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Mode of Action of Vitamin D. The Effect of Vitamin D Deficiency in the Rat on Anaerobic Glycolysis and Pyruvate Oxidation by Epiphyseal Cartilage

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The role of vitamin D in calcification of bone still awaits elucidation; this is not to be wondered at since the mechanism of normal calcification is still far from clearly understood. The demonstration of alkaline phosphatase in bone and hypertrophic cartilage by Robison (1923) and Robison & Soames (1924) led to the suggestion that the enzyme promoted calcification by causing a local increase in concentration of inorganic phosphate. That this hypothesis did not explain fully the process of calcification was recognized by Robison, who postulated the presence of a second mechanism which was susceptible to the action of cyanide, fluoride and iodoacetate (Robison, MacLeod & Rosenheim, 1930; Robison & Rosenheim, 1934). Though Robison & Rosenheim (1934) did not make any suggestion about the nature of this second mechanism, in the light of the later knowledge of the action of the above-mentioned inhibitors in the intermediate metabolism of carbohydrates, it may be permissible to conclude that Robison's second mechanism must be very intimately connected with glycogen metabolism in hypertrophic cartilage.

Gutman & Gutman (1941) provided experimental evidence associating glycolysis with calcification occurring in calcifying cartilage. They demonstrated the presence of phosphorylase in significant concentration in the epiphyses of growing rats and rabbits and suggested that this enzyme could initiate conversion of the glycogen of hypertrophic cartilage cells into phosphoric ester substrates for cartilage phosphatase. Later Gutman, Warrick & Gutman (1942) observed that the addition of 0.0005 M phloretin, which is a specific inhibitor for

phosphorylase, completely blocked the calcification of rachitic cartilage in solutions containing calcium and inorganic phosphorus in concentrations of 8 and 5 mg. respectively/100 ml. of the medium. The addition of glucose 1-phosphate (G-1-P) restored the capacity of the cartilage to calcify even in the presence of phloretin. The authors concluded, therefore, that phosphorylase was necessary in the calcification mechanism. Further work with the use of inhibitors like cyanide, iodoacetate and fluoride suggested that the calcification of cartilage involved phosphorylative glycogenolysis at least to the point of phosphopyruvate formation.

Additional evidence in support of the important role of glycogenolysis in initiating calcification was forthcoming from the work of Albaum, Hirschfeld & Sobel (1952*a*) who observed that adenosine triphosphate (ATP), which is essential in these enzymic processes, was present in epiphyseal cartilage of rat tibiae at the same level as was normally found in liver and kidney (9.2–17.2 µg./rat, i.e. two tibiae). Albaum, Hirschfeld & Sobel (1952*b*) further demonstrated the presence of most of the glycogenolytic enzymes in epiphyseal cartilage and several of the phosphorylated intermediates by a chromatographic technique.

Although the role of glycogenolysis in calcification had thus been clearly demonstrated, current opinion appears to view this process only from the standpoint of its being able to provide phosphoric esters, which under the influence of alkaline phosphatase would provide the requisite concentration of inorganic phosphorus for the deposition of the bone salt. This concept, which lays emphasis on the

concentrations of calcium and inorganic phosphorus alone, does not fully explain the observed facts.

Dikshit & Patwardhan (1952) have shown that the epiphyseal cartilage of rachitic rat after treatment with vitamin D can undergo calcification *in vitro* in media in which the cartilage of untreated rats fails to calcify. These authors suggested that, on administration of vitamin D, some changes as yet ill understood take place in the cartilage which impart to the latter the capacity to calcify. A histochemical examination of rachitic epiphyseal cartilage from rats before and after treatment with vitamin D has recently been made by Ramalingaswami, Sriramachari, Dikshit, Tulpule & Patwardhan (1954). As a result of these studies, the authors concluded that failure of calcification in vitamin D deficiency probably was due to a disturbance or arrest of the metabolism of glycogen in the rachitic cartilage. Experimental evidence that such disturbance does in fact take place in induced rickets in rats is described in the present communication.

EXPERIMENTAL

The anaerobic breakdown of glycogen and hexose phosphates and the aerobic oxidation of pyruvate by the epiphyseal cartilage were studied by the Warburg manometric technique with the use of standard Warburg apparatus using flasks of 15 ml. capacity with centre well and one side chamber. The anaerobic respiration of cartilage was studied in the atmosphere of nitrogen and for aerobic oxidation of pyruvate, O_2 was used as the gas phase. All observations were made at 37°.

Preliminary trials with slices and homogenates of the epiphyseal cartilage of normal young rats showed that homogenates gave results which were not reproducible probably owing to inadequate homogenization of the cartilage. Hence all experiments described in this paper have been carried out with cartilage slices. Utter, Wood & Reiner (1945) had observed that the addition of ATP, diphosphopyridine nucleotide (DPN), Mg^{2+} and nicotinamide to buffered substrate was necessary to evoke maximum glycolytic activity of cell-free extracts and homogenates prepared from the central nervous system. Preliminary experiments showed that the same cofactors favourably influenced the glycolytic activity of cartilage and hence, they were incorporated in the reaction medium in concentrations given below. In experiments with pyruvate, cytochrome c, with ATP and DPN, was found to improve the rate of oxidation. The reaction mixtures for glycolysis and aerobic oxidation experiments were made up as follows:

(a) *Anaerobic experiment: gas phase, nitrogen*

	ml.
(i) Na-K phosphate buffer, pH 7.3 (0.03M)	1.0
(ii) $NaHCO_3$ (0.36M)	0.2
(iii) $MgSO_4$ (0.24M)	0.1
(iv) ATP (0.0021M)	0.1
(v) DPN (0.005M)	0.1
(vi) Nicotinamide (0.6M)	0.1
(vii) Fructose 1:6-diphosphate } or Glycogen } or G 1-P } or G 6-P }	(0.0075M) 0.2

The total volume was made up to 3.2 ml. with distilled water. Temperature equilibration was obtained by shaking the reaction mixtures for 10 min. before the cartilage slices were tipped into the chamber from the side arm. Readings were taken every 5 min. for half an hour. Enzyme activity was expressed as $Q_{CO_2} = \mu l. CO_2$ liberated/mg. dry wt./hr.

(b) *Aerobic experiment: gas phase, oxygen*

	ml.
(i) Krebs-Ringer bicarbonate buffer without $CaCl_2$ (pH 7.4)	1.0
(ii) ATP (0.0021M)	0.1
(iii) DPN (0.005M)	0.1
(iv) Cytochrome c ($3 \times 10^{-5}M$)	0.1
(v) Sodium pyruvate (0.05M)	0.3

The total volume was made up to 3.0 ml. with distilled water; 0.2 ml. of 10% (w/v) aqueous KOH was placed in the centre well. The cartilage slices were added to the main chamber from the beginning. Equilibration time was 10 min. Readings were taken every 20 min. for 1 hr.

The enzyme activity is expressed as $q_{O_2} = \mu l. of O_2$ consumed/100 mg. dry tissue/hr. The results of pyruvate oxidation with the epiphyseal cartilage of normal 4- to 5-week-old rats are given in the last column of Table 2.

Experimental animals. 180 young and healthy albino rats weighing about 45 g. and between 24 and 28 days of age were caged individually and kept in a room where no direct or reflected sunlight could penetrate. All the animals were fed rachitogenic diet (Dikshit & Patwardhan, 1947) with Ca:P as 10:1. The control rats received 25 i.u. of vitamin D/rat/day. At intervals of 5, 10, 15 and 20 days a fixed number of rats in each group was killed, tibial cartilages were dissected out, cut longitudinally in thin slices and immediately used for manometric observations. In rachitic animals the glycolytic activity was estimated on cartilage slices of individual rats since sufficient amount of cartilage could be obtained from each animal, but in case of vitamin D treated controls the cartilage from two animals had to be pooled for each trial. On the other hand, owing to very low oxidative capacity of the cartilage, slices from two animals in the rachitic group had to be pooled for experiments with pyruvate.

RESULTS AND DISCUSSION

Bywaters (1936a, b), Dickens & Weil-Malherbe (1936), Hill (1940) and Laskin, Sarnat & Bain (1952) have studied the respiratory activity in cartilage with the use of manometric technique. Their results pointed to the fact that the glycogenolytic process in the cartilage was predominantly anaerobic. Even so, the level of glycolytic activity of the epiphyseal cartilage of the tibia observed in the present investigation (Table 1) was much higher than that reported by the above mentioned workers. This might be due to the fact that ATP, DPN and Mg^{2+} were added by us to the reaction medium in suitable concentrations and that glycogen and phosphorylated hexoses were used as substrates. Hill (1940) and Laskin *et al.* (1952) observed a fourfold increase in the oxygen uptake of rabbit and horse articular cartilage when methylene blue was added to the reaction mixture. They also produced evidence to

show that the formation of pyruvate takes place in the metabolic process.

The results in Table 1 indicate that in the initial stages of rachitogenesis, the glycolytic activity of rachitic cartilage with all the substrates tried was considerably higher than that exhibited by the cartilage from vitamin D treated rats. However, as rickets became well established, a fall in glycolytic activity to the level of vitamin D treated controls was witnessed on the 15th day. There were fairly large variations in the values of Q_{CO_2} obtained at different stages of rachitogenesis in comparable animals. On the whole, however, the observed differences between the anaerobic glycolytic activity of cartilage in the rachitic and vitamin D treated animals were not found to be statistically significant. If anything, the values of Q_{CO_2} with G 1-P and G 6-P as substrates were slightly higher after 15 days in rachitic cartilage than in that of vitamin D treated animals.

One can tentatively conclude from these experiments that the mechanism of anaerobic breakdown of glycogen in cartilage is not disturbed in vitamin D deficiency.

The results of experiments dealing with pyruvate oxidation are summarized in Table 2.

As mentioned earlier, q_{O_2} (pyruvate) was very much less than the glycolytic activity displayed by rachitic cartilage. There was a difference in the early stages of rachitogenesis between the endogenous respiration of the cartilage and the rate at which the added pyruvic acid was oxidized. Statistical analysis of the results shows that this difference was more marked in vitamin D treated rats than in deficient animals ($P < 0.001$), as observed on the fifth day after placing the rats on rachitogenic diets. As the rats continued on the diet and with the establishment of rickets the capacity to oxidize pyruvic acid suffered a decrease in rachitic rat cartilage and after 20 days on the rachitogenic diet, the rachitic cartilage seemed to have lost altogether the capacity to oxidize pyruvic acid. On the other hand, in rats treated with vitamin D,

pyruvate oxidation remained mostly unimpaired, as would be evident from a comparison of these results with those on cartilage of normal young rats given in Table 2. These results, therefore, clearly indicate that in vitamin D deficiency the rachitic epiphyseal cartilage of rats is unable to oxidize pyruvic acid, thus confirming the suspicions expressed in the earlier paper by Ramalingaswami *et al.* (1954).

Pyruvate oxidation trials were carried out with liver and kidney homogenates prepared according to the method of Green, Loomis & Auerbach (1948) from rachitic and vitamin D protected rats. The results showed that the capacity of these organs to oxidize pyruvate was not impaired in vitamin D deficiency. It appears possible, therefore, that the effect of vitamin D deficiency described in this paper is restricted to bone.

The antirachitic action of citrate demonstrated by Shohl (1937) and the essential similarity of histochemical changes in rachitic cartilage induced under the influence of vitamin D on the one hand and citrate on the other as observed by Ramalingaswami *et al.* (1954) had indicated that in vitamin D deficiency one was more likely to find an arrest or disturbance in the oxidative phase of glycogen metabolism than in the earlier and anaerobic stages of glycogen breakdown. The experiments described here have fulfilled these expectations. It is not yet clear, however, how the orderly progress of glycogen metabolism would be essential to calcification. Two tentative suggestions can be made at this stage. It is possible that the supply of citrate itself is inadequate in rickets owing to a progressive inability of the cartilage to deal with pyruvate. That citrate is of importance to bone can be concluded from the observation of Dickens (1941) that 90 % of citrate in the animal body is to be found in the skeleton. The same author has shown that in rickets citrate in bone is reduced. Further, the relationship between the state of vitamin D nutrition and citrate metabolism has been well brought out by the work of Harrison & Harrison (1952), who found that in

Table 1. *Glycolytic activity of rat epiphyseal cartilage*

		$\mu\text{l. CO}_2/\text{mg. dry wt./hr. with substrate}$ (averages only are given)			
		Glycogen	Glucose 1-phosphate	Glucose 6-phosphate	Fructose 1:6-diphosphate
Five days on rachitogenic diet	Without vitamin D (16 rats)	15.1	16.6	13.8	24.1
	With vitamin D in diet (10 rats)	6.3	7.8	7.2	7.1
Fifteen days on rachitogenic diet	Without vitamin D (10 rats)	5.8	6.8	7.6	7.0
	With vitamin D in diet (12 rats)	4.6	3.9	3.5	6.5

Table 2. Oxidation of pyruvate by epiphyseal cartilage of rat tibiae in rachitic and vitamin D protected animals

	Rats on rachitogenic diets			Rats on rachitogenic diets and vitamin D			Normal rats on colony diets (8)* S.E.
	Five days (32)* S.E.	Ten days (12)*	Twenty days (24)* S.E.	Five days (32)* S.E.	Ten days (12)*	Twenty days (20)* S.E.	
(a) q_{O_2} with substrate in $\mu\text{l./100 mg. dry tissue/hr.}$	191.2 $\pm$ 16.57	187.4	132.1 $\pm$ 15.53	252.5 $\pm$ 13.85	288.4	257.5 $\pm$ 15.72	284.2 $\pm$ 14.41
(b) q_{O_2} without substrate in $\mu\text{l./100 mg. dry tissue/hr.}$	137.3 $\pm$ 10.68	161.3	138.1 $\pm$ 10.41	129.2 $\pm$ 8.30	154.7	150.7 $\pm$ 9.72	118.5 $\pm$ 5.86
q_{O_2} (pyruvate) (a - b)	53.9	26.1	-6.0	123.3	133.7	106.8	165.7
Student's 't'	2.7358	—	0.3187	7.6409	—	5.8880	10.6479
P	<0.02	—	0.8	<0.001	—	<0.001	<0.001

The significance of differences in q_{O_2} pyruvate within and between groups on 5th and 20th days is tabulated below:

(1) Rachitic 5th and 20th days	$t = 4.6244$ (26)†	$P, < 0.001$
(2) Vitamin D 5th and 20th days	$t = 0.9296$ (24)	Not significant P between 0.3 and 0.4
(3) Rachitic and vitamin D on 5th day	$t = 4.4318$ (30)	$P, < 0.001$

* Figures in parenthesis indicate the number of animals.

† Degrees of freedom.

rachitic infants the concentration of citrate in serum was significantly lower than in normal infants, and that it was raised on administration of vitamin D. Dixon & Perkins (1952) have shown the existence of enzyme systems, viz. citrogenase, aconitase and isocitric dehydrogenase in bone. It may not be unreasonable to conclude that the formation of citrate is inhibited in rickets, a conclusion of which experimental confirmation is now being sought.

On the other hand, other possible roles which pyruvate oxidation may play in calcification will have to be examined. Much of the energy needed for calcification must be derived from the progressive oxidation of pyruvic acid. Then again, our earlier observations (Ramalingaswami *et al.* 1954) suggested a possible defect in the formation of cartilage matrix in rickets which seems to undergo a qualitative change after treatment with vitamin D. It is possible that the formation of the appropriate matrix may be closely related to pyruvate metabolism in cartilage.

SUMMARY

1. Anaerobic breakdown of glycogen and phosphorylated hexoses and the oxidation of pyruvate by epiphyseal cartilage of the tibiae of rats kept on rachitogenic diets with and without vitamin D has been studied by the Warburg manometric technique.

2. In rachitic rats the glycolytic activity of the epiphyseal cartilage was almost the same as that observed in vitamin D protected rats in comparable experiments.

3. In well established rickets the epiphyseal cartilage of the rat loses the capacity to oxidize pyruvate.

4. The significance of the above findings in the mechanism of calcification has been discussed.

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The Carbohydrate Metabolism of the Isolated Diaphragm of the Rat. Glucose Uptake and Body Size

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Since Gemmill (1940, 1941) and Gemmill & Hamman (1941) have shown that the isolated diaphragm of the rat is a suitable tool for the experimental study of the glucose uptake and glycogen synthesis by muscle tissue, and demonstrated that these processes are stimulated by *in vitro* addition of insulin many investigators have used the method to study the hormonal control of carbohydrate metabolism. More recently, attempts have been made to estimate small amounts of insulin by that method (Groen, Kamminga, Willebrands & Blickman, 1951–2). The experimental conditions under which reproducible results may be expected have been the subject of several studies (Stadie & Zapp, 1947; Krahl & Cori, 1947; Krahl & Park, 1948), but for some time the stimulation of the glucose uptake by insulin could not be repeated in this laboratory. Similar difficulties were encountered by Perlmutter, Weisenfeld & Mufson (1952). Therefore it was felt necessary to re-examine the effect of some factors on the carbohydrate metabolism of the isolated diaphragm of the rat and on the stimulating effect of insulin on its glucose consumption.

The present paper deals with the relation existing between the size of the rats and the uptake of glucose measured. It was found that the uptake of glucose per g. of tissue was high if small rats were used, and low if large rats were used. Possible explanations are presented. Differences between insulin effects obtained with animals of different sizes could not be demonstrated. A preliminary note has appeared elsewhere (Liébecq, 1953). Later work will show the effect of other factors on the extent of the insulin effect.

METHODS

All rats were obtained from Brofo, Zevenaar, Holland, and were of the Epymus Albino Wistar strain. They were fed a mixture of oat flakes (50%), skimmed dried milk (35%), wheat germ (6%), dried yeast (6%), CaCO_3 (1.5%), NaCl (1%) and cod-liver oil (0.5%) *ad lib.*, supplemented with fresh vegetables. This diet contains 25–27% protein, 5–6% lipid, 60–65% carbohydrate and 5–6% ash. Rats were fasted 20–24 hr. before use.

The animals were killed by decapitation and the two hemidiaphragms were carefully excised and immediately transferred into two Warburg flasks kept in ice both containing 1 ml. of glucose (1.4 mg./ml.) in Krebs & Henseleit's (1932) bicarbonate medium (but with 6 mg. calcium/100 ml. instead of 10). Excess of Novo insulin (5 $\mu\text{g.}$, i.e. approx. 0.1 unit/ml.) free of hyperglycaemic factor was present in one flask only. The flasks were filled with 95% O_2 + 5% CO_2 and incubated for 30 min. at 37° while shaken at 140–145 strokes of approx. 5 cm./min., then cooled. For glucose determination, 0.3 ml. samples (in duplicate) were diluted with 5 ml. water and deproteinized by addition of 0.45 ml. 0.3N- Ba(OH)_2 and 0.40 ml. 0.15M- ZnSO_4 (9 ml. of the Ba(OH)_2 solution added dropwise should neutralize 10 ml. of the ZnSO_4 solution in the presence of 100 ml. water and 10 drops of 0.1% (w/v) phenolphthalein in ethanol). Glucose was determined on 1 ml. of the filtrate, in duplicate, by the method of Nelson (1944) as modified by Somogyi (1952). The hemidiaphragms were blotted on filter paper at the end of the experiment and weighed. The uptake of glucose was calculated in mg./g. of moist tissue/hr.

All solutions were prepared with reagents of analytical grade purity and with glass-distilled water. To remove residual insulin, stainless steel instruments were soaked in a concentrated solution of a commercial preparation of alkaline detergent, pipettes in 2N-NaOH, then washed carefully and dried in an oven at 120–130°. Warburg flasks were boiled in the solution of alkaline detergent for at least 10 min. before washing.