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STUDIES ON THE METABOLISM OF THE ORAL TISSUES ACTION OF PHOSPHORYLATED VITAMIN D ON THE OXYGEN CONSUMPTION ON RAT PALATAL MUCOSA FROM NORMAL AND VITAMIN D DEFICIENT ANIMALS

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As part of a general study dealing with the effects of vitamin deficiencies on mammlian oral tissues, vitamin D was the first vitamin chosen for investigation. Although the exact biochemical role of vitamin D has not yet been established, there is evidence to suggest that in vitamin D deficiency two aspects of cellular metabolism might be affected, namely carbohydrate metabolism and cellular respiration. An inhibitory action by vitamin D on aconitase isolated from Aspergillus niger was noted by Bruchmann [1958]. According to Pincussen [1941] the administration of vitamin D to rats led to an increased glycogen deposition in liver and muscle as well as an increased carbohydrate/lactic acid ratio in blood, liver and muscle. Observations have indicated that the shift from anaerobic to aerobic metabolism occurring in muscles with high energy output was delayed in rachitic rabbits [Räihä *et al.*, 1937]. The capacity for work in muscles of rachitic dogs was depressed, but returned to normal almost immediately after the administration of vitamin D [Peitsara, 1944]. The glycolytic activity of the epiphyseal cartilage of rachitic rats was reported to be almost of the same order as that observed in vitamin D protected rats, However, in well established rickets this tissue lost its ability to oxidize pyruvate. This effect was not found in liver and kidney tissues of the same animal [Tulpule and Patwardhan, 1954]. An inhibition of glycolysis in rat liver slices by ergosterol and ergocalciferol was noted by Festenstein [1955]. Rachitic rat cartilage was shown to produce more lactic acid and less citric acid from glucose and glycogen than normal cartilage [Picard and Cartier, 1960]. In vitro addition of vitamin D_2 to the rachitic cartilage caused citrate production to be significantly increased. In the normal rat this effect was not observed, hence the influence of vitamin D was limited to the vitamin-deprived tissue only.

Concerning the role of vitamin D in respiratory metabolism, Zetterström and Ljunggren (1951) demonstrated an activation of aerobic oxidation in healthy rabbit kidney mitochondria by phosphorylated vitamin D_2 in the presence of glutamic acid as oxidizable substrate. A greater activation by phosphorylated vitamin D_2 was observed in experiments utilizing kidney mitochondria from rachitic rats. The observations of De Luca *et al.* [1957a, b] revealed an inhibition of citrate and isocitrate oxidation in rat kidney mitochondria by vitamin D.

Since contradictory reports concerning the mode of action of vitamin D on intermediary metabolism are apparent from the literature, it was decided to investigate the in vitro influence of vitamin D on the citric acid cycle of oral tissue slices and homogenates obtained from normal and vitamin D deficient rats. A short note concerning the effect of vitamin D on the respiration of rat oral tissue has already been published [Zar and Irving, 1959].

MATERIALS AND METHODS

The materials and techniques utilized in this study with the exception of phosphorylated vitamin D_2 and the preparation of tissue homogenates have been described previously [Mendelsohn, 1961].

Preparation of phosphorylated vitamin D

Water soluble vitamin D was prepared according to the method of Zetterström [1951]. Biological activity of the compound was periodically tested in the following manner: rachitic rats were given an oral dose of approximately 30 units phosphorylated vitamin D_2. Four days later the animals were sacrificed and the epiphyses of the long bones examined radiologically and histologically to establish whether healing had occurred. The same tissue taken from rachitic rats which had not received any phosphorylated vitamin D acted as controls. Histological examination (the "line test") was carried out as described by Coward [1947]. It was found that the phosphorylated vitamin D when stored over calcium chloride at 4°C and protected from light retained its biological activity for at least 18 months.

Preparation of tissue homogenates

Approximately 150 mg. fresh rat palatal mucosa was immediately placed in chilled Krebs-Ringer solution and finely sliced using a sharp scalpel blade. The tissue was homogenized with 0·5 ml. chilled 0·25M sucrose for 4 minutes. Thereafter, 4 ml. of 0·25M sucrose was added taking care to remove all adhering particles from the plunger. One ml. aliquots of the homogenate were added to each Warburg flask. The flasks had previously been prepared for standard manometric measurements and kept on cracked ice until the homogenate was ready to be added.

After the necessary manometric readings had been taken the contents of the main compartments were poured into weighed 15 ml. centrifuge tubes. The flasks were rinsed three times with distilled water and the washings added to the contents of the centrifuge tubes. Five ml. 10% trichloracetic acid was then added and the tubes allowed to stand at room temperature for one hour. This was followed by centrifugation at 2,000 r.p.m. for a further hour. The clear supernatant fluid was decanted and the centrifuge tubes containing the precipitate dried overnight at 100°C. After cooling, the tubes were again weighed and the dry weight of the homogenates thus obtained. The results were expressed as microlitres oxygen taken up per mg. dry weight homogenate per hour.

RESULTS

Tables I and II show that the addition of phosphorylated vitamin D in vitro caused no alteration in basal oxygen consumption of both normal and rachitic tissue. The Qo_2 of normal tissue on the incorporation of any of the members of the citric acid cycle remained essentially unaltered in the presence of phosphorylated vitamin D. However, in the case of mucosa from rachitic rats, vitamin D produced a further rise in the Qo_2 in the presence of Krebs cycle intermediates. The action of vitamin D was noted only in fresh tissue; after storage for one day at 4°C in Krebs-Ringer solution no such effect on the oxygen uptake could be detected, although the metabolites still increased the oxygen consumption above the basal value (Table III).

The following experiments were then performed to determine whether the effect of vitamin D in causing an elevation of the Qo_2 of rat oral tissues in the presence of Krebs cycle intermediates was due to the vitamin affecting the permeability of these metabolites across the cell membrane, or whether the phosphorylated vitamin D exerted its influence within the cell. Experiments were carried out in a similar manner

TABLE I

Effect of phosphorylated vitamin D on the Qo_2 of fresh normal rat palatal mucosa.

Metabolite added	Qo_2	
	No phosphorylated vitamin D incorporated	Phosphorylated vitamin D incorporated
Control	1·96±0·29 (50)	2·13 (20)
0·02M Pyruvate	2·45 (3)	2·64 (3)
0·01M Oxalacetate	2·51 (3)	2·72 (3)
0·02M Citrate	2·01 (3)	2·29 (3)
0·02M Aconitate	2·57 (4)	2·73 (4)
0·04M Isocitrate	2·84 (3)	2·74 (3)
0·04M Oxalosuccinate	1·73 (4)	1·66 (4)
0·02M *a*-ketoglutarate	1·69 (4)	1·65 (4)
0·02M Succinate	3·34 (4)	3·53 (4)
0·02M Fumarate	1·88 (3)	1·69 (3)
0·02M Malate	1·94 (4)	2·29 (4)

The reaction mixture consisted of: 2 ml. Krebs-Ringer-phosphate solution, pH 7·4; either 0·05 ml. of a 0·5% aqueous phosphorylated vitamin D solution or 0·05 ml. distilled water; 20-30 mg. wet weight tissue. When substrate was added 1 ml. Krebs-Ringer-phosphate solution and 1 ml. substrate (final concentration indicated in the table) were used. The figure in brackets indicates the number of observations. Where only a few determinations were made, the mean value is given, followed by the standard deviation in the case of the control.

The "t" test was applied to the results which were shown not to differ by significant amounts.

TABLE II

Effect of phosphorylated vitamin D on the Qo_2 of fresh vitamin D deficient palatal mucosa.

Metabolite added	Qo_2	
	No phosphorylated vitamin D incorporated	Phosphorylated vitamin D incorporated
Control	1·92±0·2 (15)	1·94 (10)
0·02M Pyruvate	2·47 (4)	3·0 (4)
0·01M Oxalacetate	1·73 (3)	2·46 (3)
0·02M Citrate	2·02 (6)	3·34 (6)
0·02M Aconitate	2·5 (4)	3·17 (4)
0·04M Isocitrate	1·91 (4)	2·94 (4)
0·04M Oxalosuccinate	1·76 (5)	2·26 (5)
0·02M *a*-ketoglutarate	1·84 (3)	2·5 (3)
0·02M Succinate	2·43 (3)	3·92 (3)
0·02M Fumarate	1·72 (3)	2·02 (3)
0·02M Malate	2·54 (3)	4·49 (3)

The additions to each flask and the explanation of the table are the same as described in Table I.

The "t" test was applied to the pairs of results. The differences were found to be significant at the following levels: 5% succinate; 4% pyruvate and oxalacetate; 3% malate and fumarate; 2% citrate, aconitate, isocitrate and *a*-ketoglutarate; 1% oxalacetate.

TABLE III

Effect of phosphorylated vitamin D on the Qo_2 of normal and vitamin D deficient rat palatal mucosa stored one day.

Type of Tissue	Metabolite added	Qo_2 No phosphorylated vitamin D incorporated	Qo_2 Phosphorylated vitamin D incorporated
Rachitic Rat Mucosa	Control	1·23±0·17 (12)	1·37 (6)
	0·02M Pyruvate	1·85 (3)	2·18 (3)
	0·01M Oxalacetate	1·61 (3)	1·43 (3)
	0·02M Citrate	2·94 (3)	2·89 (3)
	0·04M Isocitrate	1·9 (3)	1·71 (3)
	0·04M Oxalosuccinate	1·7 (3)	1·6 (3)
	0·02M α-ketoglutarate	1·43 (3)	1·54 (3)
	0·02M Succinate	3·8 (3)	3·82 (3)
	0·02M Fumarate	1·73 (3)	1·76 (3)
	0·02M Malate	2·16 (3)	2·23 (3)
Normal Rat Mucosa	Control	1·39±0·30 (20)	1·55 (6)
	0·02M Citrate	2·64 (3)	2·5 (3)
	0·02M Succinate	2·66 (3)	2·71 (3)
	0·02M Malate	1·77 (3)	1·84 (3)

The contents of each flask and the explanation of the table are the same as described in Table I. The "t" test indicated that the oxygen consumption of stored palatal mucosa was not significantly altered on the addition of phosphorylated vitamin D in the presence of citric cycle intermediates.

TABLE IV

Effect of phosphorylated vitamin D on the Qo_2 of rat palatal mucosa homogenates.

Type of Tissue	Metabolite added	No. of determinations	Qo_2 No phosphorylated vitamin D added	Qo_2 Phosphorylated vitamin D added
Normal Mucosa Homo-genate	0·02M Aconitate	3	0·524	0·529
	0·02M Citrate	3	0·261	0·252
	0·02M Succinate	6	0·473	0·445
	0·02M Fumarate	3	0·373	0·392
	0·02M Malate	3	0·20	0·207
Rachitic Mucosa Homo-genate	0·02M Aconitate	3	0·141	0·281
	0·02M Citrate	3	0·341	0·61
	0·02M Succinate	6	0·26	0·45
	0·02M Fumarate	4	0·351	0·485
	0·02M Malate	3	0·251	0·375

To the main compartment of each Warburg flask was added the following: 1 ml. Krebs-Ringer-phosphate solution, pH 7·4; 1 ml. metabolite (final concentration indicated in the table); 0·3 ml. of 0·01M adenosine triphosphate; 0·05 ml. aqueous 0·5% phosphorylated vitamin D solution or 0·05 ml. distilled water (control); 1 ml. tissue homogenate.

The "t" test was applied to the results. The action of phosphorylated vitamin D on normal tissue homogenates was shown not to be significant, whereas rachitic mucosa homogenate results revealed a significance at the 1% level for succinate; 3% level for aconitate; 5% level for citrate, fumarate and malate.

to those described for tissue slices, with the exception that tissue homogenates were used. It was noted that phosphorylated vitamin D had the same effect when added to homogenates as described for tissue slices, i.e. it was without action on the oxygen consumption of homogenates prepared from normal tissue, but increased the Qo_2 of rachitic mucosa homogenates. These results are indicated in Table IV.

DISCUSSION

The use of phosphorylated vitamin D in biological experiments has been criticized by Moore [1953] on the grounds that no natural phosphate of vitamin D is known to exist. However, Werner [1958] has shown that phosphorylated vitamin D (whether prepared by the direct phosphorylation of vitamin D_3 or by the irradiation of phosphorylated cholesterol) was able to cure rickets in rats and activate alkaline phosphatase activity in bone and kidney. In the present investigation the biological activity of non-phosphorylated and phosphorylated vitamin D as measured by the curative action on rickets in rats was found to be identical. Since experiments with propylene glycol and Tween 80 as solvents for non-phosphorylated vitamin D produced erratic results in our hands, the water soluble derivative of the vitamin was used throughout this study.

Observations on the influence of vitamin D on oxidative and related processes seem to be at variance. Some investigators have described an activation of aerobic oxidation by phosphorylated vitamin D_2 using glutamic acid as oxidizable substrate [Zetterström and Ljunggren, 1951]. Others have observed a diminution in oxidation of citrate and isocitrate by vitamin D in rat kidney homogenates and mitochondrial preparations [De Luca *et al.*, 1957a, b].

The results presented here are not in agreement with the findings of Bruchmann [1958] who reported that vitamin D inhibited the action of aconitase obtained from Aspergillus niger. They are also at variance with the findings of De Luca *et al.* [1957a] that vitamin D inhibited the oxidation of citrate and isocitrate in rat kidney homogenates. However, the discrepancies noted above are probably due to the fact that vitamin D has a differential action on various tissues. This is further borne out by the observations of De Luca *et al.* [1956, 1957b], who failed to demonstrate the same effect of vitamin D in kidney and liver tissues of animals under comparable conditions.

In the present investigation a noteworthy enhancement of oxygen consumption in the presence of citric acid cycle metabolites on the addition of phosphorylated vitamin D was noted in the case of rachitic palatal mucosa only. Picard and Cartier [1960] similarily observed that the action of vitamin D was confined solely to rachitic tissue. They demonstrated that vitamin D (when added in vitro) increased citrate formation from carbohydrate in rachitic cartilage but had no action on normal cartilage.

From the results with tissue slices it was difficult to determine whether the vitamin exerted its action directly in the regulation of the oxidation of substrates via the citric acid cycle or whether it was acting indirectly by affecting the permeability of the cell wall to the metabolites. However, homogenates prepared from normal and vitamin D deficient oral tissues still differed in their response to the in vitro addition of phosphorylated vitamin D. These findings strongly suggest that vitamin D produces its effect by influencing the activity of certain intracellular enzyme systems directly rather than by increasing cell membrane permeability to substrates. The possibility that the vitamin might have an effect on mitochondrial membrane permeability has not been excluded by the present investigations. It is of interest to note that De Luca *et al.* [1960] concluded that vitamin D affected mitochondrial structure. A lack of vitamin D was shown to produce swollen, morphologically damaged mitochondria

in rat liver. These workers further suggested that the reduction by vitamin D in citrate oxidation by rat kidney preparations might not be due to an enzymic inhibition but might rather be caused by a physical inhibition of the penetration of the citrate ion into the mitochondrion.

The problem as to the precise mode of action of vitamin D on cells remains unsolved. It is possible that the differences in the effect of vitamin D that have been observed depend primarily on the role played by the vitamin in enzymatic processes concerned with vital intra-cellular activities. Besides influencing calcium, phosphorus and bone metabolism, it probably serves an important function in the regulation of other metabolic reactions. The findings of this investigation strongly suggest that vitamin D might be an important factor in the regulation of aerobic metabolism in certain tissues.

SUMMARY

The in vitro action of phosphorylated vitamin D on the enzymes of the citric acid cycle of normal and vitamin D deficient rat oral mucosa (tissue slices and homogenates) was studied.

Vitamin D did not alter the basal Qo_2 of either type of tissue nor did it influence the oxygen consumption of normal tissue on the addition of components of the tricarboxylic acid cycle.

Vitamin D significantly increased the Qo_2 of fresh rachitic palatal mucosa in the presence of intermediates of the Krebs citric acid cycle in both tissue slices and homogenates.

It is suggested that vitamin D probably exerts its effect directly on intracellular oxidative metabolic activities, although the possibility of a further action on mitochondrial permeability has not been excluded.

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