

Activation of Aerobic Oxidation in Kidney Mitochondria by Phosphorylated Vitamin D₂

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Despite the great number of investigations concerning the mode of action of vitamin D the problem still remains obscure. Besides influencing the development of the skeleton the vitamin is known to affect the metabolic patterns of other tissues. It seems probable that the different effects of vitamin D primarily depend on the part played by the vitamin on the enzymatic processes concerned with cellular function.

In broadest aspect vitamin D stimulates growth¹. The retardation of growth by D-avitaminosis is probably due to quite a fundamental disturbance in the metabolic processes. Pathological changes in the carbohydrate metabolism have been observed in D-avitaminosis (rickets)²⁻⁵. Values on the content of hexose phosphates in resting muscle and in muscle after tetanic contraction, given by Rähkä and co-workers⁶, indicate that the shift from anaerobic to aerobic metabolism, occurring in muscles with high energy output⁷, is delayed in rachitic rabbits. The capacity of work is depressed in muscles from rachitic dogs but returns almost immediately to normal after administration of vitamin D⁸. These muscle experiments seem to give evidence that in D-deficiency there is an impairment of the oxidative phase of carbohydrate metabolism in muscles and that vitamin D plays a role in cellular respiration.

The activity of different enzyme systems in the presence of the phosphorylated water-soluble vitamin D₂⁹ is investigated as a means of obtaining information concerning the vitamin's action on the energy transfer system. In an earlier report¹⁰ evidence has been presented that phosphorylated vitamin D₂ (D₂P) activates purified alkaline phosphatase from kidney, intestine and bone.

This communication deals with results obtained from experiments to determine the effect of D_2P on the oxygen uptake of the respiratory enzyme complex in mitochondria which is known to catalyze all the reactions of the Krebs tricarboxylic acid cycle and fatty acid oxidation¹¹⁻¹².

EXPERIMENTAL

Preparation of the mitochondria (washed granules):

The granules were prepared by a method similar to that developed by Slater (personal communication) for heart muscle mitochondria. The animals (rabbits or rats) were killed by decapitation and the kidneys were immediately removed. All subsequent operations were performed at 2–4° C. In about 100 ml of an ice-cold 0.11 *M* potassium chloride solution, being 0.05 *M* with respect to sodium fluoride, the kidney cortex was cut into small slices with a pair of scissors. After three washings with 100 ml of the potassium chloride solution containing fluoride the tissue was ground to a fine paste with quartz sand in two volumes of a solution of the following composition: one part of 0.15 *M* KCl, one part of 0.2 *M* NaF and two parts of 0.065 *M* phosphate buffer of pH 7.3. The preparation was then centrifuged in an International refrigerated centrifuge for five minutes at about $600 \times g$. The residue was then ground once again with two volumes of the same solution as before and thereafter recentrifuged at the same gravity. The supernatants were combined and admitted to high speed centrifugation at about $20\,000 \times g$ for 15 minutes. The supernatant was discarded, and the residue fraction washed once by resuspension in a 0.11 *M* KCl solution, being 0.05 *M* with respect to NaF, followed by resedimentation for 15 minutes at $20\,000 \times g$. The supernatant was carefully decanted and the washed mitochondria were taken up in a sufficient amount of ice-cold 0.2 *M* glycylglycine buffer of pH 7.3 to yield a suspension suitable for experimental purpose.

Other materials: Vitamin D_2 was phosphorylated by the method described earlier⁹. Since the phosphorylated vitamin, especially when stored in water solution, is extremely labile, the D_2P was tested before use by means of its ability to activate purified alkaline kidney phosphatase. Destroyed D_2P is not capable to give such an effect.

Glucose-6-phosphate of purity 0.90 was obtained as follows: glucose-1-phosphate was prepared according Sumner and Somers¹³, the phosphate ester obtained was then incubated with phosphoglucomutase, purified according to Najjar¹⁴; the glucose-6-phosphate formed was finally purified as the Ba-salt.

Adenosine-5-phosphate was obtained from the Sigma Chemical Company.

Hexokinase was prepared according to Berger, Slein, Colowick and Cori¹⁵.

Components of the incubation mixture: Incubations with the suspension of mitochondria in glycylglycine were made aerobically and in the presence of K^+ , Mg^{++} , fluoride, adenosine-5-phosphate and inorganic phosphate. Glutamate was used as oxidizable substrate and glucose served as phosphate acceptor. Hexokinase was added in order to transfer esterified phosphate to glucose. The pH of the mixture was 7.3; temp. 30° C.

ANALYTICAL METHODS

Manometric measurements of oxygen uptake were made at 30° C in Warburg vessels of conventional design with air as the gas phase. The center well contained 0.2 ml 2 *N* KOH. Glucose and D_2P were pipetted into the side-arms. Flasks were equilibrated

for 15 minutes prior to closing of the taps. Glucose and D_2P were tipped in after closing. Readings were mostly made every ten minutes.

Phosphate was determined by the method of Fiske and Subbarow¹⁶ after fixation of the sample in 7% (wt./vol.) trichloroacetic acid (TCA).

RESULTS

The effect of D_2P in a concentration of $2 \times 10^{-5} M$ on the oxygen uptake of the mitochondria suspension is shown in Fig. 1. In comparison to the control experiment there is a considerable activation*.

The maximum effect of the vitamin is exerted immediately after it is mixed with the enzyme complex; thus it must be rapidly associated to the enzyme system. After 30 minutes of incubation, the oxygen uptake is almost the double in comparison to the experiment without D_2P . After the lapse of about 90 minutes, the uptake per unit of time is the same in both experiments. The velocity of the reaction thus becomes the same in the two incubation mixtures after the initial activation caused by D_2P at the start of the experiment.

The effect of D_2P on oxygen uptake when incubating mitochondria in the presence of limited amounts of substrate is shown in Fig. 2. The relative initial activation given by D_2P is about the same as in the experiments with unlimited amounts of substrate. When the substrate is almost completely oxidized the rate of oxygen uptake declines considerably and the curve of the uptake comes rather rapidly to an asymptote being almost of the same level in both experiments. The asymptote, however, was reached quite a bit earlier in the experiment with D_2P , indicating that the entire oxidation of glutamate to carbon dioxide, water and ammonia was performed at a more rapid rate when D_2P was present. Since the oxygen uptake thus was the same in both experiments, the increased oxygen uptake after addition of D_2P cannot be due to an oxidation of the vitamin. Such conditions may also be denied by the low amount of D_2P present (5×10^{-2} micromoles).

The experiments referred in Figs. 1 and 2 have been performed with mitochondria from healthy rabbits. Preliminary investigations, presented in Table 1, have shown a much greater activation by D_2P in experiments with kidney mitochondria from rachitic rats**.

* Throughout this paper the term activation has been used to indicate an increase in the rate of oxygen uptake in the entire system.

** Rickets was produced by giving young rats, weighing 40–50 g, a rachitogenic diet¹⁷ for a period of six weeks.

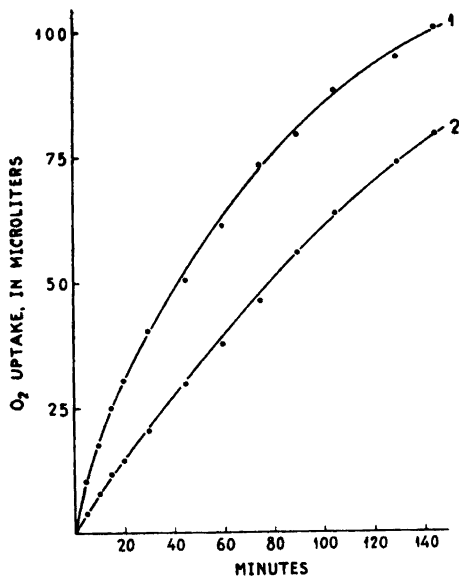


Fig. 1. Stimulation of respiration of rabbit kidney mitochondria by D₂P. Curve 1 represents an incubation with D₂P. Curve 2 represents an incubation without activator.

The initial volume in the main compartment of each Warburg vessel was 2.0 ml. The mixture contained 0.3 m of the enzyme suspension (the mitochondria from two kidneys had been suspended in 8 ml 0.2 M glycylglycine buffer of pH 7.3), 80 micromoles of KCl, 6 micromoles of MgCl₂, 100 micromoles of NaF, 5.0 micromoles of orthophosphate, 0.4 micromoles of adenosine-5-phosphate*, 30 micromoles of sodium glutamate and 0.01 ml hexokinase solution. In the side-arms 30 micromoles of glucose and in the experiment with D₂P 5 × 10⁻² micromoles of the activator. The volume in the side-arms 0.5 ml. The taps were closed after an equilibration period of 15 minutes. The contents in the side-arms were then tipped into the main compartment. Thus, the final volume of the incubation mixture was 2.5 ml. The vessels were incubated at temp. 30° C with air as the gas phase.

On aerobic incubation of intact mitochondria in the presence of an oxidizable substrate the oxidation is coupled to formation of phosphate bound energy^{18,19,12}, the phosphate esterification accompanying the oxidation being roughly proportional to the amount of substrate oxidized²⁰. In the presence of a suitable phosphate acceptor and a phosphate transfer system an accumula-

* The very low concentration of adenosine-5-phosphate has been used since it has been shown by Lindberg and Ernster (to be published) that this concentration is optimal and most physiological for oxidative phosphorylation in mitochondria.

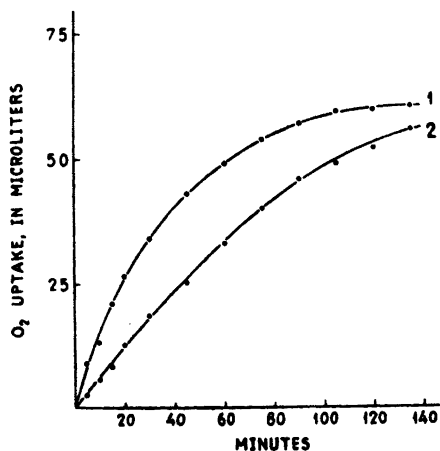


Fig. 2. The activation of oxygen uptake of kidney mitochondria by D_2P with a small amount of oxidizable substrate.

Curve 1 represents an incubation with D_2P .

Curve 2 is from an experiment without activator.

The incubations were performed as in the experiments in Fig. 1 with the exception that each Warburg vessel contained only 0.6 micromoles of sodium glutamate.

Table 1. Stimulation of respiration of kidney mitochondria from rachitic rats by D_3P .

Mitochondria prepared from 8 kidneys were suspended in 4 ml 0.2 M glycyglycine buffer of pH 7.3. Each Warburg vessel contained in the main compartment 0.5 ml enzyme suspension, 80 micromoles of KCl, 8 micromoles of $MgCl_2$, 100 micromoles of NaF, 5.0 micromoles of orthophosphate, 0.3 micromoles of adenosine-5-phosphate, 30 micromoles of glutamate, and 0.05 ml hexokinase solution. In the side-arms 30 micromoles of glucose and in the experiment with activator 7×10^{-2} micromoles of D_3P . The experiments were made in the same way as in Fig. 1. Final volume 2.5 ml. Temp. 30° C.

Microlites of O ₂ taken up during 15 minutes after equilibration	
With D ₂ P	Without activator
22 ^a	5
22	5.5
23.5	4.5

^a Each value represents one determination.

Table 2. The effect of varying concentrations of D_2P on the esterification of phosphate and on the oxygen uptake in aerobic incubations of rabbit kidney mitochondria.

The incubations were made in Warburg vessels. The experimental conditions were the same as in Fig. 1. Manometric readings were made every ten minutes.

Amount of D_2P added. In micromoles	O_2 taken up/10 min. during the first 60 min. In microliters	O_2 taken up/10 min. during the period 60–135 min. In microliters	Amount of phosphate esterified. In micromoles
—	5.9	5.4	3.6
1.10^{-3}	7.1	6.1	3.5
5.10^{-3}	7.7	6.1	3.1
$1.5.10^{-2}$	8.8	5.9	2.2
5.10^{-2}	10.2	5.5	1.0

tion of esterified phosphate takes place. Thus, if the activation of oxygen uptake in mitochondria after addition of D_2P is due to a more rapid utilization of substrate and if no dissociation of electron transport and phosphorylation occurs, the increased activity should increase phosphorylation.

This was, however, not the case, as shown in Fig. 3. The maximal esterification was reached in both experiments after a period of about 25 minutes. After that time the curves showed a tendency to decline. During the first minutes of incubation the phosphate uptake was about the same with and without D_2P . Later on, however, a difference between the two sets of condition was obtained. The net esterification of phosphate was less in the experiment with D_2P than in the other, and this difference seemed to be gradually increasing. The oxygen uptake as controlled on a part of each sample in a Warburg vessel was satisfactory in the two sets of conditions throughout the whole experimental period.

Table 2 shows the effect of different concentrations of D_2P on the esterification of phosphate in an experiment continued for a long period. The net uptake decreased considerably with increasing concentration of D_2P . With the highest concentration of D_2P applied ($2 \times 10^{-5} M$) the amount of phosphate esterified was less than one third of that in the experiment without addition of vitamin. The same table also shows the effect on oxygen uptake with varying concentrations of D_2P . Even a concentration as low as $4 \times 10^{-7} M$ gave a significant increase in activity. The activation increased with increasing concentrations of D_2P . In all cases the activation occurred during the first period of incubation, whereas in the later phase there was no significant difference

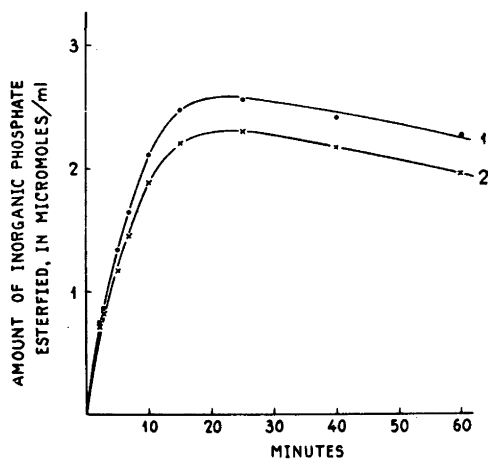


Fig. 3. Net esterification of phosphate in an aerobic incubation of rabbit kidney mitochondria under the influence of D_2P .

Curve 1 represents an experiment without D_2P .

Curve 2 represents an experiment with D_2P in a concentration of $1.2 \times 10^{-5} M$.

The incubations were performed under constant stirring in two open Florence flasks of 250 ml, the surface of the incubation mixtures being large enough to permit steady oxygen uptake. Samples of 1 ml were removed and immediately fixed in ice-cold TCA at times given in the figure. The total volume in each flask was 12 ml. Each incubation mixture contained 2.5 ml mitochondria suspension in 0.2 M glycylglycine buffer (mitochondria from two kidneys were suspended in 5.5 ml of the buffer solution), 520 micromoles of KCl, 36 micromoles of $MgCl_2$, 600 micromoles of NaF, 40 micromoles of orthophosphate, 1.5 micromoles of adenosine-5-phosphate, 30 micromoles of sodium glutamate, 180 micromoles of glucose, and 0.1 ml of the hexokinase solution. The pH of the mixtures being 7.3. Temp. 30° C.

between the five groups. Thus, the ratio of esterified phosphate to oxygen taken up decreased with increasing amounts of D_2P added.

The decreasing tendency of the amount of esterified phosphate after a long period of incubation (*cf.* Fig. 3) indicates the presence of a non-inhibited phosphatase in the enzyme preparation. Swanson²¹ has demonstrated a phosphatase specific for glucose-6-phosphate in mitochondria preparations. Since D_2P has been shown to activate alkaline phosphatase from different organs¹⁰, it may be possible that the diminished net uptake of phosphate is due to an activated break down of phosphoric esters.

Table 3 shows that in the mitochondrial suspension there was a marked initial increase in the phosphatase activity towards glucose-6-phosphate after the addition of D_2P . The activation was of the same "initial" type as that

Table 3. The effect of D_2P on the phosphatase activity in rabbit kidney mitochondria.

The incubations were made in closed test tubes. The final volume in each experiment was 7.0 ml. Samples of 1.0 ml were taken with time intervals given in the table. 0-values were calculated from the amount of orthophosphate present in the mitochondria suspension and in the glucose-6-phosphate solution before mixing. Each mixture contained 1 ml mitochondria suspension in 0.2 M glycylglycine buffer of pH 7.3 (the mitochondria from two kidneys were suspended in 6 ml of the buffer solution), 580 micromoles of KCl, 20 micromoles of $MgCl_2$, and 20 micromoles of glucose-6-phosphate. In the experiment with D_2P the vitamin was present in a concentration of 1.3×10^{-5} M. Temp. 30° C.

Time min	Amount of orthophosphate formed ^a	
	Without D_2P	With D_2P
5	1.6	4.0
10	1.1	8.8
20	5.9	16.0
40	17.0	17.2

^a In micrograms/ml incubation mixture.

obtained when incubating purified alkaline kidney phosphatase with phenyl phosphate in the presence of D_2P .

DISCUSSION

The exact nature of the activation of oxygen uptake by D_2P cannot be fully understood by means of the results given in this report.

Many steroid hormones, supposed to influence the metabolic patterns by affecting the function of enzyme systems, such as desoxycorticosterone, testosterone, progesterone and others, inhibit the oxygen uptake of tissue slices ²², of homogenates ²³, and of isolated enzyme systems such as succinoxidase ²⁴ and D-amino acid oxidase ²⁵. The specificity of these actions has been questioned, since physiologically inactive steroids also are capable of inhibiting the same systems. It has been supposed that the steroids in some way, specifically or non-specifically, interact with the proteins ²⁴.

A change in the activity of the electron transport system can, according to Keilin and Hartree ²⁶, be caused *in vitro* by compounds which either react directly and specifically with one or more components in the dehydrogenase-cytochrome system, or by those which, by modifying the colloidal state of the enzyme preparations, may markedly increase or decrease the activity. Decreased rate of oxygen uptake in a system, due to suboptimal concentration

of orthophosphate, can, by addition of denaturated globin or of Ca^{++} or Al^{+++} , be brought to the same level as with optimal phosphate concentration; the effect being due to a change in the colloidal structure²⁶.

If the activation by D_2P is only due to a change in the colloidal state the activation would remain constant for the whole period of incubation; particularly with regard to the fact that the net uptake of phosphate is depressed and thus the phosphate concentration remains nearer the optimum. The concentration of D_2P necessary to induce activation of oxygen uptake was very low. Since D_2P also was shown to interact with the net uptake of phosphate the effect of D_2P is probably not the result of a change in the colloidal state of the system.

The increased oxygen uptake obtained after the addition of D_2P takes place in the first period of incubation, *i. e.*, when the esterification of inorganic phosphate coupled to the electron transport process is most intense. Further on, when the rate of esterification has declined, there is no longer any activation. In long period experiments (*cf.* Table 2) the amount of esterified phosphate is considerably decreased when D_2P is added; after a short period of incubation, however, the difference between the two sets of condition is very little (*cf.* Fig. 3). *I. e.*, the P : O ratio decreases gradually both with and without D_2P . The decline is, however, much greater in experiments with D_2P . In the *in vitro* system applied, the amount of esterified phosphate represents a resultant between the synthesis of new phosphate bonds coupled to the oxidation and the action of splitting enzymes such as phosphatases and ATPase. The balance between these two processes must be changed after the addition of D_2P , since the phosphatase activity is increased. Therefore, it might be possible that the absolute amount of esterified phosphate in the initial phase of the incubation is greater with D_2P than without.

Phosphorylation can be completely inhibited without any effect on the oxidation^{27,28}. Thus esterification of inorganic phosphate is not an essential part of oxidation. It might be assumed, however, that an increased possibility for phosphorylation gives a corresponding possibility for increased oxidation. Earlier the hypothesis has been advanced²⁹ that certain dinucleotide splitting enzymes are directly engaged in the mechanism of phosphorylation. Phosphate transferring enzymes, necessary for oxidative phosphorylation, might *in vitro* in the absence of a suitable acceptor split esters, thus acting as ordinary phosphatases (*cf.* Meyerhof and Green³⁰). It seems reasonable that D_2P through activation of enzymes acting as phosphatases when tested *in vitro* but under physiological conditions, *i. e.*, *in vivo*, having a function other than to split phosphate esters, might increase the possibility for aerobic phosphorylation, involving a stimulation of oxidation.

SUMMARY

Phosphorylated vitamin D₃ (D₃P) added to a suspension of kidney mitochondria, containing glutamate as oxidizable substrate, gives an obvious initial activation of oxygen uptake. The net esterification of phosphate coupled to oxidation is almost the same with or without D₃P in the first phase of incubation. Further on, when the phosphorylation rate is declining the net uptake becomes less in the presence of D₃P depending on an activation of phosphatase. The significance of these findings has been discussed.

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