VITAMIN D AND ENDOCHONDRAL OSSIFICATION IN THE RAT AS INDICATED BY THE USE OF SULFUR-35 AND PHOSPHORUS-32

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PLATES 5 AND 6

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Following the demonstration by McCollum and his coworkers (1, 2) that a condition resembling human rickets can be established in rats maintained on diets deficient in vitamin D, numerous attempts have been made to elucidate the mode of action of this vitamin.

By the use of calcium-45 in rats, Greenberg (3), Harrison and Harrison (4), and Underwood, Fisch, and Hodge (5), showed that vitamin D promotes the absorption of calcium from the gastrointestinal tract. Evidence has been presented that this vitamin also influences the deposition of calcium in bone (3, 5). Migicovsky and Emslie (6), on the other hand, found that although more calcium-45 was taken up by the femurs of normal chicks than by the femurs of rachitic chicks when the isotope was administered orally, after intramuscular administration it was retained to the same extent in both groups of chicks. They concluded that vitamin D affects the absorption of calcium from the gastrointestinal tract of chicks, but that it does not affect the mineralization of bone. Perhaps there is a species difference. Evidence for an effect of vitamin D on the mineralization of rat bone, using phosphorus-32, has been reported by Cohn and Greenberg (7) and by Morgareidge and Manly (8). An increased deposition of phosphorus in rachitic bone was apparent 54 to 72 hours after administration of vitamin D and this increased deposition was not ascribed to an increased absorption of phosphorus from the gastrointestinal tract.

Ossification in most instances involves calcification in regions of matured cartilage cells (9, 10); endochondral ossification. In rickets there are a proliferation and apparent maturation of cartilage cells, the rate of their disintegration and elimination, however, is lower than in normal animals. As a result, epiphyseal cartilage plates increase in width far beyond that seen in normal animals of the same age. Since there is a concurrent decrease in calcification of the bones, the question arises whether the accumulation of the apparently mature cartilage cells is a result of impaired calcification, or whether the calcification is imparied by a defect in the cartilage. In the present work an attempt has been made to answer this question, using sulfur-35 and phosphorus-32 in rachitic rats. The

experimental results detailed in this paper suggest that there is a primary defect in the cartilage, namely an impaired utilization of chondroitin sulfate for the subsequent processes of calcification.

Procedure

In the first set of experiments 36 albino rats of the Sherman strain were maintained on a vitamin D-deficient diet (1) for 21 days, starting with the 22nd day of life. Each of 18 animals then received 25γ of vitamin D_2[1] in 0.1 ml. of ethyl laurate by intraperitoneal injection, whereas the remainder of the animals received only the ethyl laurate. Immediately thereafter, each of the 36 animals was given in water[2] by intraperitoneal injection 0.3 μc. per gm. of body weight

TABLE I

Summary of Second Set of Experiments with Vitamin D-Deficient Rats

Experiment No.	Hrs. between injection of vitamin D_2 and $Na_2S^{35}O_4$					
	6	12	18	24	36	48
	No. of animals used at each time interval*					
1	8	8	8	8	8	6
2	8	12	8			
3‡	10	10	10	10	10	10

* In each case only half of the indicated number received vitamin D_2.

‡ For each time interval the skeletons of 5 control animals (except for the femurs and tibiae) were pooled for the isolation of sulfomucopolysaccharides as previously described (12, 13). Similar pools were made for each group of skeletons from the animals which received the vitamin D_2. The pelts were similarly grouped, pooled, and analyzed.

of sulfur-35 as sodium sulfate. 6 animals from each group were sacrificed 24, 72, and 120 hours later. Sera, urines, femurs, and tibiae were analyzed as described in preceding papers (11–13).

In the second set of experiments the procedures and the analyses were the same as outlined above except that the sulfur-35 was given at intervals of time after the vitamin, instead of being given immediately thereafter, and all the animals were sacrificed 12 hours after administration of the isotope. Experiments in this pattern were performed three times; they are summarized in Table I.

In the third set of experiments 24 vitamin D-deficient rats received ethyl laurate by intraperitoneal injection; each of an equal number of the rats was similarly given 25γ of vitamin D_2 in ethyl laurate. Phosphorus-32 as disodium phosphate[2] in water was injected intraperitoneally into 4 rats from each of the two groups 6, 12, 18, 24, 36, and 48 hours later. The dose was 1 μc. of phosphorus-32 per gm. of body weight. The animals were sacrificed 12 hours after receiving the isotope and their sera, urines, femurs, and tibiae were analyzed as described in the preceding paper (13).

To have rats as comparably deficient as possible, the deficiency was produced at the same

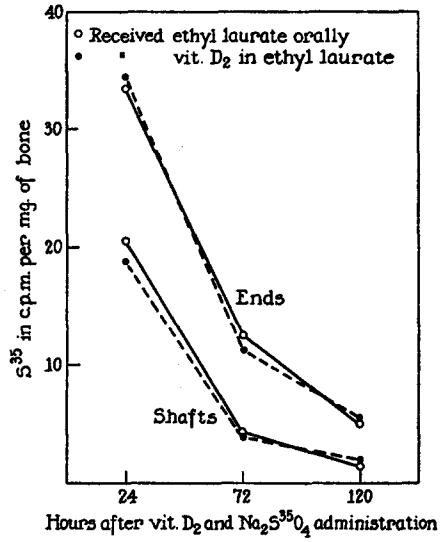
[1] The vitamin D_2, calciferol, was a commercially available crystalline product. Its molecular extinction coefficient at 265 mμ was found to be 18,600, using absolute ethanol as the solvent.

[2] The sulfur-35 and phosphorus-32 used in this investigation were supplied by the Oak Ridge National Laboratory on allocation from the United States Atomic Energy Commission. The dosage used was calculated on the basis of the data available from the supplier.

time and under the same conditions in the rats that subsequently received phosphorus-32 and in some of the rats that were given sulfur-35. In addition, to help assess the extent of the deficiency, sections of the tibiae of all the animals were stained with alizarin red (14).

RESULTS

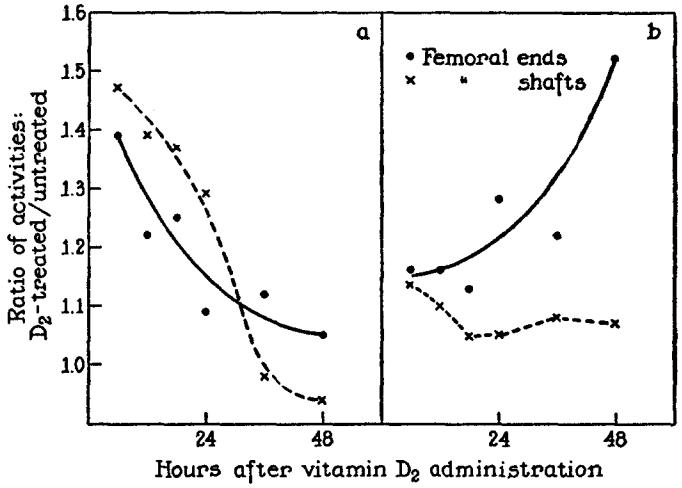
The Immediate Effects of Administering Vitamin D_2 to Deficient Rats.— The administration of vitamin D_2 concurrently with sulfur-35 was not reflected by any change in the amount of sulfur-35 excreted in the urines or in the concentra-



TEXT-FIG. 1. Apparent absence of an effect of vitamin D_2 on the disposition of simultaneously administered sulfur-35 in the ends and shafts of femurs in vitamin D-deficient rats. Each rat received by intraperitoneal injection 0.3 μc. of carrier-free sulfur-35 as sodium sulfate per gm. of body weight at the same time that either ethyl laurate or 25γ of vitamin D_2 in ethyl laurate were administered. Each value is the mean of 6 determinations.

tions of the isotope in the sera and in the femurs of the vitamin D-deficient animals. The radiochemical data on the femurs are graphically presented in Text-fig. 1. The radioautographs produced by sections of tibiae, however, did reveal that vitamin D_2 had some influence on sulfate metabolism in the skeletons (Figs. 1 to 12): The total concentration of isotope in the tibiae of the treated rats is similar to that in the tibiae of the untreated rats but the distribution of the sulfur-35 in the former is different from that in the latter. It can be seen that in the vitamin-treated animals the epiphyseal cartilage plate decreased progressively in width, whereas in the untreated rats the width of the cartilage plate remained about the same. This is well illustrated by a comparison of Fig. 2 with Fig. 5, and of Figs. 4, 5, and 6. In addition, it can be seen that at the 24th hour the epiphyseal cartilage plates of the vitamin-deficient rats,

Fig. 4, elicited a slightly greater reaction on the photographic film than did those of the vitamin D_2-treated rats, Fig. 1, while at 72 and 120 hours after the injection of vitamin D_2 the reverse was the case (Fig. 2 compared with Fig. 5, and Fig. 3 with Fig. 6). In the vitamin D_2-treated rats there was also a progressively greater deposition of sulfur-35 in regions of the diaphysis subjacent to the epiphyseal cartilage plate than in the same regions of the diaphyses from the untreated rats. Figs. 7, 8, and 9 are to be compared with Figs. 10, 11, and 12.



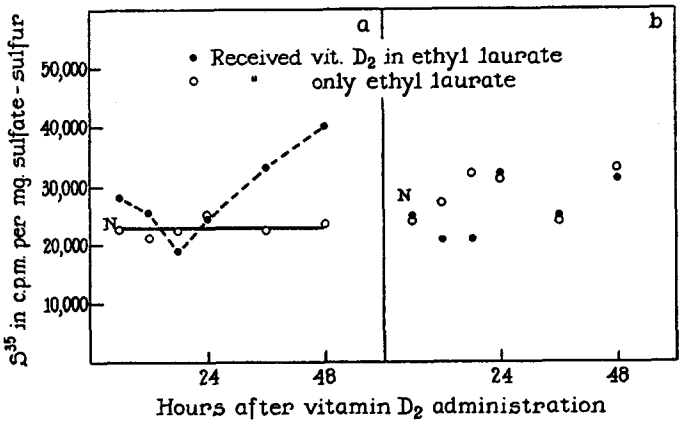
TEXT-FIG. 2. Effect of vitamin D_2 on the concentration of sulfur-35 and phosphorus-32 in the femurs of vitamin D-deficient rats. The rats received each by intraperitoneal injection 0.1 ml. of ethyl laurate or 25γ of vitamin D_2 in 0.1 ml. of ethyl laurate. Thereafter, at intervals of time as indicated on the abscissa each animal was given by intraperitoneal injection per gram of body weight (*a*) 0.3 μc. sulfur-35 as sodium sulfate or (*b*) 1 μc. of phosphorus-32 as disodium phosphate. The animals were sacrificed 12 hours after isotope administration. In the experiments with sulfur-35 the activity of the isotope was calculated as counts per minute per milligram of bone. In the experiments with phorphorus-32 the specific activities were calculated, counts per minute per milligram of phosphorus. The value of each point is the ratio of the means on all the animals examined at that time interval.

The inorganic sulfate-sulfur concentration in the sera of the vitamin D-deficient rats was found to be 2.6 to 3.5 mg. per cent as compared to 2.0 $\pm$ 0.1 mg. per cent for normal animals of this age. It was not altered following administration of vitamin D_2.

The Delayed Effects of a Single Dose of Vitamin D_2 Given to Deficient Rats as Reflected in the Uptake of Sulfur-35 and Phosphorus-32.—In the experiments in which sulfur-35 was administered at intervals of time after vitamin D_2, Text-fig. 2 *a*, about 40 per cent more sulfur-35 was taken up by the femurs of animals given the vitamin 6 hours before the isotope than by the femurs of the untreated rats. Thereafter, as the interval of time between administration of vitamin D_2

and sulfur-35 increased the amount of sulfur-35 taken up decreased; and, in the animals that were given the isotope 48 hours after the vitamin, the uptake was found to be only slightly greater than in the controls.

In Figs. 13 through 16 it can be seen that following vitamin D_2 administration the epiphyseal cartilage plate produced a denser autographic image than that produced by the cartilage plate of the tibiae from deficient rats, Fig. 17.



TEXT-FIG. 3. The specific activity of the sulfate in mucopolysaccharides isolated from the skeletons and pelts of vitamin D-deficient rats following administration of sodium sulfate-S^{35}. At intervals of time following the intraperitoneal administration of 0.1 ml. of ethyl laurate or of 25γ of vitamin D_2 in 0.1 ml. of ethyl laurate each rat received by intraperitoneal injection per gm. of body weight 0.3 μc. of sulfur-35 as sodium sulfate. The time indicated on the abscissa is the time after vitamin administration when the isotope was given. 12 hours later the tissues were taken for analysis.

(*a*) The samples of chondroitin sulfate from the skeletons resembled each other in composition. On analysis about 3.3 per cent of sulfate-sulfur, about 4.5 per cent of nitrogen, about 24 per cent of hexuronic acid, and about 20 per cent of hexosamine were found.

(*b*) The samples of the sulfomucopolysaccharides from the pelts also resembled each other in composition which was found by analysis to be about 2.9 per cent sulfate-surfur, about 5.3 per cent nitrogen, about 20.5 per cent hexuronic acid, and about 27 per cent hexosamine.

There was, however, a progressive decrease in the width of the cartilage plate, which explains, in part, the observation that the total sulfur-35 concentration in femoral ends following administration of vitamin D_2 approached that in the femurs of the untreated rats. It is of interest to note that an increased utilization of sulfate was also indicated as taking place in the diaphysis subjacent to the epiphyseal cartilage plate, Figs. 18 through 21 compared with Fig. 22.

The effect of vitamin D_2 on the specific activity of the chondroitin sulfate samples from the skeletons is shown in Text-fig. 3 *a*. As can be seen, the value for the specific activity was of greater magnitude in the samples isolated from the skeletons of the animals that had received vitamin D_2 6 hours before the

sulfur-35 than in samples from the untreated rats. It seems likely that thereafter the values fell as a concomitantly increased utilization of chondroitin sulfate took place. In turn either a further increase in synthesis or a decrease in utilization of chondroitin sulfate occurred some 36 to 48 hours after vitamin administration, so that values for the specific activity once again became greater than in the untreated rats. It is of interest that the values for the specific activity of the samples from the untreated deficient rats approximated the value for the sample isolated from the skeletons of normal rats of the same age.

The values of the specific activity of the sulfomucopolysaccharides from the pelts of the vitamin D_2-treated animals did not differ significantly from the corresponding values for the D-deficient control animals, Text-fig. 3 *b*. Furthermore, they did not diverge systematically from the value of the sample isolated from the pelts of normal animals.

The concentrations of sulfur-35 and of inorganic sulfate-sulfur in the sera were the same whether vitamin D_2 was or was not administered. The value for the inorganic sulfate-sulfur was again found to be higher than the value in normal rats. The fractions of the dose of sulfur-35 excreted in the urines as total sulfate-sulfur were also similar in the two groups.

In contrast to the immediate effect on the metabolism of sulfate in the skeletons, a definite effect on the metabolism of phosphate was not discernible until 24 hours or more after administration of the vitamin, Text-fig. 2 *b*. The effect was seen in the femoral ends but not in the shafts. In general, the same impression was obtained from the radioautographs produced by sections of tibiae from these rats, Figs. 23 through 27, as from the radiochemical data.

An increased staining with alizarin red was observed in regions of the diaphysis subjacent to the cartilage plate when the animals had received vitamin D_2 at least 24 hours previously.

No significant change in the excretion of phosphorus-31 in the urine or in the concentration of the acid-soluble fraction of the serum was produced by the injection of vitamin D_2 into either normal or deficient animals.

DISCUSSION

The results of the experiments with phosphorus-32 are in agreement with the results reported by others (7, 8). These in conjunction with the results of the experiments in which sulfur-35 was used, indicate that the impaired calcification in vitamin D-deficiency is not due to a decreased synthesis of chondroitin sulfate, (Text-fig. 3 *a*). Such a suggestion accords with the observations that overproduction of cartilage in this vitamin deficiency occurs in regions of active endochondral ossification. However, an interdependence of the process of calcification and the metabolism of chondroitin sulfate probably does exist, since in vitamin D deficiency there is an impaired utilization of chondroitin

sulfate (Figs. 1 to 12). It is likely that this aspect of the turnover of chondroitin sulfate is the one primarily influenced by vitamin D. One can further interpret the data as indicating that, secondarily, an increased synthesis of chondroitin sulfate follows and the rate at which it is synthesized, if the stimulus is of sufficient magnitude, may exceed the rate in normal animals, Text-fig. 3 *a*. Calcification may also be stimulated slightly immediately after vitamin D administration. An unmistakable effect on calcification, however, is not apparent until 24 to 48 hours later. It has also been observed repeatedly that when calcification is revived in rickets, it is first noted at the edge of the cartilage plate, which in tibiae may be separated from the diaphysis by a wide rachitic metaphysis (1, 2, 15, 16). The histological evidence as to the site of calcification and the observations that calcification is restored relatively slowly in rickets suggest that calcification is directly dependent upon a metabolic process in cartilage, which in its turn is directly dependent upon an adequate supply of vitamin D. These considerations may be regarded as additionally suggestive that vitamin D directly influences the step in which chondroitin sulfate is used in endochondral ossification.

SUMMARY

The concentration of inorganic sulfate-sulfur in the serum of vitamin D-deficient rats, 2.6 to 3.5 mg. per cent, was found to be higher than that in the serum of normal rats of the same age, 2.0 mg. per cent. No change was observed following the administration of 25 γ of vitamin D_2.

In accord with the results of others, it was found that a definitely increased deposition of phosphorus in femurs and tibiae had occurred 36 to 48 hours after the administration of vitamin D_2 to vitamin D-deficient rats. An immediate increase in the uptake of sulfate by the skeleton was found using sodium sulfate-S^{35}.

As measured by the specific activity of sulfate-sulfur in samples of chondroitin sulfate isolated from the skeletons of the vitamin D-deficient animals and from normal controls receiving equal doses of sulfur-35, the rate of synthesis of chondroitin sulfate in rachitic rats is similar to the rate in normal rats of the same age. Likewise, the incorporation of labelled sulfate into the sulfomucopolysaccharides of the pelts was found to be equal at 12 hours to that in normal rats. Following the administration of vitamin D_2 to deficient animals an increase in the rate of synthesis of the chondroitin sulfate of the skeletons was noted.

The radiochemical and radioautographic evidence suggest that there is in vitamin D-deficient rats an impaired utilization of chondroitin sulfate and that vitamin D_2 is able to accelerate this process.

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EXPLANATION OF PLATES

PLATE 5

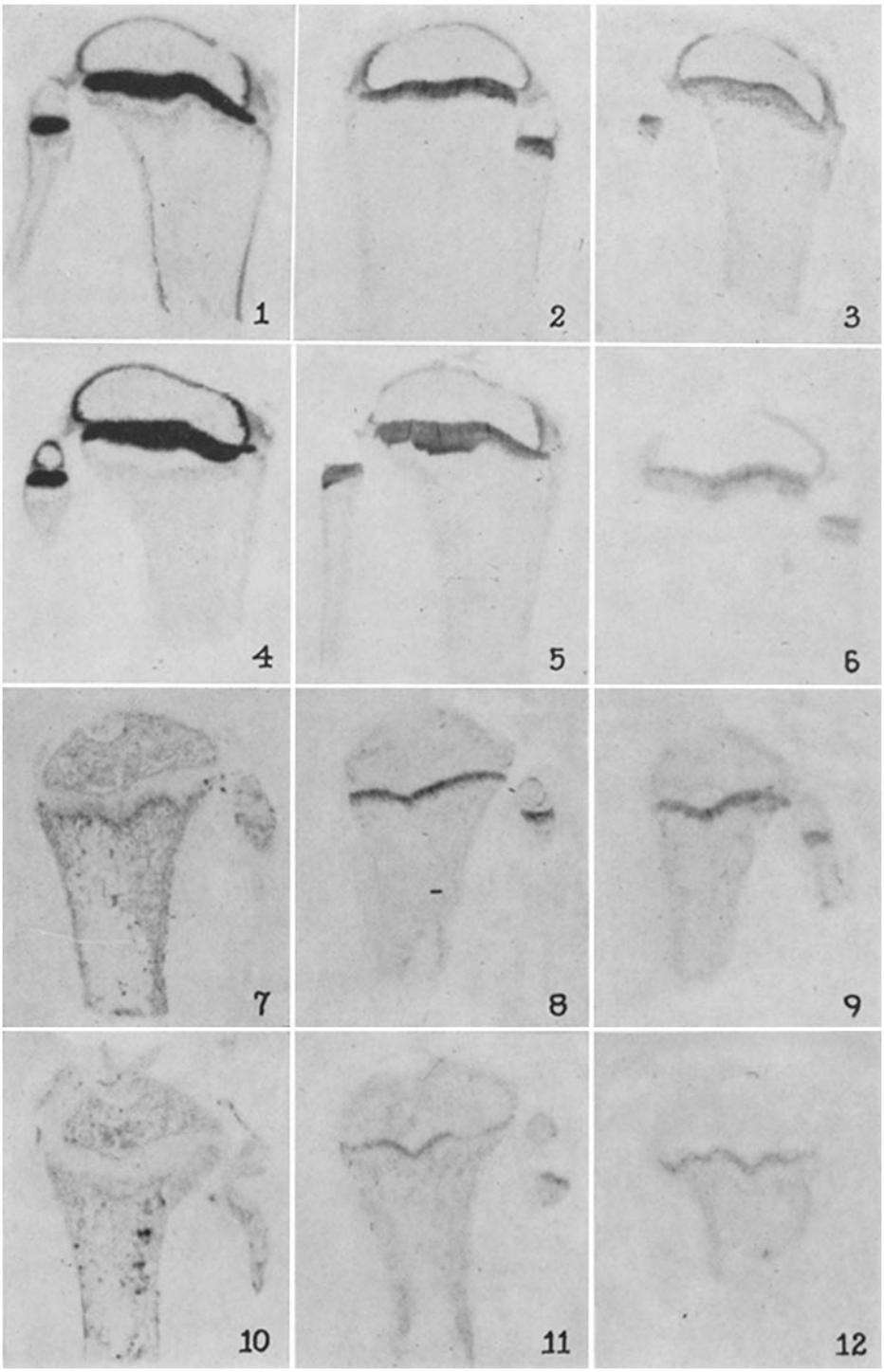
Radioautographs produced by sections of the proximal ends of tibiae fixed for 48 hours at 25°C. in a 3.7 per cent solution of formaldehyde (Figs. 1 through 6) or in a 3.7 per cent solution of formaldehyde previously saturated with barium hydroxide (Figs. 7 through 12). Kodak contrast process ortho film was exposed to the sections for 2 and 4 weeks, respectively. Each rat received 0.3 μc. of sulfur-35 as sodium sulfate per gm. of body weight.

FIGS. 1, 2, and 3. Photographs of radioautographs produced by sections of tibiae removed from vitamin D-deficient rats 24, 72, and 120 hours after the concurrent intraperitoneal injection of 25γ of vitamin D_2 in ethyl laurate and sulfur-35. × 5.

FIGS. 4, 5, and 6. To be compared with Figs. 1, 2, and 3, respectively. They are from vitamin D-deficient rats that were concurrently given only ethyl laurate and sulfur-35.

FIGS. 7, 8, and 9. Photographs produced by sections of tibiae removed 24, 72, and 120 hours after the concurrent intraperitoneal injection of 25γ of vitamin D_2 in ethyl laurate and sulfur-35. × 5.

FIGS. 10, 11, and 12. To be compared with Figs. 7, 8, and 9, respectively. They are from vitamin D-deficient rats that received a concurrent injection of ethyl laurate only and sulfur-35. × 5.



(Dziewiatkowski: Vitamin D and endochondral ossification)

PLATE 6

Radioautographs produced by sections of the proximal ends of tibiae fixed for 48 hours at 25°C. in a 3.7 per cent solution of formaldehyde (Figs. 13 through 17), in a 3.7 per cent solution of formaldehyde previously saturated with barium hydroxide (Figs. 18 through 22), and in a 3.7 per cent solution of formaldehyde previously saturated with magnesium carbonate (Figs. 23 through 27). Kodak contrast process ortho film was exposed to the sections for 2 weeks, 4 weeks, and 1 week, respectively.

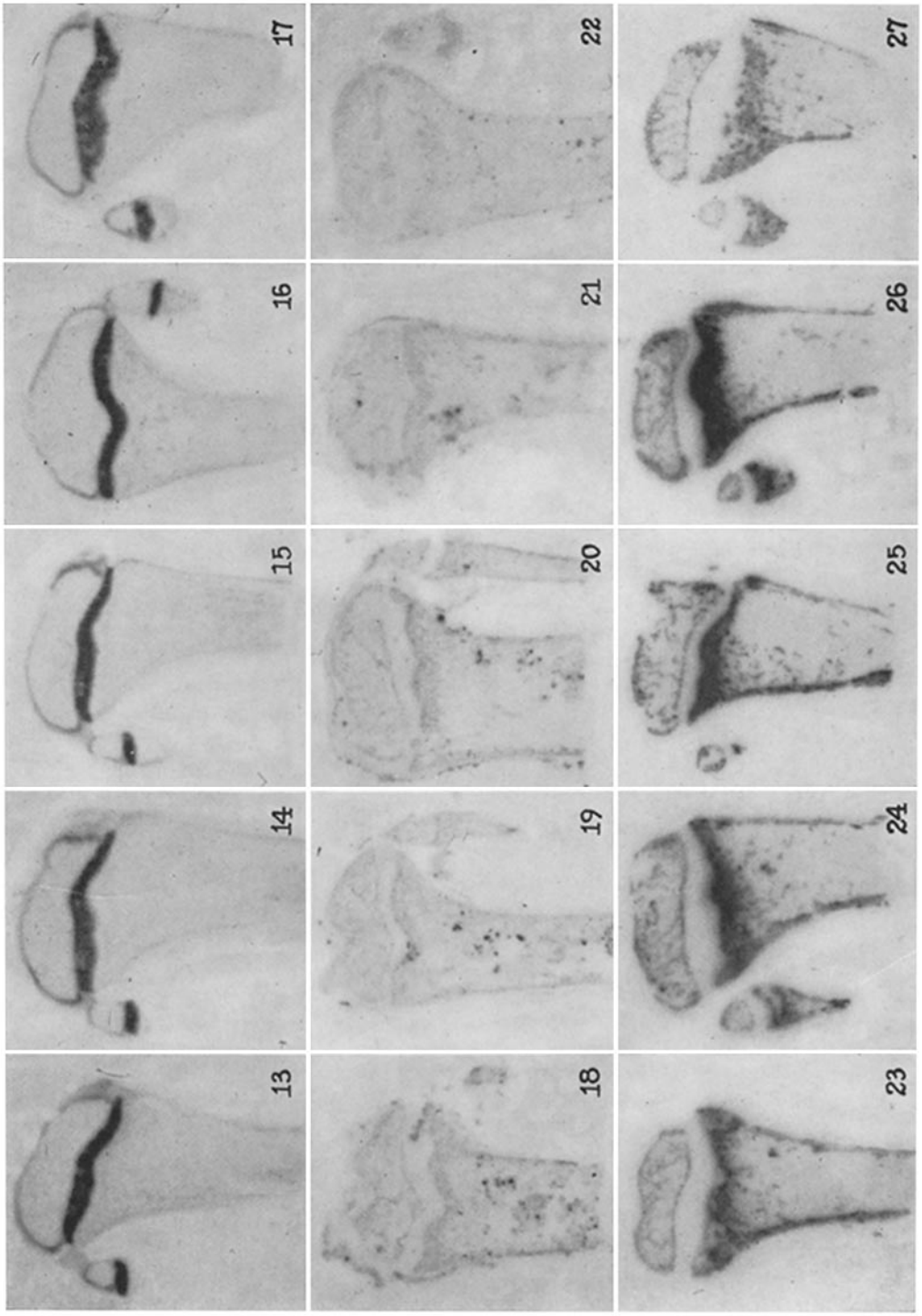
FIGS. 13, 14, 15, and 16. Photographs of radioautographs produced by sections of tibiae from vitamin D-deficient rats that received vitamin D_2. Administration of sodium sulfate-S^{35} followed 12, 24, 36, and 48 hours later, respectively. The tibiae were removed 12 hours after the administration of the isotope. × 5.

FIG. 17. For comparison with Figs. 13 through 16. It is representative of the autographs produced by sections of tibiae from untreated vitamin D-deficient rats that 12 hours before sacrifice also received 0.3 μc. sulfur-35 per gm. of body weight. × 5.

FIGS. 18, 19, 20, 21, and 22. Photographs of the radioautographs produced by sections of tibiae removed from the rats that furnished the materials for Figs. 13, 14, 15, 16, and 17, respectively. The tibiae in this instance were fixed in the formaldehyde solution previously saturated with barium hydroxide. × 5.

FIGS. 23, 24, 25, and 26. Photographs of radioautographs produced by sections of tibiae from vitamin D-deficient rats that received vitamin D_2. The rats then received 1 μc. of disodium phosphate-P^{32} per gm. of body weight 12, 24, 36, and 48 hours later. 12 hours after administration of the isotope the tibiae were removed. × 5.

FIG. 27. Included for comparison with Figs. 23 through 26. It is representative of the autographs produced by sections of tibiae from untreated vitamin D-deficient rats when they received 1 μc. of phosphorus-32 as phosphate per gm. of body weight 12 hours before sacrifice. × 5.



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