Effects of Age on Citrate Metabolism in Bone and on the Distribution of Skeletal Fluoride* (33572)

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When the effects of fluoride ingestion on the responses to vitamin D were studied, we observed that small differences in the age of the rats had a marked effect on the citrate response at various bone sites. Fluoride decreases bone citrate (1, 2); vitamin D increases citrate in some sites, but not in others (2, 3). The concentration of citrate in normal bone (1) and the amount of fluoride deposited in bone also varies with the age of the animal and the type of bone (4, 5). Since rapid growth occurs earlier in some bones than in others (6), it seemed possible that the effects at different bone sites might depend on the rate of growth and the age at which the site matured. The present investigation was undertaken to obtain further information on changes in composition which take place with age in normal bone, and to determine whether these changes could be correlated with the effects of age on deposition of fluoride and on the responses to vitamin D. The concentrations of citrate, calcium, and phosphate in different segments of long bone, calvaria, and serum were determined at frequent intervals in male rats from 31 to 170 days of age. The observed changes were compared with various measurements reported by other workers for assessing rates of growth and maturity in these sites (7–9).

The results reported here indicate that active growth in each site was associated with characteristic changes in citrate, with the citrate leveling off to a relatively constant concentration at the age when growth in the site was essentially complete. Differences in the effects of vitamin D and the deposition of fluoride in young and mature rats were related to the stage of development of a given site at the time of treatment.

Distribution of skeletal fluoride appeared to be related to the calcium deposited in the site during fluoride ingestion as well as to the stage of maturity of the site.

Methods. A large lot of weanling, male Sprague-Dawley rats all born on the same day were randomized by weight, housed 8 per cage and fed Rockland laboratory diet. Rats from one cage were sacrificed at each of the following ages: 31, 38, 45, 52, 59, 66, 76, 84, 91, 113, 140 and 170 days. In another experiment, young (23 day old) and mature (113 day old) rats received 125 ppm fluoride as NaF in the drinking water for 4 and 8 weeks; 8 control rats given distilled water instead of fluoride were sacrificed along with each group of treated rats. Serum and bone from the following 4 areas of each animal were analyzed: *calvaria,* membranous flat bone known to reach maturity earlier than most bone (6); *epiphyses,* cancellous bone of the joint area which should reach maturity at about the same time as growth in width of tibia and femur is complete; *metaphyses,* cancellous bone at the proximal end of the tibia and the distal end of the femur; the "growing zone" (10) adjacent to the epiphyseal disk which should remain active even in adult rats [long bone in the rat never stops growing and epiphyseal closure does not occur (11)]; and *diaphyses,* compact bone of the shaft of the tibia and the femur.

The rats were fasted 18 hr, anesthetized with ether and bled to death from the abdominal aorta.[1] The bones were removed and

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[1] Great care was exercised in drawing blood for citrate measurements. If the rat struggled during anesthesia or became cyanotic or stopped breathing momentarily during collection of the sample, citrate

rapidly stripped of flesh. The calvaria was trimmed along the frontonasal suture to include frontal, parietal, and interparietal bone. The epiphyseal portion above the cartilage plate of the tibia and femur was carefully broken away from the shaft, freed as completely as possible of tendons and cartilage, and combined for analysis. The fresh tibia was weighed and measured. Tibia and femur were split open and the blood and marrow were sponged out. The metaphyses (the trabeculae) from the proximal end of the tibia and the distal end of the femur were combined. The metaphyseal portion at the other end of the bones was discarded. The shaft of the femur and tibia were combined and are hereafter referred to as the diaphyses. The bones were cut into small pieces and placed in 95% alcohol. They were then extracted with frequent shaking over a period of at least 5 days; twice with ethanol and 3 times with ether and dried for 3 hr at 105°. The dry bones were pulverized in 15–30 sec in a Crescent dental Wig-L-Bug using a tungsten carbide capsule with a single large ball. A 30–35-mg sample of powdered bone was dissolved overnight in 1 ml of 4 *N* hydrochloric acid, diluted to 5 ml with distilled water, and centrifuged. Aliquots of the supernatant were analyzed for citrate, calcium, and phosphate as previously described (12). Another sample of 10–15 mg of powdered bone was analyzed for fluoride by the microdiffusion method of Wharton (13). Serum was analyzed for calcium, phosphate, and citrate as described elsewhere (12). Serum and the bone sites from each rat were analyzed separately except in very young rats where it was sometimes necessary to pool calvaria or epiphyses fom 2 or 3 rats.

Results. Growth. Weights of the rats throughout the experimental period from age 31 to 170 days are compared with tibia weight and length in Fig. 1.

Serum. The concentration of calcium in serum remained relatively constant between 11.1 and 10.5 mg/100 ml. Phosphate dropped from 11.6 to 6.8 mg/100 ml at 84 days (Fig. 2). Citrate decreased from 12.4 to 6.4 mg/100 ml at 76 days and remained relatively constant thereafter (Fig. 1).

Bone. The concentration of calcium (Fig. 1) increased with age in all bone sites and most of the increase occurred before 66 days of age when the rate of elongation of tibia decreased. Phosphate increased also, but was most pronounced and significant ($p<.001$) in epiphyses prior to 66 days of age (Fig. 2).

Citrate first changed in a manner characteristic for each site and then leveled off to a relatively constant concentration. In each site the period when citrate changed corresponded very well with the period of active growth described by others. The age at which citrate leveled off in each site agreed with the age at which growth was essentially complete. This can be seen from the following comparisons:

In epiphyses, citrate increased sharply from the initial concentration (34 μmoles/g) at 31 days up to 59 days when it leveled off at a concentration of 42–44 μmoles/g bone[2] (Fig. 1). Tapp (8), using tetracycline labeling methods in rats 14–91 days old, reported that the rate of growth in width of tibia (which parallels growth in width of epiphyses) decreased rapidly after 28 days and leveled off at 56 days of age.

In diaphyses and metaphyses, citrate decreased sharply (from 41 and 37 μmoles/g, respectively) until the interval between 91 and 113 days and leveled off at a concentration of 21–22 μmoles/g bone (Fig. 1). Tapp reported that the rate of growth in length of tibia, as measured by radiological and tetracycline methods (8), decreased between 28 and 56 days, increased slightly between 63 and 77 days and then decreased and leveled off near 91 days when the experiment was terminated.

Moss (9) reported that the two sections of rat calvaria developed at different rates. Growth of the neurocranial portion (parietal and posterior portion of the frontal bones) was essentially complete at 34 days, but growth in the visceral-cranial portion contin-

concentrations were invariably low. This was also true if the rats were not fasted at least 17 hours.

[2] All measurements unless otherwise indicated are for dry, fat-free bone.

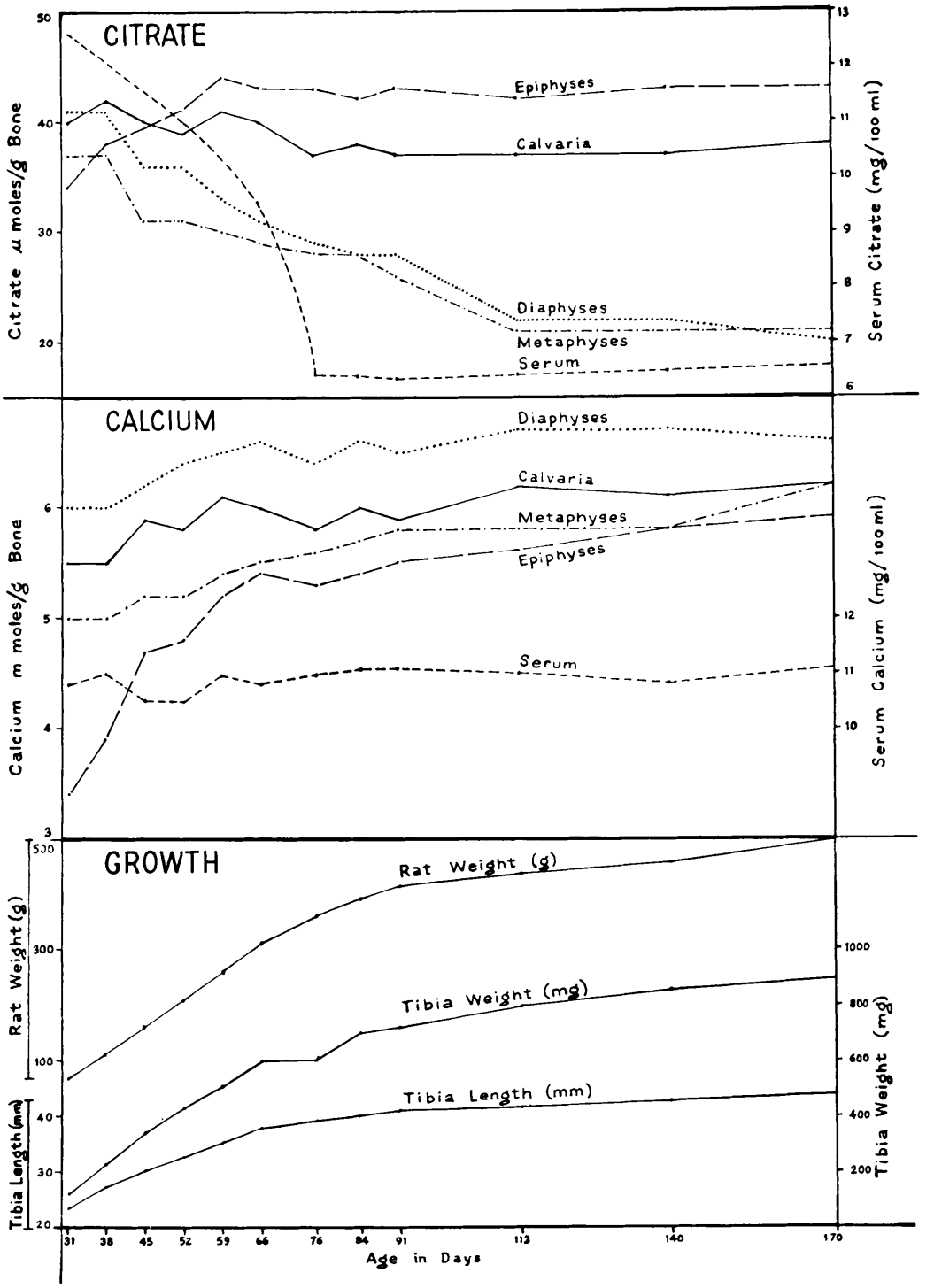

FIG. 1. Effects of age on growth and calcium and citrate in serum and various bone sites.

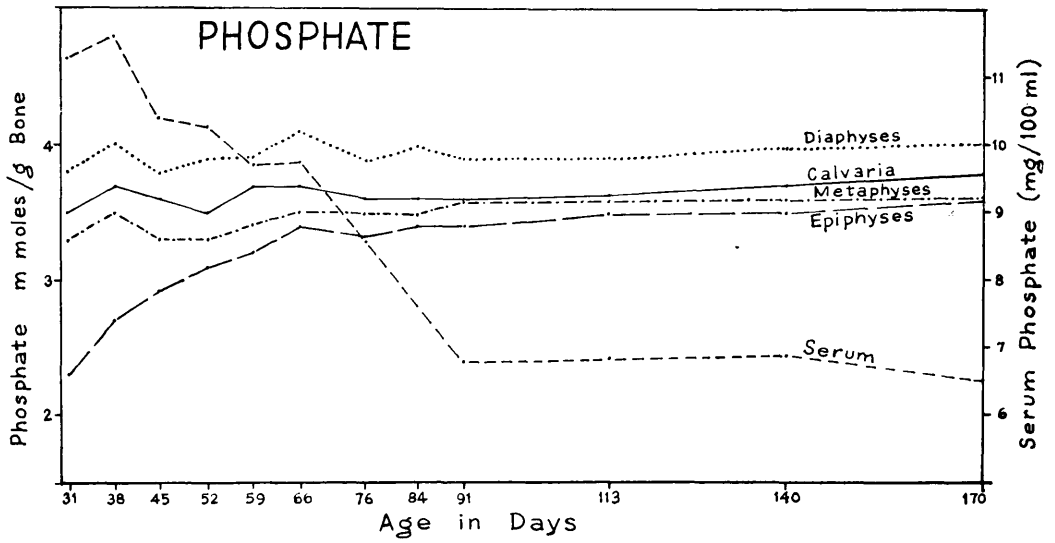

FIG. 2. Effect of age on phosphate in serum and various bone sites.

ued for some time after this, with osteogenesis evident on all surfaces up to 60 days of age. Citrate in calvaria fluctuated for a while (after growth was complete in one portion of calvaria) and then declined and leveled off at 76 days of age at a concentration of 36–38 μmoles/g of bone (Fig. 1).

The agreement between the period of active growth, the changes in citrate, and the age at which active growth and changes in citrate leveled off were in remarkably good agreement: in epiphyses, citrate leveled off between 59 and 66 days—growth in width of tibia at 56 days; in calvaria, citrate leveled off at 76 days—osteogenesis continued to at least 60 days; in diaphyses, citrate level off between 91 and 113 days—growth in length of tibia at 91 days; and in metaphyses citrate leveled off at 91–113 days, but some reduced activity is to be expected after this time since long bone continues to grow slowly even in old rats (11).

Additional information is needed on the changes in citrate prior to 31 days when most active growth occurred in all sites, and in metaphyses of animals where epiphyseal closure and growth in long bone ceases, but the results here indicate that it may be possible to estimate the age of maturity in various bone sites from changes in citrate.

Effects of vitamin D. Many of the perplexing differences in the effects of vitamin D at different bone sites now can be related to the age at which a given site matures. Apparently vitamin D produces an increase in citrate only if the site is actively growing at the time of treatment. In another study (3) we reported differences between sites with respect to the increase in citrate induced by treatment with vitamin D. When massive doses of the vitamin were given for 2 or more weeks, starting at 38 or 46 days of age, citrate was increased in all sites and in serum. At that time, according to the present results, all sites were actively growing. When vitamin D was started at age 53 days, at a time when growth in epiphyses was nearing completion (59 days), citrate was increased in all sites except epiphyses. When vitamin D was started at 81 days, there was no increase in citrate in either epiphyses or calvaria (which matures at about 75 days). In metaphyses, there was still a significant increase in citrate at 113 days, but the increase was not as large (26%) as at 53 days (37%) or at 46 days (62%). Serum citrate was not increased in mature rats, but serum calcium was elevated at all ages. It is now quite clear that vitamin D did not produce an increase in citrate in any bone site after growth in the site was complete, or in serum after growth in most sites was complete (113 days).

TABLE I. Fluoride Deposition and Decrease in Bone Citrate in Young and Mature Rats.[a]

Bone site	Fluoride concentration[b] (μmoles/g)		Citrate in untreated controls (μmoles/g)		Citrate decrease[c] controls vs F-treated (%)	
	Young	Mature	Young	Mature	Young	Mature
	Fluoride (125 ppm) ingested 4 weeks					
Epiphyses	85 ± 2	22 ± 1	41 ± 1	43 ± 1	22	8
Metaphyses	74 ± 2	50 ± 1	31 ± 1	21 ± 0.5	20	20
Diaphyses	73 ± 2	19 ± 1	36 ± 1	22 ± 0.5	18	9
Calvaria	57 ± 1	16 ± 1	39 ± 1	38 ± 1	16	6
	Fluoride (125 ppm) ingested 8 weeks					
Epiphyses	104 ± 2	46 ± 1	43 ± 1	44 ± 1	33	18
Metaphyses	91 ± 2	81 ± 2	28 ± 1	22 ± 0.4	35	22
Diaphyses	93 ± 2	41 ± 1	29 ± 1	20 ± 0.4	34	15
Calvaria	70 ± 2	35 ± 1	37 ± 1	38 ± 1	33	16

[a] Young rats ingested 125 ppm fluoride as NaF in the drinking water from age 23 to 51 days (4 weeks) and 23 to 78 days (8 weeks); mature rats from 113 to 140 days and from 113 to 170 days. Untreated controls received distilled water during the same periods. The commercial diet contained less than 0.002% fluoride and the highest concentration of fluoride in any bones of the untreated rats was 8 μmoles/g.

[b] Concentrations of fluoride and citrate are μmoles/g of dry, fat-free bone ± SE.

[c] The percentage decrease in citrate due to fluoride ingestion was calculated by comparing the concentration of citrate in untreated controls with that in the rats treated with fluoride.

Distribution of skeletal fluoride also appears to depend on the stage of maturity of the bone site at the time of ingestion of fluoride. In Table I, the concentration of fluoride deposited in various bone sites is shown for young (23 day old) and mature (113 day old) rats given 125 ppm fluoride in the drinking water for 4 and 8 weeks. In young rats, fluoride deposition was higher in all sites than in mature rats, and 78–80% of the total fluoride taken up by the bones was deposited during the first 4 weeks (age 23–51 days) when all sites were actively growing. In mature rats, growth in calvaria, epiphyses, and diaphyses was essentially complete prior to the administration of fluoride and approximately equal amounts of fluoride were deposited during the first (age 113–140 days) and second (140–170 days) periods of ingestion. In metaphyses, where growth continued though at a reduced rate, 62% of the total fluoride taken up was deposited during the first period. This is in agreement with the view that there are two distinct phases of fluoride deposition (14), a rapid one attributed to ionic exchange of fluoride with the hydroxyapatite system, and a slower one due to the appearance of new exchangeable sites produced by osteoclastic and osteoblastic activity (15).

The distribution of fluoride in long bones of young rats was in the order: epiphyses > metaphyses = diaphyses, whereas in mature rats it was in the order: metaphyses > epiphyses > diaphyses. The significantly higher concentration of fluoride in epiphyses than in the other actively growing sites in young rats may be due to the higher rate of calcification in this site at the time. The concentration of calcium increased 59% in epiphyses as compared with 9–11% in the other sites (Fig. 1).

The distribution of fluoride in long bones of young rats was in agreement with that reported by Zipkin and Schow (16) in 20-day-old rats given smaller amounts of fluoride intraperitoneally for 31 days. They concluded that deposition of fluoride could not be correlated with the degree of calcification of the different sections of long bone. They

did not think that the differences in fluoride deposition could be due entirely to vascularity of the sites (17), and pointed out that distribution of skeletal fluoride was similar to that of radioisotopes of calcium, phosphorus, and sulfur, and changed in a similar way with age.

A relationship between bone vascularity and fluoride incorporation and the importance of bone growth and mineralization have been stressed repeatedly, but the precise relationship between the age of bone and its fluoride content was not clear despite several investigations (5). If fluoride deposition is higher during active growth and dependent upon the state of maturity of a site as well as on the calcium deposited *during* the period of fluoride ingestion, fluoride might indeed act like the radioactive markers. Much more work is needed to establish such a relationship, but it would explain most of the reported differences in the distribution of skeletal fluoride.

The decrease in bone citrate produced by fluoride ingestion was higher in actively growing sites than in mature sites, Table I. In metaphyses where growth continued even in mature rats, the percentage decrease in citrate during the first 4-weeks ingestion was the same as in young rats (20%) although the concentration of fluoride in mature rats was lower. After 8-weeks ingestion in young rats, the percentage decrease was the same in all sites (33–35%) even though the concentrations of citrate and fluoride were different in the various sites. Concentrations of citrate were in decreasing order: epiphyses, calvaria, diaphyses = metaphyses; concentrations of fluoride in decreasing order were : epiphyses, metaphyses = diaphyses, calvaria. This has been observed repeatedly even when more fluoride was ingested during the same period or for longer periods (3). Posner *et al.* (18) suggested that fluoride reduced the specific surface of the crystal and this prevented the substitution of citrate. It may be that this reduction in surface area can prevent substitution of only a third of the citrate.

None of the rats in any of these experiments had severe toxic symptoms. Whether the percentage decrease in citrate will be greater and different in various sites in severe crippling fluorosis is currently under investigation.

Summary. In calvaria and long bones of the rat the concentration of citrate changed in a pattern characteristic for the bone site during active growth; it remained relatively constant after growth was essentially complete (epiphyses 60 days, calvaria 75 days, diaphyses 90–113 days). In metaphyses (the growing zone of long bones) there was still reduced activity after this time since growth in long bone continues even in old rats. This procedure may be useful in determining maturity in other bone areas where rate of growth is difficult to assess by other methods, and in selection of the proper age and bone site for evaluation of the effects of various treatments. Differences in the citrate response to vitamin D and in the deposition of fluoride at various bone sites in growing rats could be correlated with the age at which growth in a given site was complete. Fluoride deposition also appears to be related to deposition of calcium in the site during fluoride ingestion. Most of the differences in distribution of skeletal fluoride can be explained on this basis. Fluoride acts much as the radioactive tracers and it may be that fluoride deposition could be used to measure calcium accretion during the bone remodelling process.

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The Adjuvant Effect of Pertussis Vaccine in Experimental Thyroiditis of the Rat* (33573)

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Bordetella pertussis vaccine was first shown to increase the encephalitogenic potency of a central nervous system antigen–complete Freund adjuvant emulsion in mice by Lee and Olitsky (1) and later in rats by Levine and Wenk (2). Subsequent work revealed that the potentiating action of pertussis vaccine was such that it could effectively replace complete Freund adjuvant in the sensitizing injection (3, 4). Because of the impressive results obtained using pertussis vaccine in the allergic encephalomyelitis system, we investigated its effect on the production of another autoimmune disease in rats, experimental thyroiditis. Parallel findings have been obtained by Paterson and Drobish (5) who observed that allogeneic thyroid extracts plus complete Freund adjuvant together with pertussis induced thyroiditis within 3 weeks in 100% of either Lewis or CFN rats. Allogeneic or xenogeneic extracts without pertussis gave little or no thyroiditis at 16 or 21 days in either test strain.

Material and Methods. Female Lewis (Microbiological Associates, Inc., Bethesda, Md.) and Fischer 344 (Simonsen Labs, Gilroy, Calif.) rats between the ages of 6–12 weeks were used in the following experiments. Saline extracts were prepared from random bred Wistar rat thyroids (Pel Freez Biologicals, Rogers, Arkansas). The thyroids were shipped in the frozen state and were stored in a freezer until the time when extract was prepared. These antigen preparations (RaTE) were adjusted to a protein concentration of 30 mg/ml.

The immunization regimen consisted of a single injection of 0.2 ml of RaTE (6 mg) emulsified in an equal volume of complete Freund adjuvant. The complete Freund adjuvant was prepared from incomplete Freund adjuvant (Difco) with the addition of 4 mg/ml of *Mycobacterium tuberculosis* $H_{37}Ra$. This material was administered intradermally in the four foot pads and intramuscularly in the thighs. Where noted a simultaneous injection of 0.5 ml of *Bordetella pertussis* vaccine was given on the dorsum of each foot. The pertussis vaccine was an aqueous preparation containing 200 billion organisms/ml.

Serum antibody titers were measured in a microtitration system using the tanned cell hemagglutination procedure of Rose and Witebsky (6). Upon sacrifice of the animals, the portion of trachea containing thyroid tissue was removed *in toto* and fixed in a phosphate buffered formalin solution. Histological sections were prepared and stained with he-

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