Bone Formation in Normal and Vitamin D-Treated Rachitic Rats During the Administration of Polyphosphates.* (31480)

JAMES T. IRVING,† DONAT SCHIBLER, AND HERBERT FLEISCH
(Introduced by W. D. Armstrong)
Laboratory of Experimental Surgery, Schweizerisches Forschungsinstitut, Davos, Switzerland

There is now considerable evidence that condensed phosphates are able to inhibit calcification *in vivo* as well as *in vitro*(1,2, 3,4). Since these compounds have proved to be effective against experimentally-induced aortic(3) and skin(4) calcification, it is possible that they might be used therapeutically to prevent ectopic calcification. In view of this possibility we have investigated whether the doses required to inhibit pathological calcification have an influence of the normal mineralization process in bone.

Two experiments were carried out: the first was to test the effect of polyphosphates on normal bone formation, the second to investigate whether this salt had any effects on the healing of rickets with vitamin D.

Material and methods. Experiment 1. Normal rats. Eighteen young female rats of the Wistar strain, weighing approximately 60 g, were fed stock rat diet (Altromin, Lage, Germany) throughout the experiment. They were injected daily with Graham salt (J. A. Benckiser, Ludwigshafen/Rhein, Germany) at a dose level of 10 mg P/kg body weight given subcutaneously. Three animals were killed at the beginning of the experiment and their tissues examined. Subsequently, 3 were killed after 2 days of Grahan salt injection, and 4 each after 4, 7 and 14 days.

The upper ends of the tibias were removed. One was decalcified, embedded in paraffin, and sections were stained either with hematoxylin-eosin or by the alcian blue or periodic acid Schiff (P.A.S.) technique. The other tibia was treated for Sudan black staining by Irving's method(5) in the following way: The bone was extracted with benzol and after decalcification was embedded in gelatin and sectioned with a freezing mictrotome. Sections were stained with Sudan black and mounted in glycerin jelly.

Experiment 2. Rachtic rats. Forty-eight female rats of the Wistar strain, weighing from 60 to 80 g, were housed throughout the 35 days of the experiment in a darkened room and fed a high calcium, low phosphate rachitogenic diet obtained from Altromin, Lage, Germany. The composition of the diet is given in Table I. The animals were divided into 3 groups, each of 16 rats, and were treated as follows:

Group 1. After 28 days 3 animals were killed for examination. The other 13 animals were given a single dose of 50 i. u. of vit. D_3 by stomach tube and maintained on the rachitogenic diet for a further 7 days. They were then killed and examined.

TABLE I. Composition of the Rachitogenic Diet (Altromin).

Soya meal (50% protein)	30%
Rice starch	56%
Olive oil	2%
Cellulose powder	4%
Mineral mixture*	6%
Vitamin " †	2%

* 60 g (in 1000 g of the diet) contains: $CaCO_3$, 30 g; K-acetate, 10 g; NaCl, 8 g; $NaHCO_3$, 6 g; $MgSO_4 \cdot 7H_2O$, 5 g; Fe gluconate $\cdot 2H_2O$, 1480 mg; $MnSO_4 \cdot 4H_2O$, 450 mg; $ZnCO_3$, 40 mg; $CuSO_4 \cdot 5H_2O$, 20 mg; KI, .5 mg; $Na_2MoO_4 \cdot 2H_2O$, .5 mg; NaF, 10 mg.

† 20 g (in 1000 g of the diet) contains: DL-methionine, 1200 mg; vitamin A, 15000 I.U.; vitamin E, 150 mg; vitamin K_3, 10 mg; vitamin $B_1 \cdot$ HCl, 20 mg; vitamin B_2, 20 mg; vitamin $B_6 \cdot$ HCl, 15 mg; vitamin B_{12}, 30 μg; pantothenic acid (Ca salt), 50 mg; nicotinic acid, 50 mg; choline chloride, 1000 mg; folic acid, 10 mg; biotin, 200 μg; inositol, 100 mg; p-aminobenzoic acid, 100 mg; vitamin C, 20 mg.

Total Ca = 1.28%. Total P = 0.26%.

* This work has been supported by the Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung; USPHS grant AM:0726-03 from Nat. Inst. of Arthritis & Metab. Dis.; USPHS grant DE-01592 from Nat. Inst. of Dent. Res.; and the Sandoz-Stiftung zur Förderung der medizinisch-Biologischen Wissenschaften. Statistical analysis of the results by Dr. E. E. Hunt is gratefully acknowledged

† J. T. Irving was on research leave from Forsyth Dental Center, Harvard School of Dental Med., Boston

TABLE II. Effect of Graham Salt, Vitamin D_3, and Graham Salt and Vitamin D_3 on Growth Rate in Rats.

Treatment	Graham salt			Vitamin D_3			Graham salt and vit D_3		
	Number	Mean wt (g)	S.D.	Number	Mean wt (g)	S.D.	Number	Mean wt (g)	S.D.
Pre-treatment	13	73.2	9.4	13	75.0	6.4	13	75.9	9.6
Gain during treatment	13	44.8	13.0	13	35.6	9.6	13	37.2	15.3

Group 2. These rats were given from the 26th day to the end of the experiment a daily injection of Graham salt subcutaneously at a dose level of 10 mg P/kg body weight. On the 28th day, 3 were killed for examination, and the rest were sacrificed 7 days later.

Group 3. These rats were treated with Graham salt in the same way as those in Group 2. On the 28th day, 3 were killed and examined and the rest given a single dose of 50 i. u. of vit. D_3 by stomach tube. Seven days later, the remainder were killed and examined.

The treatment with Graham salt in Groups 2 and 3 was started 2 days before vit. D administration, because this procedure had been found to prevent vit. D-induced ectopic calcification(3,4). All the animals were weighed twice weekly during the experiment.

The tibias of all animals were removed and treated as follows: One was fixed in formol-saline, decalcified, embedded in paraffin and stained with hematoxylin and eosin, alcian blue or by the PAS method. The other was fixed in 5% formaldehyde solution, and cut in half longitudinally with a scalpel. One half was treated with silver nitrate and assessed for rickets or healing using Sobel's method(6), and the other was sectioned on a freezing microtome, stained with silver nitrate, treated with sodium thiosulfate and mounted in balsam. In Sobel's method, the degree of calcification across the metaphysis is assessed from 0, or no calcification, to ++++, a complete line of calcification. The calcification of the metaphysis below the line is assessed from 0 to 4, or maximum calcification.

Results. Experiment 1. Effect of Graham salt in normal animals. These rats gained weight at a normal rate over the experimental period, none died, and all appeared healthy. The injections of Graham salt had no detectable effect upon bone formation in the epiphyseal region and did not affect the appearance with any of the stains. The cartilage cell arrangement was unchanged and capillary invasion proceeded normally throughout the experiment. Sudan black staining of the matrix, a procedure which indicates an early stage in calcification, was present in all sections round the hypertrophic cartilage cells. The P.A.S. reaction stained all the sections a uniform pink, but the matrix of the epiphyseal cartilage and the cores of the trabeculae of the primary spongiosa were stained a little more deeply. The alcian blue method stained the cartilage matrix and the cartilage cores faintly in a similar way in all sections. No changes, either of a rachitic type, or those characteristic of inanition, were seen. Graham salt, at the level given, had thus had no detectable effect on the histology of bone formation.

Experiment 2. Effect of Graham salt in rachitic animals. Two of these rachitic animals died at an early stage of the experiment; the rest remained in good health throughout. Details of the growth data are given in Table II. An analysis of variance showed that the increments of growth were not significantly different ($p>.05$) in the 3 groups. *Calcification.* Three animals were killed from each group at the 28th day. All showed marked rickets as seen by Sobel's method 0(0). The animals killed at 35 days showed the following. *Group 1.* All these animals, treated with vit. D_3 only, exhibited very marked healing of rickets, as indicated by an average score of 3.5(++++). The lowest score, seen in 2 animals, was 3(++++). *Group 2.* Nearly all these animals, given Graham salt alone, continued to show marked rickets; their average score was 0.5(+). The highest score was, in one animal, 1(++), and 7 had scores

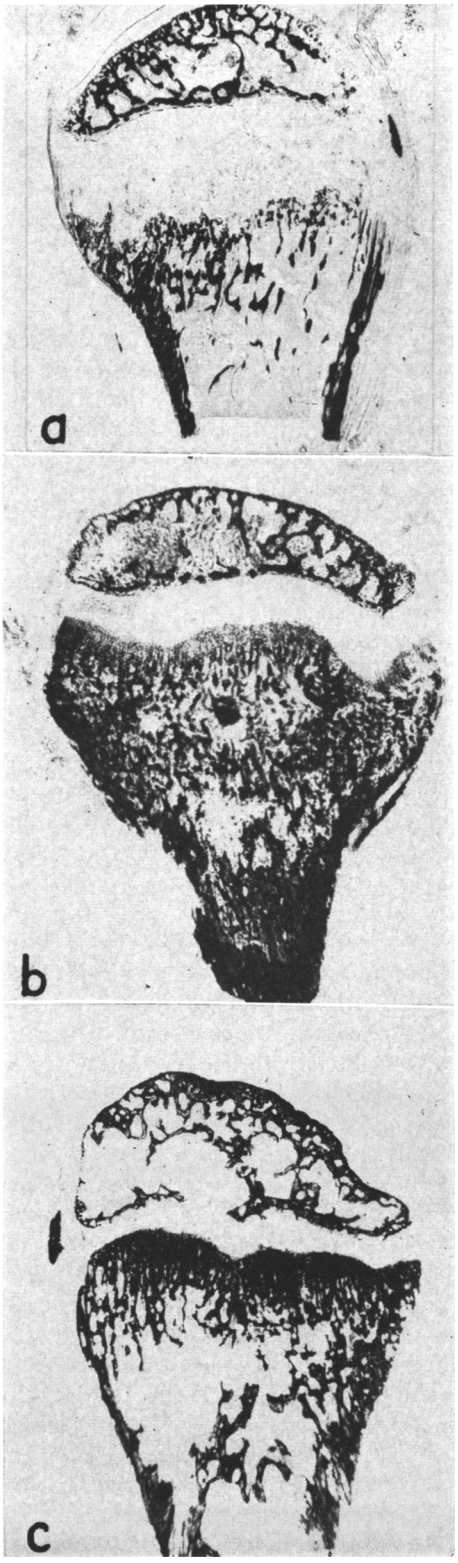

FIG. 1. Epiphyses of upper ends of tibias stained with silver nitrate. All rats were on a rachitogenic diet for the 35 days of experiment. a) Given Graham salt from 26th to 35th day. Note advanced rickets, 0(0) on the Sobel scale(6). b) Given a single dose of vit. D on the 28th day of the experiment. Note complete healing of the rickets 4(++++). c) Given Graham salt from 26th to 35th day, and a single dose of vit. D on the 28th day. Note healing of rickets similar to that in Fig. b, 4(++++). Magnification × 15.

of 0(0). *Group 3.* The healing of rickets in these animals, given Graham salt as well as vit. D_3, was as marked as that in the animals receiving vit. D_3 alone. The average score was 3.5(++++); the lowest, seen in one animal, was 2(+++). Typical photographs of epiphyses from the 3 groups are shown in Fig. 1. Thus Graham salt did not accelerate or slow down the healing of rickets. *Matrix stains.* The P.A.S. reaction was similar to that observed in the first experiment. In both the rachitic and healed animals, whether treated with Graham salt or not, the matrix between the cartilage cells and the cartilage remains in the trabeculae stained a little more intensely than the rest of the section. In contrast with the appearance in the first experiment, alcian blue stained all cartilage cells a deep blue, both in the rachitic and healed animals, and again Graham salt had no effect on the staining.

Discussion. These results, when compared with those reported previously(7), show that polyphosphates have remarkably different effects on ectopic compared with normal calcification. Thus they are able completely to prevent the calcification and associated matrix changes in the aorta which are caused by toxic doses of vit. D_3(3). They are also able to prevent calciphylaxis in skin(4). In the present experiments polyphosphates were given at dose levels sufficient to cause the above effects. Under these conditions they had no inhibitory influence on normal bone formation or on the ability of vit. D in physiological doses to heal rickets. Results obtained by Dr. I. Antener (personal communication) indicate that similar or larger doses of Graham salt given subcutaneously do not inhibit the uptake of radio Ca under the influence of vit. D. Graham salt alone (Group

II) had no effect upon the rachitic status. It is not claimed that polyphosphates will not at higher dose levels inhibit normal calcification. Indeed, calcification of chick embryos cultivated *in vitro* has been found to be inhibited by pyrophosphate(2); but such an inhibition was only attained when pyrophosphate was added to the incubating medium in relatively high concentrations (between 4 and 16 μg P/ml), thus over 20 times the concentration normally found in human plasma. Such concentrations are not obtained by injecting rats with the dose of Graham salt used in our experiments (unpublished observations), at a level which inhibits ectopic calcification but does not affect normal calcification.

There must be some fundamental significance in this difference with respect to tissues which normally calcify, and those which only do so in pathological circumstances. The simplest explanation is that these differences reflect variations in the tissue levels of enzymes breaking down condensed phosphates. Although polyphosphates(8,9,10), and polyphosphatases(11,12) have been described in mammalian tissues, detailed investigations of their distribution in calcifying and non-calcifying tissues are not available. The present results suggest that bone, in contrast to soft tissues, contains sufficient amounts of these enzymes to counteract the inhibitory effects of the administered polyphosphates. There is some evidence to suggest that one of the polyphosphatases of bone might be alkaline phosphatase(13) since several enzymes with classical phosphatase activity are now known to be able to function as pyrophosphatase(14, 15,16).

These findings that polyphosphates have a lesser action on bone calcification may have clinical significance. If polyphosphates prove to be useful in the treatment of pathological calcifications *e.g.*, arteriosclerosis and calcinosis universalis, it is important to know that doses which prevent aortic calcification and calciphylactic reactions in skin have no effect upon normal calcification sequences in bone.

Summary. Rats were given Graham salt (a long chain polyphosphate) at dose levels which prevent calciphylactic reactions in skin and the ectopic calcification of the aorta caused by large doses of vit. D. Graham salt given at this dose level to growing rats had no effect upon the normal calcification sequences in the epiphyses, nor did it affect the cure of rickets by physiological doses of vit. D. The significance of these results is discussed.

1. Fleisch, H., Neuman, W. F., Am. J. Physiol., 1961, v200, 1296.

2. Fleisch, H., Straumann, F., Schenk, R., Bisaz, S., Allgöwer, M., ibid., in press.

3. Fleisch, H., Schibler, D., Maerki, J., Frossard, I., Nature (London), 1965, v207, 1300.

4. Schibler, D., Fleisch, H., Experientia, submitted.

5. Irving, J. T., Arch. oral Biol., 1963, v8, 735.

6. Sobel, A. E., Ann. N. Y. Acad. Sci., 1955, v60, 713.

7. Irving, J. T., Schibler, D., Fleisch, H., Proc. Soc. Exp. Biol. and Med., 1966, v122, 852.

8. Grossmann, D., Lang, K., Biochem. Z., 1962, v336, 351.

9. Lynn, W. S., Brown, H. R., Biochem. Biophys. Res. Commun., 1963, v11, 367.

10. Penniall, R., Griffin, J. B., Biochim. Biophys. Acta, 1964, v90, 429.

11. Mattenheimer, H., in Kondensierte Phosphate in Lebensmitteln, Springer-Verlag, Berlin-Göttingen-Heidelberg, 1958, 45.

12. Berg, G. G., J. Histochem. Cytochem., 1964, v12, 341.

13. Russell, R. G. G., Lancet, v ii, 461.

14. Nordlie, R. C., Arion, W. J., J. Biol. Chem., 1964, v239, 1680.

15. Stetten, M. R., Taft, H. L., ibid., 1964, v239, 4041.

16. Cox, R. P., Griffin, M. J., Pancet, 1965, v ii, 1018.

Received March 22, 1966. P.S.E.B.M., 1966, v123.