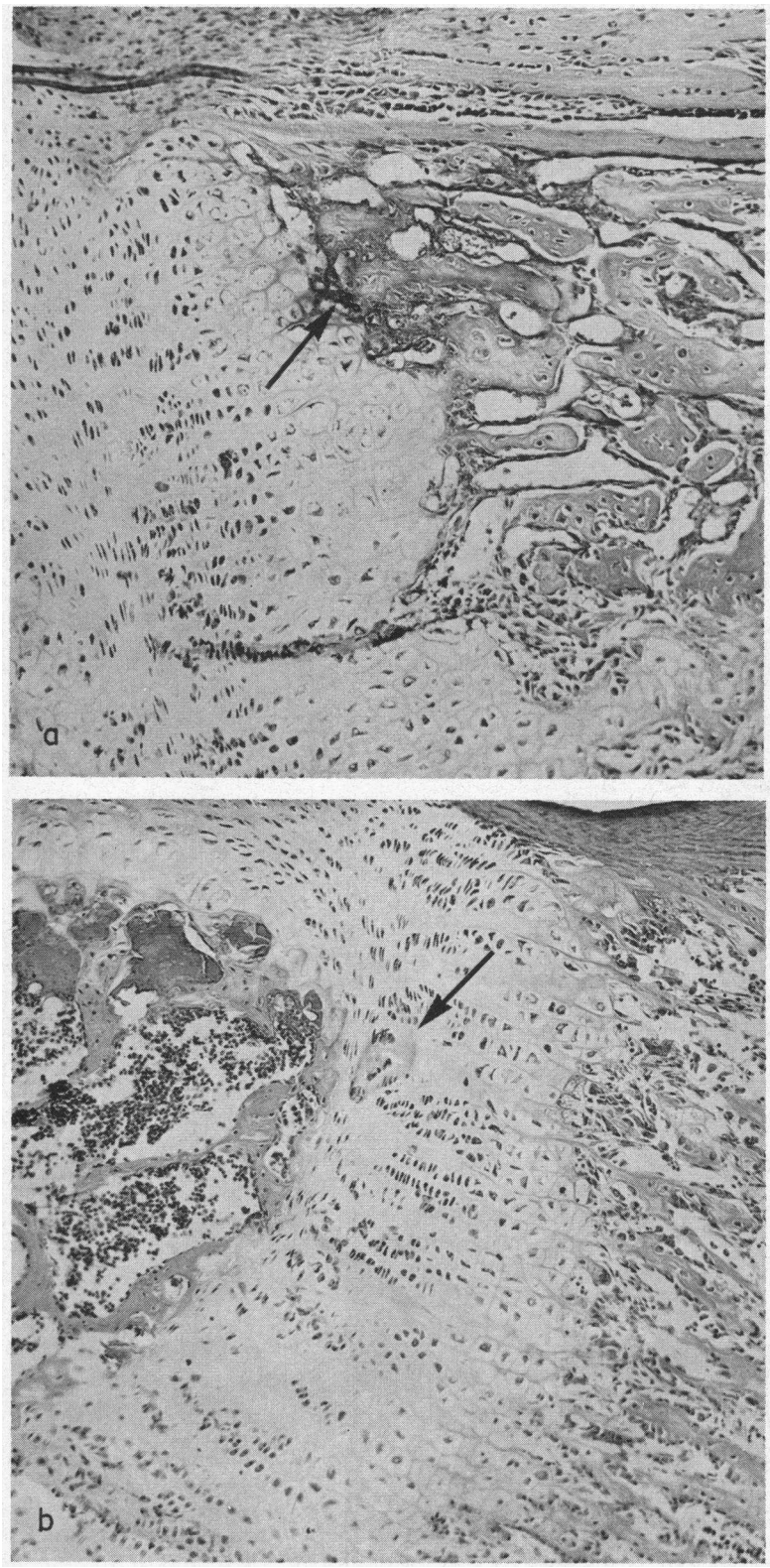

FIGURE 2 Histological section from upper tibial epiphyseal plate of a rachitic rat. Ferric chloride marker is shown at sites of micropipette tips. (*a*) Typical site of C_{fl} collection in the hypertrophic cell zone. (*b*) Micropuncture site in the resting cell zone near the cartilage surface. × 90.

capillary pipette. After ultracentrifugation, the button of sediment in the capillary tip was cut off at a level determined experimentally to include a safe margin of supernatant fluid. Analyses were made upon the remaining supernatant fluid for protein, phosphate, and pH after 4, 8, 24, and 36 hr of ultracentrifugation. Protein content in the supernatant of normal C_{fl} decreased from 0.8 ± 0.2 g/100 ml at 8 hr to <0.2 g/100 ml after 30 hr at 100,000 *g*. Decrease in supernatant concentrations of calcium and phosphate in normal C_{fl} could not be produced by increasing the time or acceleration beyond 8 hr and 130,000 *g*, respectively. Accordingly, these conditions were used for the data in Table III. Activities of calcium ($A\ Ca^{++}$) and phosphate ($A\ HPO_4^{=}$) were calculated (Table IV) according to Levinskas (21) (See appendix):

$$-\log \gamma_{Ca^{++}} = \frac{2.04\sqrt{\mu}}{1 + 2.09\sqrt{\mu}}; \quad -\log \gamma_{HPO_4^{=}} = \frac{2.04\sqrt{\mu}}{1 + 0.69\sqrt{\mu}}$$

Anatomic site of fluid origin in epiphyseal cartilage. The immediate site of collection was documented by measurements oriented in two dimensions by an ocular micrometer and by injection of a marker at or very close to the site of collection. Location of the site of collection was also facilitated by the clearly visible demarcation between metaphysis and the cartilage plate observed during collection through the dissecting microscope. C_{fl} samples were obtained with only occasional failures at a level greater than 0.5 mm deep to the cut surface (Fig. 1). Samples of lymph fluid (L_{fl}) were aspirated from peripheral resting cell and perichondrial sites less than 0.2 mm deep. C_{fl} samples from metaphyseal or epiphyseal bone or osteoid were routinely heavily contaminated with blood cells. No fluid samples were obtainable from articular or resting cell cartilage other than the special site designated in Fig. 1.

Two methodological experiments were performed to check the source of C_{fl}. The first was to determine whether the film of fluid over the cartilage surface, through which the micro capillary pipette was inserted, descended through the tract made by the pipette and contaminated the final sample. Surface fluid was labeled by flooding the surface of cartilage before micropuncture with 0.2 ml of 0.9% saline containing 5 mg of Cardio-Green/ml.[8] Oil-filled micropipettes were then submerged through the dye and aspirations made for fluid from the cartilage. These samples were diluted and read along with standards at the absorption peak for this dye (800 mμ) in the ultramicrocuvette. In samples of C_{fl} from 15 animals, dye penetrated in trace amounts indicating a contamination of cartilage fluid at a range of 1–3.5% with surface fluid, a level insufficient to interfere with these studies.

The second problem was the closeness of micropipette tips in hypertrophic cell cartilage to adjacent metaphysis during collection of cartilage fluid (Fig. 2*a*). If cartilage fluid were derived inadvertently from the latter tissue, micro-dissected cartilage separated from the metaphysis and incubated in vitro should fail to yield fluid samples. To check this point, the tibial epiphyseal plates of 19 anesthetized rats were split sagitally and one-half of the plate quickly dissected free of metaphyseal granulation, as well as adjacent cartilage tips, blotted to remove blood, and placed in a concavity slide at 37°C under oil. After 10 and 20 min, an aspiration was made for cartilage fluid. Approximately the same volume, 10–40 mμl of fluid, could be obtained from the hypertrophic cell cartilage in vitro as was aspirated in vivo. In addition, the level of P_{i_t} in rachitic C_{fl} was 1.80 ± 0.3 mmoles/liter, the same level as obtained at this site in vivo in comparison to 0.70 ± 0.4 mmole/liter in L_{fl} from similar animals. These findings demonstrated the fluid C_{fl} is derived from the hypertrophic cell cartilage and not adventitiously from surrounding tissues.

RESULTS

Data in Table II are presented to indicate that C_{fl} from rachitic and normal animals is not of cellular origin and is distinctive both from serum and perichondrial lymph (L_{fl}) (all data are expressed as the mean ± standard deviation). Values for

[8] Hynson, Westcott and Dunning, Inc., Baltimore, Md.

TABLE II
*Extracellular Nature of C_{fl}**

	Normal				Rachitic			
	No. of rats	Serum	L_{fl}	C_{fl}	No. of rats	Serum	L_{fl}	C_{fl}
Chloride, *mEq/liter*	8	102.0±2.1‡	103.0±1.7	96.0±3.8	10	103.0±2.0	102.0±2.0	92.5±4.8
Protein, *g/100 ml*	10	6.3±0.6	1.2±0.3	2.6±0.3	16	6.0±0.7	1.0±0.3	3.2±0.3
Nucleotides, *% of protein*	6	0.5±0.3	—	2.5±1.0	6	0.5±0.3	—	2.8±0.8
Hemoglobin, *mg/100 ml*	6	<50	—	<50	6	<50	—	<50

Note: mean sodium and potassium levels in three microscopic samples of normal rate cartilage fluid were found to be 140 and 4.5 mEq/liter, respectively (22).

* L_{fl}, perichondrial lymph; C_{fl}, cartilage fluid from hypertrophic cell zone (See Fig. 1 for collection sites).

‡ In this and subsequent tables, data are expressed as mean ± SD.

C_{fl} chloride were within 7–10 mEq/liter of the levels in serum or L_{fl} for both normal and rachitic animals.[9] The normal C_{fl} levels of sodium and potassium were compatible only with an extracellular source. More than trace contamination with blood or cartilage cells was further excluded by the findings of only traces of nucleotides and hemoglobin quantitated directly in the C_{fl} of normal and rachitic rats (Table II). The protein content of C_{fl} from normal and rachitic cartilage fluids was threefold greater than that of L_{fl} and approximately one-third to one-half the levels in serum, these differences being highly significant ($P < 0.001$). The composition of these proteins in C_{fl} and L_{fl} remain to be identified.

This new experimental fluid permitted for the first time direct testing of whether a mineral phase, e.g., $CaHPO_4$ or $CaHPO_4 \cdot 2H_2O$, might not be precipitated from a supersaturated solution in the cartilage septa. Evidence from in vitro studies indicates that this initial mineral phase would convert spontaneously to an apatite at pH 7.4 (3). The lowest $A\ Ca^{++} \times A\ HPO_4^{=}$ product at which spontaneous precipitation occurs in a synthetic lymph was studied by Fleisch and Neuman (23). In the latter study the minimum product required for initial separation of a mineral phase from a supersaturated solution after 72 hr was 2.7×10^{-7}. If the $A\ Ca^{++} \times A\ HPO_4^{=}$ of C_{fl} were significantly above this level in our new cartilage fluid, strong evidence would be provided for involvement of a precipitation mechanism. This consideration led to the study of Ca_t and P_{i_t} (Table I) and later to a partition of Ca_t and P_{i_t} of C_{fl} to obtain the $Ca_f \times P_{i_f}$ (Table IV).

The results of this initial survey on Ca_t and P_{i_t} measured in C_{fl} of normal, rachitic, and various healing rachitic animals is shown in Table III. In addition to the normal group, rachitic animals were studied because the large volume of hypertrophic cell cartilage in healing rickets should

[9] Due to lack of information concerning water partition in C_{fl}, all parameters measured in this report are expressed per unit total volume; small corrections for Donnan effects cannot be made.

TABLE III

*Ca_t and P_{i_t} in Serum and C_{fl} of Normal, Rachitic, and Healing Rachitic Rats**

Experimental conditions	Ca_t No. of rats	Ca_t Serum	Ca_t C_{fl}	Ca_t C_{fl}/serum	P_{i_t} No. of rats	P_{i_t} Serum	P_{i_t} C_{fl}	P_{i_t} C_{fl}/serum	$\frac{C_{fl}}{Ca_t \times P_{i_t}}$ (mmoles/liter)[2]	Histological grade of healing‡
Normal, normal food	14	2.35±0.06	1.46±0.06	0.62	17	2.80±0.15§	2.58±0.05§	0.92	3.79±0.10	
Rachitic, vitamin D_2 plus phosphate deficiency	16	2.42±0.05	1.80±0.05	0.76	22	0.71±0.05	1.58±0.05	2.23	2.87±0.13	0
Healing rachitic, 24 hr after (i.p.) 0.1 mmole Na_2HPO_4	8	2.40±0.06	1.58±0.08	0.66	10	1.23±0.07§	1.97±0.05§	1.60	3.12±0.12	+
Starved rachitic, food withdrawal for 24 hr	6	2.41±0.05	1.60±0.04	0.67	6	1.86±0.04§	2.01±0.06§	1.08	3.20±0.15	++
Rachitic, 0.1 U vitamin D_2 daily for 6 days	6	2.45±0.07	1.80±0.05	0.74	6	0.72±0.08	1.56±0.07	2.17	2.80±0.17	+
Rachitic, 1,500 U vitamin D_2 after 24 hr	8	2.58±0.08	1.91±0.06	0.74	8	1.26*±0.04§	2.06±0.06§	1.63	3.93±0.17	++
Rachitic, healing 48 hr 1500 U vitamin D_2	8	2.25±0.06	2.05±0.06	0.91	8	2.90±0.09	2.33±0.05§	0.81	4.78±0.15	+++

* Total calcium (Ca_t) and total inorganic phosphate (P_{i_t}); C_{fl}, hypertrophic cell cartilage fluid.
‡ 0, none, +, slight, ++, moderate, +++, advanced.
§ $P < 0.001$ in comparison to corresponding rachitic controls.

provide an optimal proportion of C_{fl} from sites actually undergoing calcification. Also, to examine the problem in greater breadth, regimens were employed which provided different rates of healing as judged by histological evidence of new mineral phase separation within the slightly calcified or uncalcified matrix of rachitic epiphyseal plates (Table III).

A consistent positive finding in all of the untreated rachitic animals was the high ratio of C_{fl}/serum of P_{i_t}. This ratio remained high during treatment but before the cure of rickets in all groups except the starved animals, despite an increase of serum P_{i_t} above control levels. For the starved group, as well as normal controls, the ratio C_{fl}/serum of P_{i_t} was about unity. The C_{fl}/serum Ca_t ratio remained less than unity in all experimental preparations. In regard to the $Ca_t \times P_{i_t}$ product in C_{fl} during healing of rickets, the highest level was recorded in animals receiving the massive dose of vitamin D and restoration of a normal diet for 48 hr. Accordingly, a partition of calcium and phosphate was performed on this group, as well as normal and rachitic controls (Table IV). In view of intermediate or low values of $Ca_t \times P_{i_t}$ among other experimental groups (Table III) further partitioning of calcium and phosphate in C_{fl} of these groups was not attempted. In addition to the data of Table III, Ca_t and P_{i_t} were also measured in serum and C_{fl} in six groups (5–7 rats each) of rachitic animals before as well as after (¼, ½, 1, 2, and 6 hr) intraperitoneal injection of 0.1 mmole of sodium phosphate. After sacrifice of these groups, their upper tibial epiphyseal cartilage was dissected free, sliced, and incubated in metastable calcifying solutions for 18 hr at 37°C (24). An evaluation was made for mineral seeding in the cartilage according to the criteria of Sherman and Sobel (24). First evidence of mineral seeding sites appeared in the cartilage of the groups sacrificed 2.0 hr after administration of sodium phosphate. In all of these experimental groups, including those sacrificed before 2.0 hr, the mean $Ca_t \times P_{i_t}$ of $C_{fl} < 4$ (mmoles/liter)^2 (25).

Partition of Ca_t and P_{i_t} on the basis of ultracentrifugation reveals several differences in the profiles of C_{fl} not predictable on the basis of serum proteins (Table IV). There was a substantial binding of calcium to the sedimenting fraction in the C_{fl} of rachitic rats with less binding of calcium in normal C_{fl}. Values for bound calcium in normal serum were similar to those in humans determined by Loken and his associates (20). Bound phosphate was not demonstrable in the sera of rachitic animals probably because of the extremely low levels of P_{i_t}. The finding of about 23% level of phosphate binding in C_{fl} of normal animals was a consistent one with about 17% binding in the sera of the same animals. The latter value was higher than that of human sera obtained by Loken and associates (20), and that of normal rat sera measured by an ultrafiltration method (26). Also shown in Table IV,

TABLE IV

*Partition of Ca_t and P_{i_t} in Serum and C_{fl} of Normal and Rachitic Rats by Ultracentrifugation**

Experimental conditions	No. of rats	Ca_f	Ca_f/Ca_t	P_{i_f}	P_{i_f}/P_{i_t}	pH after centrifugation	$A\ Ca^{++} \times A\ HPO_4^{=}$‡ pH = 7.40	$A\ Ca^{++} \times A\ HPO_4^{=}$‡ pH = 7.60
		mmoles/liter	%	*mmoles/liter*	%			
Normal serum	12	1.46±0.05	62.2±3.6	2.33±0.05	83.2±2.1	7.45±0.05	2.25×10^{-7}	
Normal C_{fl}	20	1.35±0.07	92.5±3.8	1.97±0.06	77.2±2.3	7.70±0.05	1.77×10^{-7}	1.96×10^{-7}
Rachitic serum	16	1.52±0.05	63.1±2.0	0.71±0.07	100.0±2.0	7.43±0.05	0.63×10^{-7}	
Rachitic C_{fl}	20	1.25±0.06	67.5+3.4	1.58±0.06	100.0±4.0	7.65±0.05	1.30×10^{-7}	1.44×10^{-7}
Rachitic serum, healing 48 hr§	6	1.53±0.08	68.0±3.0	2.45±0.07	83.6±2.4	7.43±0.05	2.48×10^{-7}	
Rachitic C_{fl}, healing 48 hr§	6	1.36±0.04	66.4±2.1	1.97±0.05	84.5±1.5	7.65±0.05	1.76×10^{-7}	1.96×10^{-7}

* Ca_f and P_{i_f}, calcium and inorganic phosphate in supernatant after ultracentrifugation at 130,000 *g* for 8 hr. For other abbreviations, see Table I.

‡ Activity coefficients calculated from Levinskas[1] Ph.D. Thesis (21); ionic strength = 0.16; temperature = 37°C.

§ Restoration of a normal diet and an oral dose of 1500 U of Vitamin D_2.

TABLE V

*Effect of the Partial Pressure of CO_2 on pH of C_{fl} In Vitro**

Experiment	Sample	Experimental conditions	P_{CO_2}‡	pH	HCO_3§	No. of rats
			mm Hg		*mmoles/liter*	
1	Normal C_{fl}	pH measurement within 15 min of starting C_{fl} collection	38	7.58 ± 0.05	35.6	10
2	Normal C_{fl}	pH measurement after P_{CO_2} in chamber adjusted to specified levels for 30–40 min	38	7.58 ± 0.04	35.6	10
			<5	8.33 ± 0.08	—	4
			19	7.81 ± 0.05	30.8	6
			57	7.40 ± 0.04	35.8	6
3	Rachitic C_{fl}	Same conditions as in experiment 2	38	7.52 ± 0.05	31.3	11
4	Normal arterial plasma	Same conditions as in experiment 2	38	7.39 ± 0.04	22.3	4

* C_{fl}, hypertrophic cell cartilage fluid.

‡ Partial pressure of CO_2 in microchamber used for pH measurements, computed from infrared analyzer data.

§ Computed from Henderson-Hasselbach equation.

are data on partition of Ca_t and P_{i_t} of C_{fl} in the group of rachitic animals healed with vitamin D and a normal diet for 48 hr. After 48 hr of healing with vitamin D_2 and a normal diet, there remained a high concentration of bound calcium similar to that of untreated rickets but there was also 16% bound phosphate, which indicated a trend toward normal C_{fl} partition of phosphate. A consistent unexplained observation in all three experimental groups (Table IV) was a 7–18% lower value for Ca_f in C_{fl} than in serum.

Finally, because of the high levels of pH of C_{fl} after ultracentrifugation (Table IV), it was of interest to measure pH on C_{fl} unaltered except in respect to arbitrarily adjusted levels of P_{CO_2} in vitro (Table V). For normal fresh C_{fl} at P_{CO_2} of 38 mm Hg the pH value was 7.58 ± 0.05. If the P_{CO_2} was increased to 57 mm Hg, pH in C_{fl} of normal animals after equilibration was reduced to 7.38. At P_{CO_2} levels studied, the computed mean bicarbonate of normal C_{fl} ranged from 30.8 to 35.6 mmoles/liter. The increment of bicarbonate C_{fl} above serum levels appeared to account quantitatively for the deficit of chloride in C_{fl} (Table II). Mean pH of C_{fl} from rachitic animals was slightly lower than from normal animals. The difference was not significant ($P > 0.05$).

DISCUSSION

In the current study, the first direct assessment of calcium and phosphate in a microscopic fluid phase from a calcifying site and the partition of these ions by ultracentrifugation has been made; the anatomical compartment from which the fluid was drawn and its extracellular nature have been defined.

The most important finding of this study is that the $Ca_f \times P_{i_f}$ of microscopic fluid samples from calcifying septa were insufficient in any of the experiments to support a mechanism of initiating calcification by spontaneous precipitation, as described by Robison (1). This conclusion is based on theoretical considerations developed in previous chemical studies of calcium phosphate precipitation in vitro (21, 23, 27, 28) applied to the partition of calcium and phosphate in C_{fl} (Table IV). Thus, the minimum total concentration product of $Ca^{++} \times HPO_4^{=}$ found concurrent with the spontaneous precipitation of a calcium phosphate salt in vitro from protein-free solutions nearly physiological in constitution has been found to be *circa* 3.8×10^{-6} (23). From this the value 2.7×10^{-7} is derived when correction is made for activity coefficients. The K_{sp} for brushite or monetite at

$\mu = 0.16$ was determined to be 2.7×10^{-7} (29). Accordingly, the view that brushite or monetite ($CaHPO_4 \cdot 2H_2O$ or $CaHPO_4$) is one of the first salts formed seems likely. However, initial precipitation of other salts is also possible, such as calcium-deficient apatite or tricalcium phosphate. This minimum activity product required for spontaneous precipitation of brushite when expressed as the corresponding product for tricalcium phosphate $A\ Ca^{++3} \times A\ PO_4^{=2}$ would be 2.3×10^{-26}, but inasmuch as no applicable K_{sp} is available for tricalcium phosphate, its spontaneous precipitation cannot be predicted. Although the existence of the latter salt has been controversial in the past (30), new evidence for its existence has recently been accrued, particularly in respect to the amorphous calcium phosphate phases in bone and cartilage (31, 32). Regardless of the actual salt or mixture of salts formed at pH 7.4–7.6, the solubility of brushite has served as a guide to whether mineral phase will or will not separate by spontaneous precipitation and its solubility is used here for this purpose (23).

Accordingly, for the current calcium and phosphate partition, the products $A\ Ca^{++} \times A\ HPO_4^{=}$ were calculated, applying to the protein-free concentration values the activity coefficients calculated by Levinskas (21) for standard conditions in vitro (Table IV). The concentration of soluble complexes of calcium which would not sediment under the conditions of this study could not be determined. Also part of the P_{i_f} of the new cartilage fluids, could be organic acid-labile phosphate. Consequently, the values for $A\ Ca^{++} \times A\ HPO_4^{=}$ shown in Table IV must be considered as the upper limit values. A correction may be made for either the normal or these healing rachitic C_{fl} values based on the assumption that chelates and complexes comprise about 7–12% of the total calcium as found in a partition of normal human plasma (3, 32a). After elimination of this fraction, $A\ Ca^{++} \times A\ HPO_4^{=}$ for C_{fl} of normal and healing rachitic animals would be reduced from identical values 1.76×10^{-7} to 1.52×10^{-7}, a level even further below the solubility product of $CaHPO_4 \cdot 2H_2O$ (23). In regard to preformed mineral, the range of dissolution products of hydroxyapatities is wide, but averages about 0.49×10^{-7} (3). Thus, for normal, rachitic, and healing rachitic cartilage fluids $A\ Ca^{++} \times A\ HPO_4^{=}$ was less than that required for spontaneous precipitation of brushite, but above that required for accretion on preformed hydroxyapatite. The $A\ Ca^{++} \times A\ HPO_4^{=}$ of normal and healing rachitic serum exceeded $A\ Ca^{++} \times A\ HPO_4^{=}$ of corresponding C_{fl} samples whereas the reverse was true for untreated rickets. A second problem is whether the $Ca_f \times P_{i_f}$ product was high in vivo but a mineral phase suspected to be present in normal C_{fl} seeded the sample during collection with resultant lowering of this product. Evidence against this happening is found in experiments[10] which indicate stability of $Ca_f \times P_{i_f}$ in the supernatant of C_{fl} after ultracentrifugation 1–24 hr after collection; in such experiments C_{fl} was incubated under CO_2 equilibrated at 37°C between collection and ultracentrifugation. Thus the data of Tables III and IV seem incompatible with a spontaneous precipitation mechanism unless the site of action were confined within cells or at cell margins, wherein changes of ion product would not register in the cartilage fluid composition.

The demonstration of a sedimenting macromolecular fraction in the C_{fl} of rickets which binds calcium might be indicative of a normal preliminary step in the process of calcification (Table IV). Of similar interest is the appearance of a phosphate-binding macromolecular fraction in normal C_{fl}. Relevant to this finding is a subsequent study (25) in which both an inhibitor of calcification and nucleating agent for mineral phase have been identified in C_{fl} sediments from normal rats. This bound phosphate of normal C_{fl} is acid-soluble and, therefore, is suspected of being part of a mineral phase suspended in C_{fl}. It is also possible that the acid-soluble phosphate is linked to some macromolecular component through the nitrogen of histidine similar to such compounds studied by Kundig, Ghosh, and Roseman (33). The fact that the partition of calcium and phosphate in C_{fl} of healing rickets in 48 hr (Table IV) is intermediate between that of rickets and normal controls supports the view that the macromolecular components binding calcium and phosphate have a functional role in calcification.

[10] To be published.

Despite the wide differences in content of bound calcium and phosphate between the normal and the advanced healing rachitic preparations, $A\ Ca^{++} \times A\ HPO_4^{=}$ upper limits were identical, a finding which is probably not fortuitous in view of the radically different treatment received by these animals (Table IV). As opposed to a precipitation in the septa, this observation is suggestive that there is a control mechanism to keep $A\ Ca^{++} \times A\ HPO_4^{=}$ within a narrow range of metastability. Downward adjustment of this product should be caused theoretically by efflux of mineral ions on to newly formed crystal surfaces or mineral embryos, as well as by buffering action of organic constituents. Also, a phosphate concentrating mechanism for upward adjustment of this activity product is possibly revealed in the high C_{fl}/serum ratio of P_{i_f} (Table IV) in rickets. Whether this high ratio C_{fl}/serum of P_{i_f} in rickets could be maintained by hydrolysis of organic phosphates after their elaboration by cartilage cells into the septa at sites preliminary to calcification is unknown. Possibly, the action of such enzymes as adenosine triphosphatase (ATPase) or other nucleotidases liberated by these cells into the matrix with action on appropriate unidentified substrates would have a functional role in producing a P_{i_f} gradient. In this connection, Krane and Glimcher demonstrated ATPase activity of collagen and binding of the monophosphate liberated in this reaction to collagen (4). It is also possible that the P_{i_f} gradient could be established by active transport of phosphate through cartilage cell membranes into surrounding septa. One source of phosphate ions would be organic components of the cells. A new layer of hypertrophic cells in the normal young rat has been calculated to reach the zone of provisional calcification every 1–3 hr (34) so that regardless of hypertrophic cell synthetic function per se, a continuous new supply of organic substrate should reach the calcifying site. Otherwise, a gradient of P_{i_f} would seem to require a continuous source of inorganic phosphate and establishment of active transport across membranes separating the plasma from the mineralizing site. Trueta postulated that invading capillary complexes may regulate mineral phase separation in the endochrondral plate (35). Possibly a continuous membrane of osteoblasts, suggested for endosteal bone by Howard (36), or capillary complexes function at the metaphyseal border to effect an ion gradient in the distal hypertrophic cell septa.

Such a membrane would also facilitate explanation of elevated bicarbonate in C_{fl} (Table V). This was another consistent finding and its validity is strengthened by the depressed chloride content of C_{fl} compared to either serum or L_{fl} (Table II). Slight elevation of bicarbonate would be predicted from the Donnan effect if C_{fl} resembles other high protein lymphs, but not the 10–12 mmoles/liter elevation computed for C_{fl} (Table V). In respiratory acidosis bicarbonate elevation develops along with the depressed chloride but in the current animals pH of serum was not reduced. Thus in the presence of an elevated P_{CO_2} restricted to local cartilage fluid, depression of pH without much alteration in bicarbonate would be predicted unless an additional mechanism were invoked even if, as in the distal nephron (37), a large P_{CO_2} gradient could be established through some unknown pathway. One likely means to establish the fluid composition of C_{fl} (Table V) would be through a membrane-governed mechanism catalyzed by carbonic anhydrase with secretion of bicarbonate into the calcifying site. Carbonic anhydrase activity has been assessed repeatedly in bones and cartilage of different animal and bird species often with the recording of high levels, but uncertainty of techniques has rendered interpretation difficult as lucidly discussed by Ellison (38). This investigator observed substantial activity of carbonic anhydrase in femoral and tibial epiphyseal plates of growing rats as well as its absence in mature epiphyses, but he tentatively assigned its location in progenitor cells of hematopoietic tissue rather than in cells directly involved with mineralization (38). Maren, Mayer, and Wadsworth, administering acetazolamide to young growing rats chronically showed no difference in the epiphyseal development between control and treated rats (39). Their data were most compatible with the absence of any important carbonic anhydrase sustained mechanism for epiphyseal growth in their experimental preparations.

However, it remains uncertain whether the invading capillary complexes of endochondral plates contain carbonic anhydrase in substantial amounts and, if so, to what extent acetazolamide penetrates critical enzyme-bearing intracellular sites in the invading capillary complexes.

An alternative explanation for the elevated pH of C_{fl} (Table V) is dissolution of mineral embryos containing carbonate or other alkaline ions at the site of C_{fl} collection. The complex composition of mineral phases formed in bone and cartilage has been reviewed by Bachra (40), and some carbonate is incorporated in epiphyseal mineral phase. The possibility of dissolution of mineral to elevate pH is complicated by the fact that at least in vitro maturation of early calcium phosphate precipitates is accompanied by release of hydrogen ion as crystalline apatites incorporate hydroxyl into crystal lattices (27). A similar maturation involving conversion of amorphous calcium phosphates to apatities is believed likely to occur in the hypertrophic cell zone of provisional calcification (31). Accumulation in cartilage fluid at calcifying sites of fixed acid metabolites from local cartilage cells as well as carbonic acid are additional factors which might impair mineral accretion in the septa without an adequate system for hydrogen ion removal. It remains to be determined whether passive diffusion in vivo suffices or whether the high pH registers an ancillary mechanism in this respect. Finally, if P_{CO_2} is not elevated in the calcifying sites, mineral phase accretion at the site of C_{fl} collection probably occurs in the presence of an elevated pH.

APPENDIX

The individual ion activity coefficients that have been used in the present report for correcting calcium and divalent phosphate molar concentrations are, respectively:

$$\gamma_{Ca^{++}} = 0.36 \quad \text{and} \quad \gamma_{HPO_4^{=}} = 0.23.$$

These values, first calculated by Levinskas (21), were applied by Strates, Neuman, and Levinskas (28) in their study with solutions prepared under nearly physiological conditions similar to ours.

The two expressions:

$$-\log \gamma_{Ca^{++}} = \frac{2.04\sqrt{\mu}}{1 + 2.09\sqrt{\mu}}; \quad -\log \gamma_{HPO_4^{=}} = \frac{2.04\sqrt{\mu}}{1 + 0.696\sqrt{\mu}}$$

given in the text under methods, have been derived by substituting in the Debye and Huckel equation:

$$-\log \gamma_i = \frac{Az_i^2\sqrt{\mu}}{1 + aB\sqrt{\mu}}; \quad \gamma_i$$

by Levinskas coefficient values; $z_i = z$, ionic charge; the combined constants A and B by their calculated values for water as solvent at 37°C (37): $A = 0.51$ mole$^{-\frac{1}{2}}$ liter$^{\frac{1}{2}}$; and $B = 0.33 \times 10^8$ cm^{-1} mole$^{-\frac{1}{2}}$ liter$^{\frac{1}{2}}$; $\sqrt{\mu} = 0.40$. In this way the average ion size parameter a was estimated.

The aforementioned expressions have been frequently used in this laboratory to compare the precipitation of calcium phosphate from solutions with different ionic strengths.

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