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Calcification XV. *In vitro* Calcification of Rachitic Bone Cartilage of Thyroparathyroidectomized Rats.*† (21269)

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Several authors have shown that the secretion of the parathyroid glands has a direct action on bone metabolism(1-3). Although Jones(4) could find no significant difference in serum calcium or phosphate levels, or bone ash, of control and thyroparathyroidectomized animals, Pappenheimer(5) found rickets more difficult to produce in thyroparathyroidectomized rats. In addition, it has been shown that bone citrogenase activity is decreased in parathyroidectomized young rats(6).

The purpose of this investigation was to compare the *in vitro* calcification of rachitic bone cartilage of thyroparathyroidectomized animals with that of control rachitic animals, and indirectly to indicate whether the ability to calcify *in vitro* is related to bone citrogenase.

Methods. Albino rats of an original Wistar strain were kept on Bills Stock Diet(7). Each litter was divided into 3 groups: control animals on the stock diet; control animals on a rachitogenic diet(8); and thyroparathyroidectomized animals on a rachitogenic diet. (It was impossible to maintain thyroparathyroidectomized animals on the stock diet as a further control, since these animals died of tetany.) The diet employed had a calcium to phosphorus ratio of 10.3, the calcium being 1.27% of the diet. The diet had a complete vitamin supplement, exclusive of vit. D. Thyroparathyroidectomy was performed 24 hours after the experimental diet had been started, when the rats were 21 to 23 days old. The effectiveness of thyroidectomy was checked by the histologic examination of the pituitary gland as described by Selye(9). If

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TABLE I. Serum Calcium and Phosphate Determinations and Weight Change in Stock Control, Rachitic Control, and Rachitic Thyroparathyroidectomized Rats.

Group	No. of animals	Calcium, mg %*	Phosphate, mg %*	Wt change, g*
Stock control	4	8.8	8.9	96.1
Rachitic "	16	9.8	2.4	15.8
Rachitic thyroparathyroidectomy	25	10.5	2.7	8.5
Statistical analysis, P values†				
Comparing stock control with rachitic control		.20	<.01	<.01
Comparing stock control with rachitic thyroparathyroidectomy		.09	<.01	<.01
Comparing rachitic control with rachitic thyroparathyroidectomy		.29	.30	.07

* Mean values.

† P = Probability that difference between 2 means is due to chance. When P $\leq$ .05, difference between means may be considered statistically significant.

the so-called "thyroidectomy" cells were found it was assumed that parathyroidectomy was complete, since in the rat the parathyroids are embedded in the thyroid gland and are constant in number, one in each lobe(10). Animals failing to show the histologic changes in the pituitary were eliminated from the experimental data. After 25 to 28 days on the experimental regime, the animals were X-rayed to check for rickets, and then sacrificed by exsanguination. Serum calcium was determined by the micromethod of Sobel and Sobel(11), and serum phosphate by a modification of the Fiske and Subbarow method (12), allowing the use of 0.1 ml of serum. The statistical analysis of the serum calcium and phosphate determinations and the weight changes were made using Fisher's test of the significance of small samples(13). The bones were examined histologically for rickets. *In vitro* calcification studies were performed on slices of tibia suspended in a calcifying solution as described by Goldenberg and Sobel (14). The composition of the calcifying solution was as follows: NaCl, 0.07 M; KCl, 0.005 M; $NaHCO_3$, 0.022 M; $CaCl_2$, 0.0025 M; and NaH_2PO_4 + Na_2HPO_4, 0.00161 M per liter. The evaluation of the degree of calcification was that described by Sobel and his coworkers(15-17) (Table II). Lack of calcification was graded 0(0); maximum calcification 4(++++); the length of the line or deposit was designated by the +'s and its thickness or width by the number preceding the parenthesis. Each figure given in the tables was the result of 3 to 6 experiments; the fractional values result from the method of averaging. Magnifications of 32× and 96× were usually employed.

Results. It is immediately apparent from Table I that both the rachitic control and the rachitic thyroparathyroidectomized animals showed markedly depressed serum phosphate levels which, compared with the stock control animals, was statistically significant. This applies also for the ability to gain weight. However, no statistically significant difference was noted for serum phosphate or weight gain between the 2 groups of rachitic animals, nor for serum calcium among any of the groups of animals.

Examination of the bones histologically revealed normal bone growth and epiphyseal cartilage in the stock control animals and active rickets in both groups of animals on the rachitogenic diet.

In vitro calcification occurred in all animals on the rachitogenic diet, these animals having shown X-ray and blood chemical evidence of rickets. No *in vitro* calcification occurred in the epiphyseal cartilage of the stock control animals. While the estimates of the degree of *in vitro* calcification may vary slightly with

TABLE II.
In Vitro Calcification of Rachitic Bones.*

Group	Degree of calcification
Stock control	No rickets
Rachitic "	1.9 (3.6+)
Rachitic thyroparathyroidectomized	2.2 (3.6+)

* Mean values.

each observer, all estimates were made by the same observer, so that any error would apply to all groups. No significant difference was noted between the rachitic control and rachitic thyroparathyroidectomized animals.

Discussion. The ability of rachitic bone cartilage of thyroparathyroidectomized rats to calcify *in vitro* was comparable to that of the control rachitic animals, since both showed the same type and degree of response. Barnicot (1), Ingalls(2), and Munson(3) have presented evidence of a direct action of the secretions of the parathyroid gland on bone metabolism, and Perkins and Dixon(6) found a diminution of the bone citrogenase of young parathyroidectomized rats. The implication is that these factors do not cause changes in the intimate system responsible for calcification from inorganic solutions. The experiments in the present study utilized an inorganic substrate. It is possible that if the system were tested with organic substrates differences might be found. Thus, one may conclude that parathyroid hormones are not essential for the production of the "local factor" of calcification, and that their absence, in spite of the diminished bone citrogenase, does not block this mechanism.

The high calcium-low phosphorus diet employed in this experiment produces rickets by failing to supply sufficient phosphate for the deposition of bone salts. Since function of the parathyroid glands with such a large phosphate deficit is low(18), the concept that the primary action of the parathyroid gland is through the control of the excretion of phosphate in the urine(19), could not be tested. Our results are in accord with those of Jones (4), in that thyroparathyroidectomy had no protective action in conservation of phosphate, as reflected in the serum phosphate levels, or in lessening the severity of the rickets. Extirpation of the parathyroid glands has a profound effect on the serum calcium and phosphate when animals are maintained on a diet with a normal calcium to phosphate ratio. Moreover, it has been shown that parathormone increases the serum citric acid level(20), the response to comparable doses of parathormone being greater in thyroparathyroidectomized animals. Thus, an influence on serum calcium and phosphate levels and the ability to develop rickets may be elicited with a diet containing a lower calcium to phosphate ratio.

Summary. 1. Rickets was produced in rats thyroparathyroidectomized at 21 to 23 days of age as readily as in control animals. Both groups were on a rachitogenic diet with a calcium to phosphorus ratio of 10.3. X-ray routine histologic examination, and blood chemical analysis for calcium and phosphate were comparable in both groups of rachitic animals. 2. Rachitic bone cartilage from thyroparathyroidectomized rats calcified to the same degree as the rachitic bone cartilage from the controls. 3. Under the conditions of this experiment, neither the secretions from the parathyroid glands nor any enzyme system such as that involving bone citrogenase is essential for satisfactory *in vitro* calcification.

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Response of Serum Inorganic Phosphate to Insulin in Normal and Diabetic Subjects. (21270)

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Wigglesworth *et al.*(1) showed that injection of insulin produced a fall in blood inorganic phosphate in rabbits. A number of investigators have confirmed these findings, both in whole blood and in serum(2-11). Similar results were obtained by Harrop and Benedict (2) and by Perlzweig *et al.*(4) after oral administration of glucose to normal subjects. Bolliger and Hartman(8) pointed out that this phenomenon did not take place in pancreatectomized dogs and attempted to apply this finding to diagnosis of diabetes mellitus(12). We worked out a procedure which we termed "Delta G/Delta P test" for the study of carbohydrate metabolism based upon changes in blood sugar and serum inorganic phosphate* from their initial level(13). The test is carried out by injecting intravenously, at a speed of about 10 ml/minute, 1 ml/kilo body weight of a 50% glucose solution, taking samples for blood sugar and phosphate analysis before and 30, 45 and 60 minutes after injection. Subjects are kept at rest for one half hour before and during test. A Delta G at 45 minutes more than + 50 mg/100 ml is taken to indicate decreased glucose removal. In normal subjects Delta P at 45 minutes is greater than —0.30 mg/100 ml. In 45 normal subjects the *averages* were + 16 for Delta G 45′ and —0.67 for Delta P 45′(14). We used this test for classifying 54 diabetic patients into 2 groups: Group I, with a defective glucose-phosphate fall and group II, with a normal fall (15). We assumed that patients from the first group did not produce insulin (69% of patients tested), whereas patients from the second group (31%) were supposed to have a normal or near normal insulin reserve. Until now, only pregnancy(16) and fever and surgical stress(17) have been shown to inhibit at least partially the glucose-phosphate fall in non-diabetic subjects. We do not imply that insulin is the only factor concerned in the glucose phosphate fall. Thus, a glucose phosphate fall has been found to occur in pancreatectomized dogs(18) and in one pancreatectomized human being(19); in both cases, however, the blood sugar had to be raised above 500 mg/100 ml before the glucose phosphate fall took place.

Thus a working hypothesis consistent with available data would seem to be that glucose phosphate fall is mainly dependent upon insulin secretion by the pancreas, although factors other than insulin probably enter also into play in the production of the phenomenon. If this hypothesis were correct, one would expect a group of diabetics showing glucose phosphate fall and a group not showing it, to exhibit roughly equal insulin glucose responses. This would support the theory further, although it would not constitute definite proof for it. We investigated therefore the effect of insulin upon phosphate of diabetic patients previously classified according to their glucose phosphate fall, comparing

* By "phosphate," we mean "serum inorganic phosphate," by "insulin-phosphate fall" the fall in serum inorganic phosphate following injection of insulin and by "glucose-phosphate fall" the fall in serum inorganic phosphate following injection of glucose.