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The Effects of Trifluoperazine on Calcifying Tissues in the Immature Rat (41856)

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Abstract. When the antipsychotic drug trifluoperazine is given to immature rats during stages of rapid bone development, severe localized derangement in their growth plate architecture results. In addition, 3 weeks of treatment with doses of 3 mg/kg body wt/day had marked effects on the anatomic distribution of bone mineral, and on the quantitative chemical composition of the bone matrix. Specifically, ash contents and Ca/P ratios were decreased and mineral crystals appeared larger and/or more perfect in the treated animals. The finding of decreased hexosamine content and alterations in bone lipid composition could be interpreted as specific effects of the drug on calmodulin activity.

Calmodulin (CaM), the "calcium-binding regulatory protein" that has been found in all eukaryotic cells in which it has been sought (1–5), has recently been localized in several mineralizing tissues including the developing tooth germ (6) and immature bone (7). The antipsychotic drug, trifluoperazine (a phenothiazine), has been used *in vitro* as a competitive inhibitor of CaM (8–11). In order to gain some knowledge of the involvement of CaM in processes related to mineralization, trifluoperazine was given to immature rats during a period of maximal growth. Histological studies and several biochemical parameters of mineralization were analyzed as an index of the effect of this drug on the mineralization process.

Materials and Methods. Fifteen female Sprague–Dawley rats, 4 weeks of age, weighing 76–82 g, were divided into three groups. Six received daily intramuscular injections (3 mg/kg body wt) of trifluoperazine-HCl (Stelazine, Smith, Kline and French Laboratories, N.Y.) for 21 days. Five were treated similarly for periods of 1 (one animal), 3 (two animals), and 5 (two animals) days. The remaining 4 animals received no drug, and served as controls. At the indicated times, animals were weighed, anesthetized with Fluothane, and sacrificed by heart puncture. Individual plasma samples were used for analyses of calcium, phosphate, cholesterol, glucose, and creatinine concentrations, and alkaline phosphatase activity (Technicon, SMA-12; analyses performed by the Hospital for Special Surgery Clinical Laboratories). All long bones, ribs, and scapulae were removed immediately after sacrifice and microradiographed. The left legs (femur, fibula, and metatarsals) were fixed in phosphate-buffered Formalin. One tibia from each animal was fixed in phosphate-buffered Formalin, embedded in methyl methacrylate, and cut into 4-μm sections; the non-decalcified sections were stained with von Kossa and hematoxylin and eosin stains. The other Formalin-fixed bones were decalcified, embedded in paraffin, and sections were stained with hematoxylin and eosin, safranin O, and the van Geisen method.

The bones from each animal, other than those used for histology, were cleaned of soft tissues and visible marrow by scraping with a fine scalpel; they were then divided under a dissecting microscope into growth plates, metaphyseal cancellous, and diaphyseal cortical bone. Comparable tissue types (e.g., all cortical bone) from a single animal were pooled, and the pooled tissues quick-frozen in liquid nitrogen and lyophilized. Because the growth plates from a single animal did not provide sufficient material for all analyses, growth plates from all 21-day-treated animals were pooled and analyzed together. Growth plates from all the control animals were similarly pooled to provide sufficient material for analysis. Lyophilized tissues were ground in a liquid nitrogen-cooled colloid-mill. The cancellous bone, which had a high blood and marrow content, was washed in ammonium-bicarbonate buffer (pH 7.8), until the wash water was hemoglobin free (Ames, Hematest), and relyophilized. (Growth plates and cortical

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TABLE I. SERUM CHEMISTRY AFTER 21 DAYS[a]

	Calcium (mg/dl)	P_i (mg/dl)	CHL (mg%)	Creat. (mg%)	Alk. P. (IU)	Glucose (mg/ml)
C (N = 4)	10.13 ± 0.32	9.50 ± 0.13	81 ± 6	1.5 ± 1.0	3.4 ± 0.6	187
T (N = 6)	8.85 ± 0.65	7.95 ± 0.6	64 ± 7	0.45 ± 0.3	3.2 ± 0.9	234
P	0.001	0.0003	0.003	0.04	NS	*b*

Note. C, control; T, treated.

[a] Mean values for calcium, inorganic phosphate, cholesterol, creatinine, alkaline phosphatase, and glucose ± SD; compared based on t test.

[b] N = 2. Sufficient serum was not available from most animals to measure glucose.

bones, lacking significant amounts of blood and bone marrow, were not washed.)

Aliquots of lyophilized tissues were subjected to replicate X-ray diffraction analyses using Cu K-alpha radiation. Where measurable, the line width of the c-axis (002) reflection was used as an estimate of crystallite size and perfection (12). Weighed aliquots of the samples which had been used for X-ray diffraction were dried at 100°C, ashed at 600°C, and used for determination of mineral content. The ashed samples were then dissolved in 3 N HCl and the Ca (13) and P (14) content of the ash determined.

The remaining lyophilized tissue was used for determination of collagen hydroxyproline (15), hexosamine (16), total lipid, and Ca-acidic phospholipid-phosphate, Ca-PL-PO_4 (17), contents. The lipid class composition of the noncomplexed lipids was determined using thin-layer chromatography (18), followed by quantitative colorimetric analysis of cholesterol and cholesterol ester (19), free fatty acids (20), triglyceride (21), and phospholipid (22). In some cases, aliquots of the phospholipids were further separated into phosphatide classes by thin-layer chromatography (23) prior to measurement of organic phosphate content (22). Ca-PL-PO_4 complexes, dissociated in formic acid (17), were analyzed for phospholipid composition in a similar manner.

Results. Trifluoperazine treatment caused rats to become lethargic relative to the nontreated but similarly handled controls. Growth was impaired and treated animals had significantly ($P < 0.05$) less weight gain (80 ± 8 g in 21 days) than controls (93 ± 13 g in 21 days). Radiographically, there were no detectable differences between treated and control bones. Biochemical examination of the serum of the treated animals showed these animals were significantly hypocalcemic, hypophosphatemic, hypocholesterolemic, and hyperglycemic (Table I). Treated animals also had reduced serum creatinine levels. During the initial 1–5 days of treatment the serum chemistries were not significantly different from the control values suggesting that changes occurred after that time.

Microscopic examination of the growth plates of the animals treated for 21 days showed, in comparison with controls, cellular disorganization and matrix disorganization. With regard to the first of these abnormalities, it can be seen in Fig. 1 that the trifluoperazine-treated animal has fewer cells than the control, and that the normal columnar arrangement of the cells is lost in the treated animals. Matrix

FIG. 1. (A) Photomicrograph of the proximal tibial growth plate in a control animal, stained at the left with hematoxylin and eosin, and at the right stained with the von Kossa stain for phosphate. From these photographs, the thickness of the calcified zone of the cartilage can be appreciated. Note, too, the orderly cellular columns of chondrocytes. (B) Photomicrograph of the proximal tibial growth plate in an animal treated with trifluoperazine for 21 days. There is disorderly arrangement of the cell columns, as compared with the controls, and in addition there appear to be fewer cells. Just above the calcified zone, there is a dissolution of the cartilage matrix, with resultant cleft formation, which is filled with blood. (C) Photomicrograph of another field of the same growth plate shown in (B). From this photograph and from (B), it can be appreciated that there is, in addition to those changes noted in (B), a loss of the normal linear regularity of the calcified zone of the cartilage and the underlying metaphyseal bone, as compared with the control (A). All figures undecalcified sections; ×84.

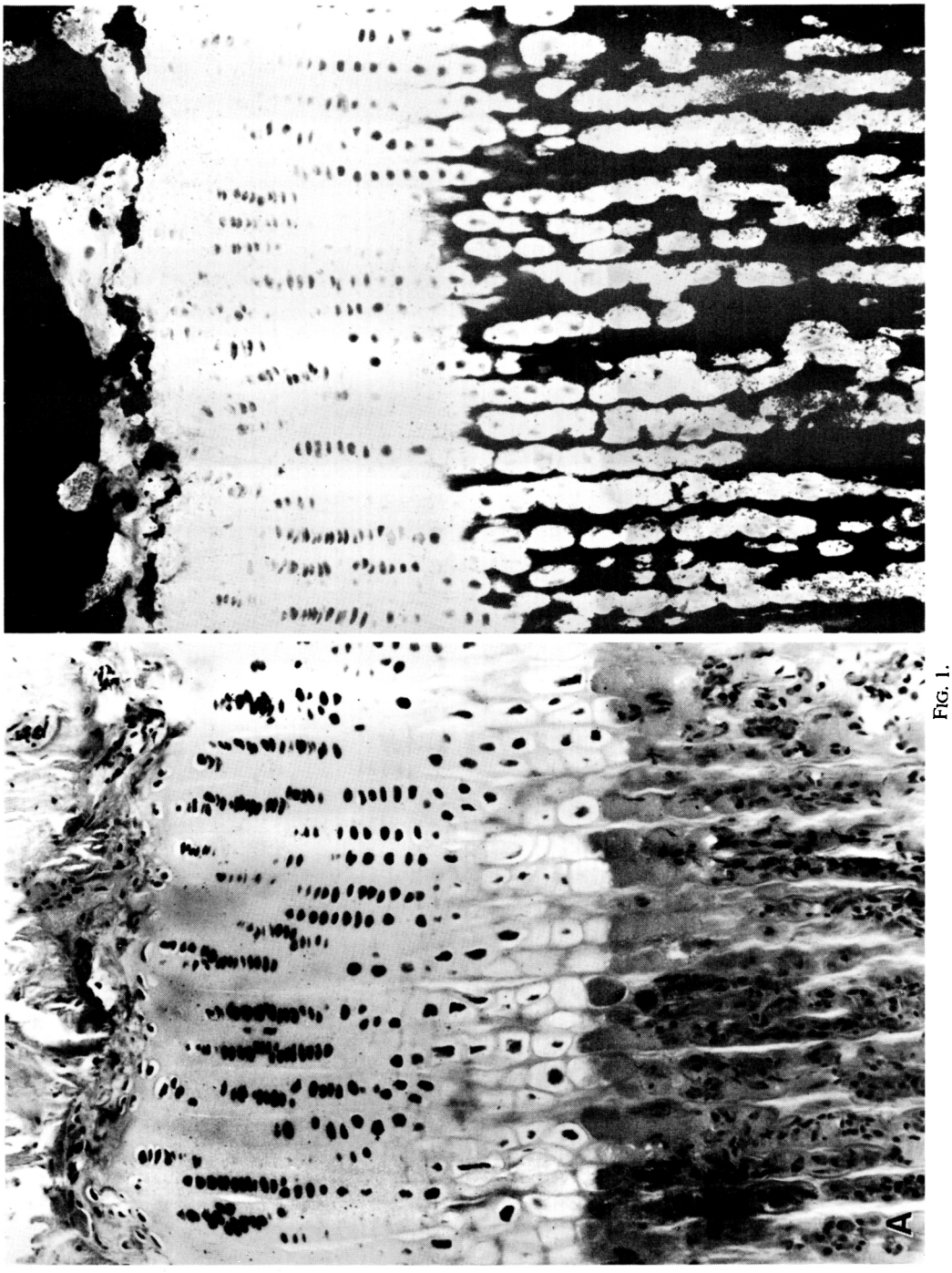

FIG. 1.

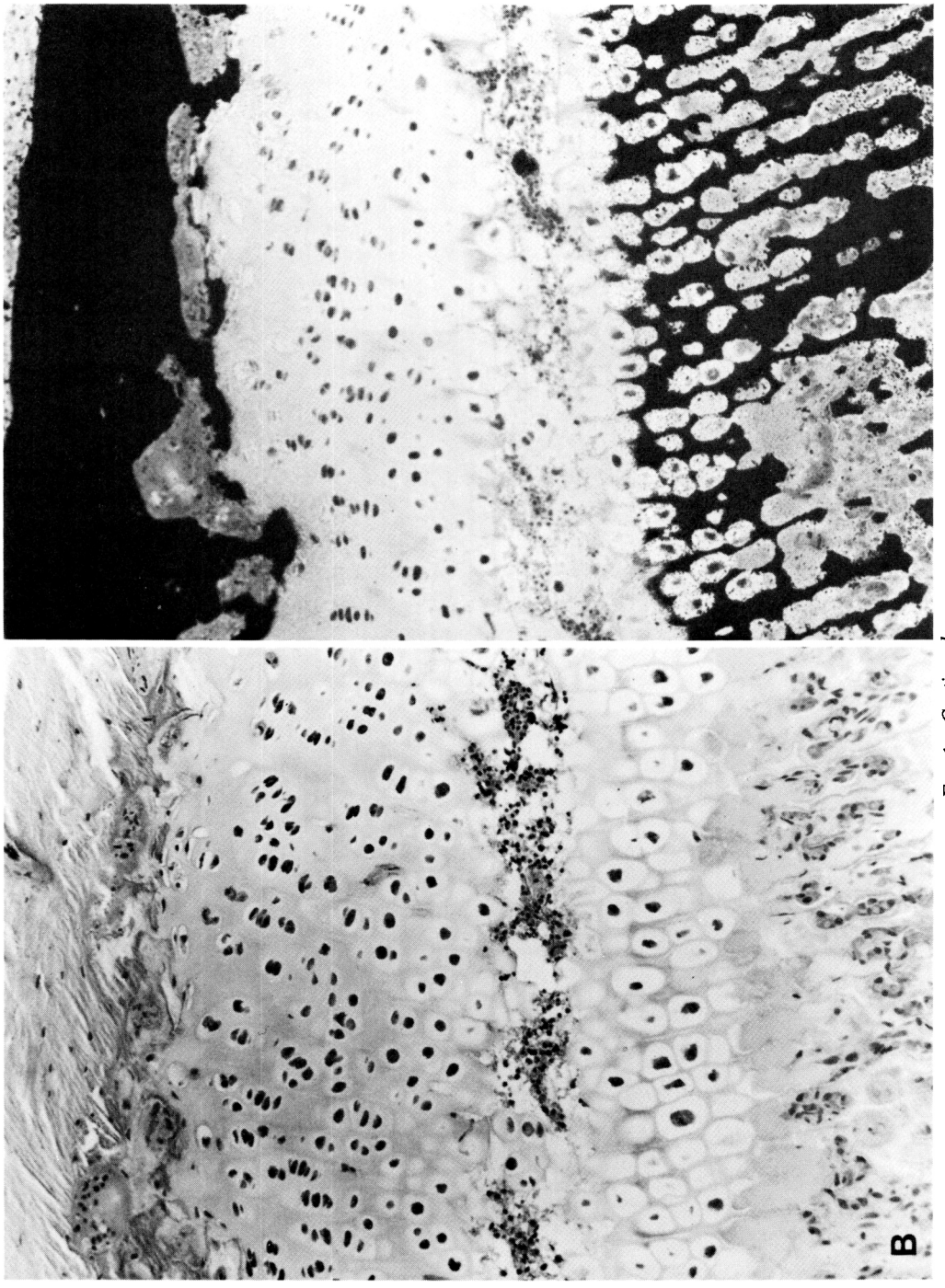

FIG. 1—*Continued.*

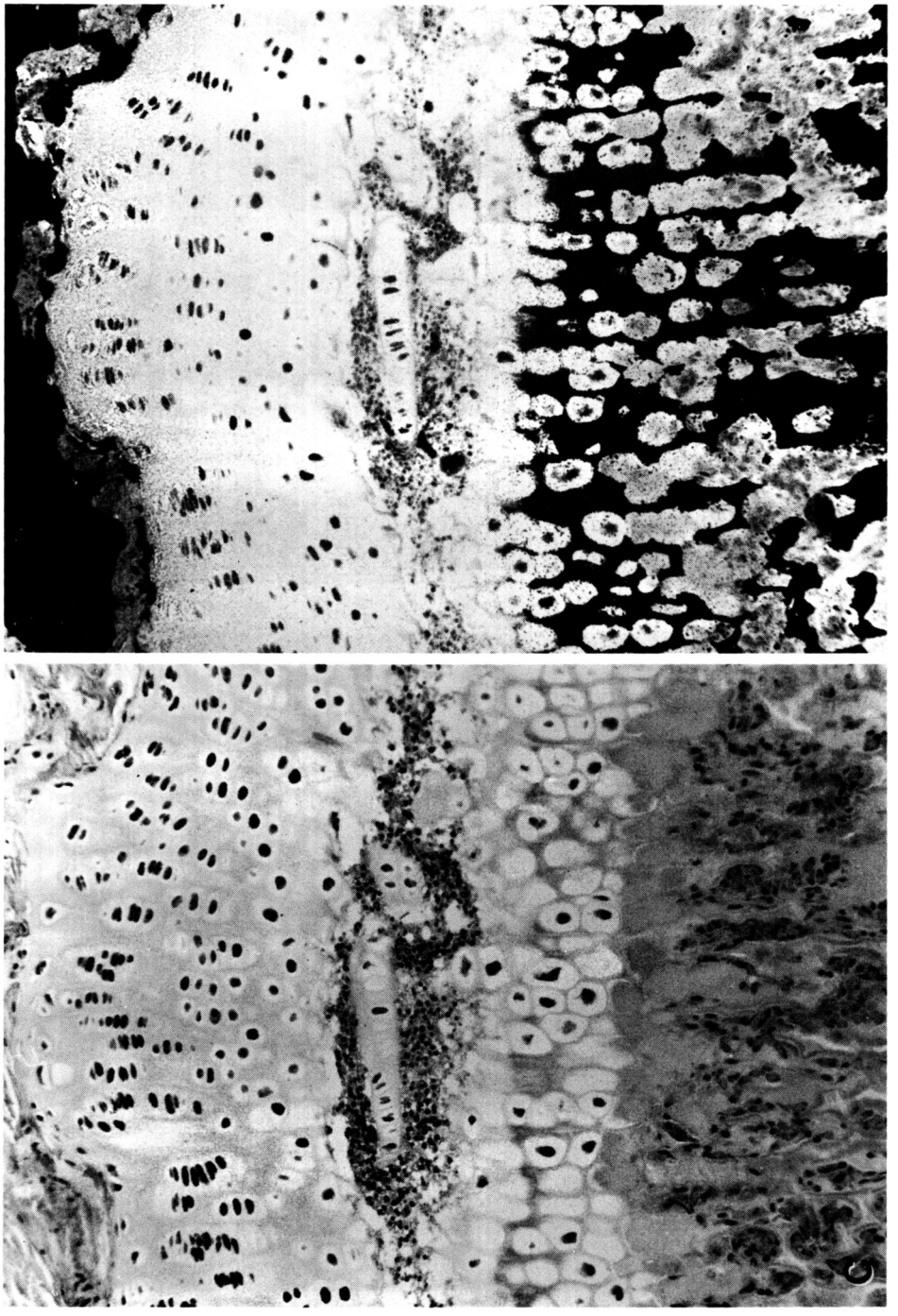

FIG. 1—*Continued.*

TABLE II. CHEMICAL COMPOSITION OF THE BONES OF TRIFLUOPERAZINE-TREATED (T) AND CONTROL (C) RATS

	Growth plates		Cancellous bone		Cortical bone	
	T	C	T	C	T	C
Mineral						
Ash (wt %)	46	49	60 ± 6*	70 ± 2	66 ± 4	70 ± 2
Ca/P (*M*)	1.61	2.58	1.53 ± 0.28*	2.18 ± 0.18	1.64 ± 0.1*	1.96 ± 0.13
β_{002}	ND	ND	ND	ND	0.54 ± 0.02*	0.56 ± 0.008
Matrix (% Demin. dry wt)						
Hydroxyproline	33 ± 5	34 ± 4	40 ± 4	43 ± 3	67 ± 9	76 ± 7
Hexosamine	10.6 ± 0.1*	12.5 ± 0.2	7.5 ± 1.9*	8.7 ± 0.5	3.8 ± 0.6	4.6 ± 1.1
Lipid	7.5 ± 0.2	7.6 ± 0.3	10.2 ± 0.2	10.4 ± 0.3	5.8 ± 1	6.4 ± 2
Ca-PL-PO_4	0.59 ± 0.2*	2.6 ± 0.2	0.65 ± 0.2	0.58 ± 0.11	0.90 ± 0.5	0.89 ± 0.2

Note. ND, Not determined. Bone data are means ± SD for six treated and four control animals.
* Significantly different from control, $P < 0.05$ based on *t* test. For growth plate means (and SD) based on replicate (three or four) determinations.

failure was seen to occur just above the calcified zone of the cartilage, and the resultant clefts were filled with blood and disorganized cartilage tissue. These changes were not apparent in the 1- to 5-day-treated animals. Safranin O and van Geison stains failed to show significant microscopic alterations in the proteoglycan and collagen of the matrix.

The chemical composition of the treated bones was not detectably altered by the 1- to 5-day treatment, but was significantly altered in animals treated for 21 days (Table II). Ash content and Ca/P molar ratios were decreased in all tissues from the treated animals. Further, the mineral crystals in the treated cortical bones, based on crystallographic analysis of the c-axis line width were either larger, and/or more perfect, than control bones. The collagen content of the treated bones was not altered, but hexosamine content was decreased in all three components of the treated bones, and calcium-acidic phospholipid phosphate content was significantly decreased in epiphyses of the treated animals. The phospholipid composition of the Ca-PL-PO_4 complexes throughout the treated and nontreated bones was constant, consisting of 33.8 ± 0.7 mole% phosphatidyl inositol and 33.2 ± 0.4 mole% phosphatidyl serine.

The lipid class composition of the growth plates and cancellous bones of TFP-treated animals was also different from that of controls, but there were no differences in cortical bone lipids (Table III). Triglyceride levels were decreased in growth plates of the treated animals, while cholesterol content decreased within the growth plates, and increased in the cancellous bone. Cholesterol ester contents were decreased in both the growth plates and the cancellous bone of the treated animals.

TABLE III. BONE LIPID COMPOSITION (wt% OF TOTAL LIPID)[a] IN TRIFLUOPERAZINE-TREATED (T) AND CONTROL (C) RATS

	Growth plates		Cancellous bone		Cortical bone	
	T	C	T	C	T	C
Triglyceride	18.9 ± 1.4*	25 ± 1.5	49 ± 2	56 ± 1	21.6 ± 0.6	20.2 ± 0.6
Free fatty acid	7.6 ± 1	7 ± 1	9.5 ± 2	7 ± 1	13 ± 3	13 ± 3
Cholesterol	2.4 ± 0.05*	5.2 ± 1	7.2 ± 1.4*	3.1 ± 0.8	2.6 ± 0.1	2.1 ± 0.07
Cholesterol ester	5.1 ± 0.2*	8.3 ± 1	9 ± 3*	16 ± 3	5.6 ± 0.5	6.1 ± 0.08
Phospholipid	16 ± 2	16 ± 3	15 ± 3	11 ± 6	14 ± 4	12 ± 4

[a] Assumes C16 free fatty acids, triolein, cholesterol palmitate, and dipalmitoyl phosphatidyl choline for calculation of weight percentage. All values are means ± SD.
* Differs from control, $P < 0.05$ based on *t* test.

Although the total phospholipid content of the bones (based on lipid phosphorus content) was not altered by the 21-day TFP treatment, the composition of the phospholipids was appreciably altered by this treatment. Thus the proportion of phospholipids (Table IV) consisting of lysophosphatides was significantly decreased in both the treated growth plates and the treated cancellous bone. Treated cortical bone similarly contained fewer lysophosphatides, although the differences between treated and control cortical ones were less pronounced (not shown).

Discussion. This preliminary study demonstrates that the antipsychotic drug, trifluoperazine, TFP, when given to immature rats, causes alteration of the normal structure of the epiphyseal growth plate, and the adjacent calcifying cartilage and cancellous bone. The cells that appeared most severely damaged were the hypertrophic chondrocytes i.e., those cells which are most intimately involved in preparation of a calcifiable matrix (24).

There has been a recent increase in interest in those factors which control the dynamic process of physiologic calcification in bone and cartilage. In this laboratory we have been particularly interested in the control systems dependent on the complexed acidic phospholipids and the proteoglycans. In this regard, the chemical composition of both the inorganic and organic matrix of the bones of the TFP-treated animals was significantly altered. Bones of treated animals had decreased mineral content, and the mineral present consisted of hydroxyapatite crystals that appeared more mature than those in control bones. The organic bone matrix in the treated animals had a normal collagen hydroxyproline content, but hexosamine content and lipid composition were significantly different from control. [With regard to the reported lipid composition, it is necessary to comment on the recovery of the total lipid pool. The composition of total lipids as shown in Table III was calculated based on several assumptions. To estimate the weight percent of total lipids belonging to each lipid class it was assumed that: all fatty acids were palmitic acid, all triglycerides were triolein, all phospholipids were dipalmitoyl phosphatidyl choline, and all cholesterol esters were cholesterol palmitate. The fact that the total lipids recovered (excluding the 15–20% (25) mono- and diglycerides whose content was not measured for the growth plate and cortical bone) was less than 60 wt% of the total lipid applied indicates these assumptions are not fully valid. However, it must be noted that these deficiencies should not affect the comparison of similar tissues. Previous experience (25) suggests that 8–12% of the total lipids applied to silica gel plates for thin-layer chromatography are lost in recovery. In the cancellous bone, where the major lipid present was triglyceride probably due to marrow and hematopoetic tissue contamination, the errors associated with these assumptions are less apparent. It should be noted that in general the overall lipid composition reported is in good agreement with the results reported for can-

TABLE IV. PHOSPHOLIPID COMPOSITION (PERCENTAGE OF TOTAL LIPID PHOSPHORUS) OF TFP-TREATED (T) AND CONTROL (C) GROWTH PLATES AND CANCELLOUS BONE

	Growth plate		Cancellous bone	
	C	T	C	T
Phosphatidyl choline	60 ± 5	60 ± 5	17 ± 2	24 ± 6*
Lysophosphatidyl choline	4 ± 1	2 ± 1	7.3 ± 0.2	6.1 ± 2
Phosphatidyl ethanolamine	14 ± 1	13 ± 5	20 ± 3	20 ± 3
Lysophosphatidyl ethanolamine	2.7 ± 0.3	1.7 ± 0.1	4.8 ± 0.7	2.8 ± 0.8*
Sphingomyelin	5 ± 1	4.5 ± 1	3.7 ± 1.8	3.1 ± 0.5
Phosphatidyl glycerol	0.6 (tr)	0 (tr)	8.4 ± 0.7	3.8 ± 0.7*
Diphosphatidyl glycerol	2.2 ± 0.3	2.4 ± 0.1	10.2 ± 0.6	11 ± 1
Phosphatidyl serine	7 ± 1	2 ± 1*	4 ± 3	4.7 ± 2
Lysophosphatidyl serine	1 ± 1	0.8 ± 0.2	6 ± 1	2.9 ± 1.1*
Phosphatidyl inositol	1.8 ± 0.1	1.9 ± 0.1	6.1 ± 0.1	5.6 ± 0.9
Phosphatidic acid	0.6 ± 1	2 ± 1	4.6 ± 1.7	5.5 ± 2
Lysophosphatidic acid	1.3 ± 0.1	1.1 ± 0.1	5.3 ± 2	2.9 ± 0.9*

Note. Values are means ± SD. tr, Not detected on all plates.
* Differs from control, $P < 0.05$ based on t test.

cellous, cortical, and epiphyseal bone by Sakai *et al.* (26). However, these investigators did find a higher fatty acid content than we report. The observed phospholipid composition for the control growth plate agrees well with that reported by Wuthier *et al.* (27); the cancellous bone phospholipids appear to be contaminated with marrow lipids, and should not be taken as indicative of cancellous bone lipid composition. However, despite the high concentration of lysophosphatides, the overall content (lysophosphatide + phosphatide) of each individual phosphatide class, with the exception of sphingomyelin, is in excellent agreement with the values reported for compact bone (28).]

There are many possible explanations for the observed matrix and mineral alterations. Some of the biochemical alterations in the bones of the TFP-treated animals may be due to the severe growth plate disruption, and the presence of blood elements from the hemorrhaging tissues. Most of the experimental observations in the TFP-treated animals can be explained in terms of calmodulin inhibition. Trifluoperazine, a phenothiazine derivative, is a competitive inhibitor of calmodulin (9–11, 29–31). It should be noted that TFP also may induce alterations in cell membrane properties, which are apparently independent of calmodulin inhibition (11, 31), due to the lipophilic (membrane-binding) character of the drug. Thus, a toxic effect of TFP on the cells, independent of calmodulin inhibition cannot be ruled out.

There are at least five calmodulin-regulated systems in bone which may be implicated in the observed biochemical and anatomic alterations:

(*1*). Calmodulin is required for cellular differentiation and mitosis (5, 32), processes required for the differentiation of cartilage cells during endochondral ossification (24, 33).

(*2*). Calmodulin stimulates the ATP-dependent transport of calcium out of the cell (30) and out of the mitochondria (10) in other tissues. It is reasonable that the same is true for cartilage cells, where such a Ca pump has been demonstrated (34), and where the flux of Ca out of the mitochondria and cell are crucial for mineralization (35). Any interference with the efficiency of this Ca pump could affect the ability of the chondrocyte to pump out calcium, and might lead to improper mineralization. The low serum Ca levels, and the decreased Ca/P ratios in the bones of treated animals could be a result of the inability of the cells to get rid of their Ca.

(*3*). Calmodulin is the Ca-dependent regulator of phospholipase A2 (36, 37), an enzyme whose activity peaks in the hypertrophic zone of the growth plate (38). Phospholipase A2 is thought to be involved in the production of extracellular matrix vesicles, which are the membrane-bound bodies believed to be the site of initial mineral deposition in cartilage (24). The decreased proportions of lysophosphatides throughout the treated growth plates and cancellous bones might be attributed to impaired activity of this enzyme. It has also been suggested that the formation, or release and formation of Ca-PL-PO_4 complexes, which are hydroxyapatite nucleators (39), is dependent on phospholipase A2 action: a hypothesis supported by decreased Ca-PL-PO_4 concentrations in the treated growth plates.

(*4*). Calmodulin is also required, perhaps associated with the activation of phospholipase A2, for exocytosis (5, 40). It is thus of interest to note that the concentration of noncollagenous proteins released into the medium by bone cells in tissue culture is decreased in the presence of TFP (41). Many of these noncollagenous proteins are involved in the regulation of hydroxyapatite deposition both *in vitro* and *in vivo* (42) and a decrease in their content in the extracellular matrix of the TFP-treated animals could contribute to the observed abnormal mineralization.

(*5*). Calmodulin is also involved in the regulation of carbohydrate metabolism (4, 5), and thus calmodulin inhibition could affect proteoglycan, and glycosaminoglycan synthesis and degradation. Decreased levels of hexosamine are indicative of such abnormalities. Since intact proteoglycans are essential for normal cartilage architecture and the control of mineral deposition (42), the observed matrix derangement, and abnormal mineralization could also be explained, in part, by proteoglycan abnormalities.

In addition, although calmodulin has not yet been shown to be required for the Ca-dependent "base exchange" enzyme (43) required for the synthesis of phosphatidyl serine, the observed decreased phosphatidyl serine concentrations might suggest such a requirement.

The observed loss of bone mineral in the TFP-treated animals might also be due to in-

creased resorption, on the basis of hyperparathyroidism secondary to the observed TFP-induced hypocalcemia, or to disuse osteoporosis in the lethargic animals. The apparent increased hydroxyapatite particle size and decreased Ca/P ratios of the mineral in the treated animals are similar to that found in some osteoporotic bones (44), and also supports the contention that increased resorption and/or decreased formation might be taking place.

It should be noted, that TFP does not, at dosages of 1.0 or 10 mg/kg/day, even over extended periods, accumulate in bones (45). However, the effects of TFP on bone, may only be observable during periods of active endochondral ossification. Further, these effects are not likely to be dependent on the accumulation of the drug in bone, but rather should be related to the effect of the drug on metabolically active cells. It should be noted that the only other study of the effect of TFP on bone (46) examined only the fluoride content of ashed bones and not the nature of the bone itself. Additional morphological and chemical studies at the ultrastructural level, and in isolated, *in vitro* systems, will be needed to clarify the short- and long-term effects of TFP on mineralizing tissues, and to verify that this effect is due to calmodulin inhibition. Nonetheless, the clinical implications of this study should not be overlooked.

In conclusion, the finding of TFP-induced mineralization defects may have clinical significance, since the dosage of drug used is comparable to the recommended adult dose, although it is higher than the starting dosage (0.05 mg/kg body wt) that is recommended for the treatment of psychotic diseases in children. However, it should be noted that there are no specific reports of growth disturbances in TFP-treated children, although there are several reports of osteopenia in individuals treated with this and other neuroleptic drugs (46–49). The immature rats in this study were given TFP during a stage of maximal endochondral growth, 4–6 weeks after birth. This time period has been widely used in our laboratory (50), and in others (51, 52), to characterize the effect of vitamin D and its metabolites on mineralization. The apparent abnormalities in the bones of these rats after as short a period as 3 weeks suggests that TFP can markedly inhibit normal skeletal metabolism especially during periods of rapid growth.

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