bearing the V2 transplantable tumor treated with the vasodilator PETN alone. Because of the longer survival time of the animals there was a greater opportunity for the development of cytotoxic antibodies. We are now developing evidence to verify or deny this assumption.

Summary. 1. When mice bearing established, solid Ehrlich carcinomas were treated with pentaerythritol tetranitrate, there was an increase in the localization of Evans blue dye in the tumors. Epinephrine caused a decrease in the dye recovered from the tumors. 2. When mice bearing solid Ehrlich tumors were treated with pentaerythritol tetranitrate, heterologous antitumor serum (adsorbed) and normal guinea pig serum, 60% of the tumors regressed if treatment was continued for 5 weeks or longer. The administration of guinea pig serum may have been unnecessary.

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Influence of Vitamin D and Starvation on Cartilage Glycogen and Serum Calcium and Phosphate. (32251)

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The presence of glycogen in the cartilage has been known for over a century(1); however, its exact role in calcification of cartilage is not yet understood. Glycogen breakdown in cartilage is suggested as (i) being involved in the synthesis of phosphoric esters which serve as substrates for alkaline phosphatase producing a local increase in phosphate concentration(2), (ii) being concerned with the formation or alteration of the cartilage matrix (3), (iii) providing energy for the eventual calcification of the cartilage(4). Histochemical methods have indicated that glycogen is present in large concentrations in the cells of the tibial epiphyseal cartilage which have most recently hypertrophied and that it suddenly decreased just prior to their calcification (5,6). Histochemical methods are useful in determining the distribution of glycogen in the various zones of the epiphyseal cartilage plate, but they are semi-quantitative. Quantitative studies have not been reported on the glycogen content of the rachitic rat cartilage at various stages of healing induced by vitamin D administration or by starvation; hence the present study was carried out. This report also deals with the utilization of glucose by rachitic cartilage following vitamin D treatment and attempts to correlate these changes with evidence of healing and alterations in serum calcium and inorganic phosphorus following vitamin D treatment or starvation.

Methods and materials. Weanling albino rats of Holtzman strain, weighing about 60 g, were fed a modified Schneider-Steenbock rachitogenic diet(7) having normal content of calcium (545 mg per 100 g diet) and a low phosphorus content (22 mg/100 g diet). At the end of 2 weeks of the regime the animals were distributed into 3 groups. Animals in Group 1 acted as rachitic controls. Animals in Group 2 were administered *per os* a single large dose of vitamin D_2 in oil (4000 I.U.) and were sacrificed seriatim at 24, 48, 72 and 96 hours following vitamin D administration. Animals in Group 3 were starved for

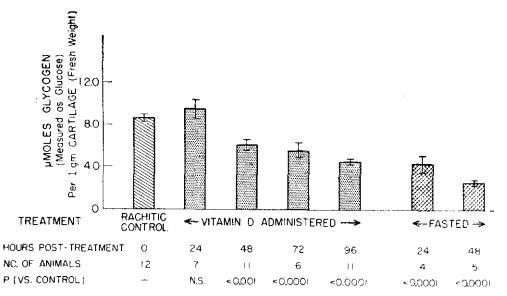

FIG. 1. Glycogen content of rachitic rat tibial epiphyseal cartilage during healing induced by vitamin D and starvation.

24 or 48 hours. All the animals were sacrificed by decapitation and blood was collected for determination of calcium(8) and inorganic phosphorus(9). The proximal head of the tibia was excised and cut longitudinally. One half of a tibia from each rat was fixed in 10% aqueous formalin solution for staining by the von Kossa method to determine the degree of *in vivo* healing. Epiphyseal cartilage from the remaining tibia was removed, cleaned of all connective tissue and quickly frozen in liquid nitrogen. The tissue was then weighed while frozen and dropped in 30% KOH solution. Glycogen was precipitated, washed with alcohol according to the method of Good, Kramer and Somogyi(10) and glucose was determined following acid hydrolysis(11).

Oxidation of glucose by the cartilage tissue obtained from rachitic controls and animals administered vitamin D 96 hours previously, was determined as follows: Pooled cartilage from 4 to 6 rachitic or vitamin D treated animals was sliced in small pieces and weighed. Between 50 and 100 mg of cartilage tissue was placed in an incubation vessel containing 2.5 ml Krebs-Ringer bicarbonate buffer medium plus 100 mg% glucose and 200 mg% gelatin.† To each vessel was added 0.4 μg glucose-1-^{14}C in 0.2 ml solution (sp. act. of labeled glucose was 10 to 15 μc per mg). The vessels were gassed with a mixture of 95% O_2 + 5% CO_2 and incubated for 3 hours at 37°C in a Dubnoff Shaker (90 to 100 oscillations per minute). Incubation was stopped by adding 2.5 ml 2 N H_2SO_4 and the carbon dioxide evolved was collected in 0.75 ml hyamine solution (purchased from Packard Instrument Co., Downers Grove, Ill.) as described earlier(12). The hyamine solution was transferred quantitatively to a counting vial using toluene (containing 0.4% PPO and 0.01% POPOP), and the radioactivity counted in a scintillation counter. From the total amount of glucose present in the medium, the total radioactivity added to it and the ^{14}C count in the CO_2 collected, the amount of glucose oxidized by the cartilage tissue was calculated(13).

Results. As shown in Fig. 1 cartilage obtained from the control rachitic rats contained 8.61 μmoles glycogen (measured as glucose) per gram wet weight of the tissue. At 24 hours following vitamin D administration there was no change in the glycogen content of the cartilage. At 48 hours following vitamin D treatment the glycogen content of the cartilage decreased significantly to 6.16 μmoles and at 72 and 96 hours after vitamin D treatment the values further decreased to 5.50 and 4.45 μmoles respectively. The decrease in glycogen content of rachitic cartilage was considerably greater during fasting. At 24 and 48 hours of fast the glycogen content was 4.45 and 2.50 μmoles respectively.

Table I shows that cartilage obtained from vitamin D treated rats utilizes glucose to a greater extent ($p = <0.02$) than that obtained from untreated controls; there being a 23.4% greater oxidation of glucose by vitamin D treated rat cartilage. In a few experiments (not shown here) it was found that glucose oxidation was enhanced by both control and vitamin D treated rat cartilage in the presence of insulin (0.1 unit per ml medium). This result confirms the findings of Bernstein *et al*(14).

Table II shows that no significant changes were observed in the serum calcium level in rachitic rats following vitamin D administration. However, there was a marked decrease in serum calcium level when the rachitic rats were fasted for 48 hours ($P = <0.0001$), confirming the finding of earlier workers (4,15).

The serum inorganic phosphorus of rats

† In several experiments (not included here) effect of insulin on the cartilage was studied, and gelatin was added to the medium to prevent adsorption of insulin on the glass surface(12).

TABLE I. Effect of Vitamin D Administration (4000 I.U.) on Oxidation of Glucose by Rachitic Rat Cartilage.

Exp No.	Rachitic controls (μmoles glucose oxidized/gram cartilage/3 hr)	Vit. D (4000 I.U.) treated (96 hr) (μmoles glucose oxidized/gram cartilage/3 hr)	Difference due to vit. D treatment (μmoles glucose oxidized/gram cartilage/3 hr)	% Increase due to vit. D	P
1	.931	1.231	.300	32.2	
2	.667	.828	.161	23.1	
3	.878	1.030	.152	17.2	
4	.982	1.189	.207	21.1	
Mean	.865 ± .069*	1.070 ± .085*		23.4	<.02

* Standard error of mean.

administered vitamin D 48 hours earlier was 5.84 mg per 100 ml compared with 4.04 mg in the untreated control; this difference was not statistically significant. This is to be accounted for by the extremely low P content of the diet. Fasting, however, produced marked elevation in the inorganic phosphorus level, probably as a result of tissue breakdown. After 48 hours of fasting the serum phosphorus level was 12.86 mg ($P = <0.0001$).

The product of calcium and phosphorus in the serum of the untreated rachitic controls was 41.6, whereas at 24, 48, 72 and 96 hours after vitamin D treatment the values were 46.1, 60.7, 60.8 and 57.1, respectively. The von Kossa silver staining and radiological examination of the metatarsals indicated that the earliest signs of "healing" were evident at or after 48 hours of vitamin D treatment. Perhaps the healing is initiated when the product of calcium and phosphorus rises to above 60. During fasting the Ca × P product was markedly elevated, at 24 and 48 hours the values were 59.6 and 66.8, respectively. Although no evidence of healing was observed at 24 hours, signs of advanced stage of healing were noted at 48 hours of fast.

Discussion. The decrease in glycogen content of the rachitic cartilage following vitamin D treatment and stravation confirms the histochemical observations of earlier workers (4,5,6). Although decrease in glycogen content occurs prior to or concomitant with the onset of healing process, it would be more pertinent to study the changes in the glycogen content of the various zones of the hypertrophic cartilage cells in order to relate the changes in glycogen with the areas where first signs of healing take place. Histochemical studies indicate that the greatest concentration of glycogen is in the cartilage cells which have most recently hypertrophied. It is in this area that the marked decrease in the glycogen reaction is observed just prior to the "first signs of healing" induced by vitamin D. Quantitative histochemical studies are in progress to compare the distribution of glycogen in the various zones of the rachitic cartilage during healing.

TABLE II. Changes in Serum Calcium and Phosphate During Healing Induced by Vitamin D Administration (4000 I.U.) and Starvation.

Hours post-treatment	Vit. D administered: 0 control	24	48	72	96	Fasted: 24	48
Serum calcium (mg/100 ml)	10.3 (6)	11.1 (11)	10.4 (6)	11.5 (10)	12.3 (6)	9.4 (5)	5.2* (8)
S.E. mean	±1.00	±.74	±.81	±.55	±.42	±.32	±.20
Serum phosphate (mg/100 ml)	4.04 (4)	4.15 (8)	5.84 (4)	5.29 (7)	4.64 (5)	6.35 (2)	12.86*(4)
S.E. mean	±.71	±.47	±1.02	±.27	±.14	±.55	±.35
Ca × P product	41.6	46.1	60.7	60.8	57.1	59.6	66.8

* Denotes that difference from control is significant ($p = <0.0001$). Number in parentheses denotes number of rats.

The increase in glucose oxidation by the cartilage obtained from vitamin D treated rat provides evidence of increased metabolic activity of the cartilage cell during healing induced by vitamin D. Elevated citrate content of the epiphyseal cartilage following vitamin D administration(16,17) may be cited in support of the importance of the glycolytic process.

Bernstein and co-workers(14) have observed that cartilage oxidizes C-1 glucose to a greater extent than C-6 glucose. In fact on the basis of the different rates of oxidation of C-1 and C-6 labeled glucose by rat cartilage, these workers suggested the presence of an active hexosemono-phosphate (HMP) pathway in the cartilaginous tissue. This pathway has been recognized as an important source of reduced TPN, which is presumably required for the synthetic activity of the cell(18). It would seem reasonable to suggest that increased glucose or glycogen utilization stimulates the cartilage cell in synthesizing the appropriate type of matrix which subsequently undergoes calcification.

The marked decrease in the serum calcium levels of fasted rachitic rats is difficult to explain. We have observed that neither the feeding of calcium to the fasting animal nor administration of vitamin D precludes this fall.

Summary. Glycogen content of the rachitic rat tibial epiphyseal cartilage is determined during healing induced by vitamin D or starvation. Rachitic rat cartilage contained 8.61 μmoles glycogen per gram net weight of tissue. Following a single dose of vitamin D (4000 I.U.) the glycogen content was not significantly altered at 24 hours, but was decreased to 6.16, 5.50 and 4.45 μmoles at 48, 72 and 96 hours respectively. Fasting caused a rapid decrease in the glycogen content to 4.45 and 2.50 μmoles at 24 and 48 hours respectively. Vitamin D treated rat cartilage oxidized glucose to a greater extent than the corresponding untreated controls. It is suggested that glucose and glycogen metabolism stimulates the formation of calcifiable matrix by the cartilage cell. Fasting for 48 hours caused a marked fall in serum calcium and a rise in serum phosphorus level.

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