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COOPERATION OF Ca²⁺ AND pH IN REGULATION OF THE ACTIVITY OF THE 2-OXOGLUTARATE DEHYDROGENASE COMPLEX AND ITS COMPONENTS FROM BOVINE KIDNEY CORTEX *

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A modified procedure for preparation of the 2-oxoglutarate dehydrogenase complex from bovine kidney cortex is presented. The enzymatic preparation obtained showed a specific activity of 18.5 μmol × min⁻¹ × mg⁻¹. This activity was dependent on Ca²⁺ (1 - 40 μm) and hydrogen ion concentration. At pH 7.6 in the absence of Ca²⁺ (<10⁻⁹ m), Sₐₙ for 2-oxoglutarate was 2.5 mm, and in the presence of Ca²⁺ it was decreased to 0.3 mm. The maximum reaction rate at this pH was increased by Ca²⁺ by 33 %. The increase of pH from 7.0 to 8.4 resulted in a 150-fold increase of Sₐₙ. The activity of 2-oxoglutarate decarboxylase, a subunit of the dehydrogenase complex, was also dependent on Ca²⁺ and pH.

The activity of 2-oxoglutarate decarboxylase, determined in the presence of ferrocyanide as electron acceptor, showed three different partial Michaelis constants for 2-oxoglutarate, low (Kₐₑ), medium (Kₐ₂) and high (Kₐ₃). At pH 6.9, Kₐ₁ was 0.11 mm, and 0.005 mm in the absence and presence of Ca²⁺, respectively. The maximum reaction rate at pH 6.9 in the presence of Ca²⁺ was by 72 % higher than in its absence. A change of pH from 6.9 to 7.6 led to an increase in Kₐ₃ from 0.005 to 0.01 mm, and Kₐ₃ from 0.11 to 0.60 mm.

Ca²⁺ had no effect on the activity of lipoamide dehydrogenase or lipoamide succinyltransferase. These results indicate that, over the pH range 6.5 - 7.2, calcium ions affect the activity of the whole complex by regulating the activity of 2-oxoglutarate decarboxylase, whereas over the pH range 7.2 - 8.4 they affect the activity of the 2-oxoglutarate dehydrogenase complex by acting on the structure of the whole complex rather than by changing the activity of 2-oxoglutarate decarboxylase.

Oxidative decarboxylation of 2-oxoglutarate is catalysed by the 2-oxoglutarate dehydrogenase complex composed of three enzymes: E1, 2-oxoglutarate decarboxylase (EC 1.2.4.2); E2, lipoamide succinyltransferase (EC 2.3.1.61); and E3, lipoamide dehydrogenase (EC 1.6.4.3). These enzymes catalyse the following reactions shown in p. 290.

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The 2-oxoglutarate dehydrogenase complex of both animal and bacterial origin is regulated by a number of factors. Garland (1964) has demonstrated that the complex from pig heart is inhibited by succinyl-CoA and NADH. Ca\(^{2+}\) and Sr\(^{2+}\) lower the \(K_m\) value of 2-oxoglutarate dehydrogenase for 2-oxoglutarate in mitochondrial extracts (Denton et al., 1978; McCormack & Denton, 1979), and ADP lowers the \(K_m\) of the enzyme from pig heart (McCormack & Denton, 1979), whereas \(K_m\) of the enzyme from bovine kidney is increased by ATP and less so by GDP (Lawlis & Roche, 1981a). The inhibitory effect of NADH and ATP is largely decreased by Ca\(^{2+}\) (Lawlis & Roche, 1980; 1981b). Lawlis & Roche (1981a) have also demonstrated an additive stimulatory effect of Ca\(^{2+}\), ADP and inorganic phosphate on the activity of the 2-oxoglutarate dehydrogenase complex from bovine kidney. In view of the complex structure of 2-oxoglutarate dehydrogenase (Tanaka et al., 1972; 1974; Pettit et al., 1973; Koike et al., 1974), it became interesting to locate the action site of Ca\(^{2+}\). Thus, the aim of the present work was to examine the effect of Ca\(^{2+}\) and pH on the activity of the whole complex from bovine kidney cortex as well as of its component enzymes. It has been demonstrated that only 2-oxoglutarate decarboxylase is activated by Ca\(^{2+}\) and that this effect, like that on the whole 2-oxoglutarate dehydrogenase complex, is pH dependent.

MATERIALS

EDTA, Triton X-100 and \(K_3[Fe(CN)_6]\) were supplied by POCh (Gliwice, Poland). Liponic acid amide, 2-oxoglutarate, EGTA [ethylene glycol bis(2-aminoethyl ether)-\(N, N'\)-tetraacetic acid] and CoA were products of Sigma Chem. Co.
(St. Louis, Mo., U.S.A). Cysteine hydrochloride, NAD and NADH were purchased from Reanal (Budapest, Hungary). Thiamine pyrophosphate (TPP), 1,4-dithioerythritol and Hepes [N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulphonic acid] were from Merck (Darmstadt, F.R.G.). Sepharose 4B was from Pharmacia (Uppsala, Sweden) and polyethylene glycol type 6000 from Serva (Heidelberg, F.R.G.).

**METHODS**

**Determination of the activity of 2-oxoglutarate dehydrogenase complex.** The incubation medium contained in a final volume of 525 µl: 50 mm-Hepes-K buffer of desired pH, 50 mm-KCl, 2.5 mm-cysteine hydrochloride, 0.5 mm-thiamine pyrophosphate, 0.12 mm-CoA, 1.0 mm-NAD, 1.0 mm-dithioerythritol, 2-oxoglutarate at varying concentrations and 0.75 µg of the enzyme. The reaction was started by adding CoA.

Activity of the dehydrogenase complex was assayed by measuring at 334 nm the amount of NADH formed and was expressed as µmol×min⁻¹×mg⁻¹. The reaction was linear with time and with the amount of enzyme protein.

**Determination of the activity of 2-oxoglutarate decarboxylase.** This was determined according to Massey (1960) by measuring the rate of reduction of Fe(CN)₆³⁻ at 436 nm.

\[ [RCHO - TPP] - E₁ + 2Fe(CN)₆⁶⁻ + H₂O → RCO₂H + 2Fe(CN)₆⁴⁻ + 2H⁺ [TPP] - E₁ \]

The enzyme activity was expressed as µmol of reduced Fe(CN)₆³⁻ × 0.5 × min⁻¹ × mg⁻¹. The incubation medium contained in a final volume of 515 µl: 50 mm-Hepes-K buffer, 50 mm-KCl, 2.0 mm-K₂[Fe(CN)₆], 2.0 mm-thiamine pyrophosphate, varying concentrations of 2-oxoglutarate and 6 µg of the enzyme protein. The reaction was started by adding 2-oxoglutarate.

**Determination of the activity of lipoamide succinyltransferase.** The activity of this enzyme was assayed by measuring the rate of NAD reduction by the dihydro-lipoamide formed from succinyl-S-lipoamide-SH according to the reaction shown below.

![Reaction Diagram]

The reaction medium contained in a final volume of 545 µl: 50 mm-Hepes-K buffer, 50 mm-KCl, 1.0 µm-NAD, 0.3 mm-CoA, 5 units of lipoamide dehydrogenase, varying concentrations of succinyl-S-lipoamide-SH and 4 µg of the enzyme. NAD was added to the medium already containing lipoamide dehydrogenase, succinyl-S-lipoamide-SH and the 2-oxoglutarate dehydrogenase complex and when the
equilibrium had been attained, the reaction was started by adding CoA. Reduction of 1 mole of NAD is accompanied by reduction of 1 mole of dihydrolipoamide (Kornfeld et al., 1978). The activity of lipoamide succinyltransferase was expressed as μmol of reduced succinyl-S-lipoamide-SH×min⁻¹×mg⁻¹.

*Determination of the activity of lipoamide dehydrogenase.* The reaction medium contained in a final volume of 550 μl: 50 mM-Hepes-K buffer, 50 mM-KCl, 0.2 mM-NADH or 1.0 mM-NAD, varying concentrations of lipoamide or dihydrolipoamide and 0.35 μg of the enzyme protein. The reaction was started by adding lipoamide or dihydrolipoamide. The activity was determined by measuring at 334 nm the rate of NADH oxidation or NAD reduction and expressed as μmol×min⁻¹×mg⁻¹.

The enzymatic activities were measured at 25°C.

The activity in the presence of Ca²⁺ was assayed in the medium containing 5 mM-EGTA + 5 mM-CaCl₂ according to Denton et al. (1978). Ca²⁺ concentration below 10⁻⁹ M was obtained by applying 5 mM-EGTA. The approximate Ca²⁺ concentrations were calculated according to Portzehl et al. (1964) from apparent association constants for EGTA calculated from true association constants for EGTA given by Sillén & Martell (1971).

*Protein determination.* This was done according to Lowry et al. (1951) with bovine serum albumin as a standard. The results of protein determinations in solutions containing Triton X-100 were corrected for interference of this compound with the Folin reagent.

*Synthesis of DL-dihydrolipoamide.* This compound was synthesized from DL-lipoamide as described by Reed et al. (1958). Purity of the preparation was checked by thin-layer chromatography on silica gel. Only a single spot of DL-dihydrolipoamide was obtained.

*Synthesis of succinyl-S-lipoamide-SH.* DL-Dihydrolipoamide, 200 mg, was suspended in 5 ml water and cooled to 4°C, then 0.15 ml triethylamine and 150 mg succinyl anhydride were added to the mixture. The reaction was run for 1.5 h with continuous stirring. The unreacted DL-dihydrolipoamide and lipoamide were extracted by chloroform. Succinyl-S-lipoamide-SH which remained in the aqueous phase was used as substrate for the enzymatic reaction. Concentration of this compound was determined enzymatically. The course of the reaction and the purity of the product obtained were followed by thin-layer chromatography on silica gel.

*Fractionation with polyethylene glycol, type 6000 (PEG).* For fractionation 50% solution of PEG was used (Polson et al., 1964). Precipitation of the 2-oxoglutarate dehydrogenase complex was followed by withdrawing samples which were then centrifuged in an Eppendorf 3200 centrifuge for 2 min at maximal speed at room temperature, and the activity of the complex was determined in the supernatant. Usually, addition of 0.09 volume of 50% (w/v) PEG was sufficient for total precipitation of the enzyme.

*Gel filtration on Sepharose 4B.* The Quickfit column (2.5×100 cm) was used. The operational pressure was 80 cm H₂O. The volume of the sample applied corresponded to 3% of bed volume of the column. The chromatographed sample was
eluted with the buffer containing 50 mM-potassium phosphate pH 7.0, 2.2 mM-EDTA and 1% Triton X-100. Fractions of 4.5 ml were collected at a rate of 10 ml/h.

Preparation of mitochondria from bovine kidney cortex. Mitochondria were isolated from 1.5 kg of bovine kidney cortex by the method of Linn et al. (1972) as modified by Roche & Cate (1977). The mitochondria obtained were suspended in 500 ml of 20 mM-potassium phosphate buffer, pH 6.5, containing 0.1 mM-EDTA and 0.2 mM-TPP, supplemented with 12 ml of rat serum, frozen in liquid nitrogen and kept at −20°C for a few days without loss of the activity of the 2-oxoglutarate dehydrogenase complex.

SDS-Polyacrylamide gel electrophoresis. The electrophoresis was run in 12.5% polyacrylamide gel in the Tris-glycine system (Laemmli, 1970). The denaturing solution contained 0.06 M-Tris/HCl buffer, pH 6.8, 2% sodium dodecyl sulphate (SDS), 5% 2-mercaptoethanol and 10% sucrose. The gel was stained with 0.25% Coomassie Brilliant Blue for 2 h (Weber & Osborn, 1969).

RESULTS

Isolation of the 2-oxoglutarate dehydrogenase complex from bovine kidney cortex. The isolation procedure was performed at 2-4°C, unless otherwise indicated. The mitochondrial suspension was thawed and diluted to 1.5 litre with 20 mM-potassium phosphate buffer, pH 6.5, containing 0.1 mM-EDTA, supplemented with 20 ml of rat serum. Then, under continuous stirring, 5 mM-NaCl was added to final concentration of 50 mM and 30% Triton X-100 to a final concentration of 3%.

After 0.5 h the solution was centrifuged at 11 000 g for 45 min. The pellet was discarded and pH of the supernatant was brought to 6.45 by adding 10% acetic acid. Then 50% PEG (0.1 volume) was added slowly, with continuous stirring, and after 0.5 h the precipitate was sedimented by centrifugation at 10 000 g for 10 min. The supernatant was discarded and the pellet was suspended in 150 ml of buffer A (50 mM-potassium phosphate, pH 7.0, 2.5 mM-EDTA, 0.1 mM-dithioerythritol) and left under continuous stirring for 14 h. The suspension was centrifuged at 25 000 g for 45 min. The supernatant was diluted with buffer A to a protein concentration of 5 mg/ml, warmed to room temperature, brought to pH 6.45 with 10% acetic acid, then 50% PEG was added slowly until the 2-oxoglutarate dehydrogenase complex became precipitated (Linn et al., 1972; Roche & Cate, 1977; Stanley & Perham, 1980). The mixture was centrifuged at 10 000 g at 18°C for 10 min and the sediment was suspended in 65 ml of buffer A supplemented with 1% Triton X-100 and left under continuous stirring for 14 h. The suspension was centrifuged at 20 000 g for 40 min. The sediment was discarded and the supernatant was diluted with buffer A supplemented with 1% Triton X-100 to a protein concentration of 8 mg/ml, and centrifuged at 140 000 g for 3.5 h. The obtained gelatinous amber-coloured sediment was transferred to 10 ml of the buffer containing 50 mM-potassium phosphate, pH 7.0, 2.5 mM-EDTA and 1% Triton X-100, and left for 14 h under mild stirring. The solution obtained was centrifuged at 24 000 g for 40 min. The
clear supernatant was submitted to Sepharose 4B filtration as described in Methods. The results of a typical separation are presented in Fig. 1. Fractions No. 60 - 70 were collected, pooled, brought to pH 6.45 and precipitated with PEG as described above. The suspension was centrifuged at 10 000 g for 15 min and the sediment was transferred to 5 ml of 50 mm-potassium phosphate buffer, pH 7.0, containing 0.1 mm-dithioerythritol and left for 6 h under mild stirring. Usually, after that time the sediment was completely dissolved. The obtained light yellow solution of the 2-oxoglutarate dehydrogenase complex was frozen and stored at -20°C. Under these conditions the preparation lost about half of its activity after one year, and after two years showed about one-third of the initial activity. The results of the procedure are presented in Table 1. The enzyme complex obtained was purified 617-fold as compared with the tissue homogenate in which the activity was 0.030 µmol×min⁻¹×mg⁻¹. The purified 2-oxoglutarate dehydrogenase complex contained 2% of the pyruvate dehydrogenase activity and no activity of lactate and malate dehydrogenases. On electrophoresis in 12.5% polyacrylamide gel, the isolated enzyme complex gave four main bands (Fig. 2) of $M_r$ 100 000 (E1, 2-oxoglutarate decarboxylase), $M_r$ 90 000 (N, unknown; Linn, 1974; Kresze et al., 1981), $M_r$ 57 000 (E3, lipoamide dehydrogenase), and $M_r$ 52 000 (E2, lipoamide succinyltransferase).

Effect of Ca²⁺ on the activity of 2-oxoglutarate dehydrogenase complex. It appeared that the initial rate ($V_0$) of the reaction catalysed by the isolated enzyme complex at pH 7.6 and at different concentrations of 2-oxoglutarate was increased in the presence of Ca²⁺ (Fig. 3). With 2.5 mm-2-oxoglutarate the respective $V_0$ values
Fig. 2. SDS-polyacrylamide gel electrophoresis of the 2-oxoglutarate dehydrogenase complex.
The amounts of the enzyme protein were: A, 15 µg; B, 10 µg; C, 5 µg; D, 30 µg.
### Table 1

**Purification of the 2-oxoglutarate dehydrogenase complex from bovine kidney cortex**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Activity</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>total</td>
<td>specific</td>
</tr>
<tr>
<td>Mitochondrial extract after centrifugation</td>
<td>1800</td>
<td>39 600</td>
<td>7326.0</td>
<td>0.185</td>
</tr>
<tr>
<td>First polyethylene glycol fractionation</td>
<td>150</td>
<td>3 750</td>
<td>6 000</td>
<td>1.60</td>
</tr>
<tr>
<td>Second polyethylene glycol fractionation</td>
<td>70</td>
<td>1 050</td>
<td>5 376</td>
<td>5.12</td>
</tr>
<tr>
<td>Ultracentrifugation at 140 000 g</td>
<td>15</td>
<td>450</td>
<td>3 465</td>
<td>7.7</td>
</tr>
<tr>
<td>Gel filtration on Sepharose 4B</td>
<td>55</td>
<td>137.5</td>
<td>3 089</td>
<td>10.8</td>
</tr>
<tr>
<td>Polyethylene glycol precipitation</td>
<td>6.0</td>
<td>132</td>
<td>2 442</td>
<td>18.5</td>
</tr>
</tbody>
</table>

in the absence and presence of Ca²⁺ were 4.62 and 12.6 μmol×min⁻¹×mg⁻¹ (Fig. 3). Ca²⁺ caused a lowering of the S₀.₅ value of the enzyme complex. At pH 7.6 this value was 0.3 mm and 2.5 mm in presence and absence of Ca²⁺, respectively.

**Fig. 3.** Effect of Ca²⁺ on the activity of the 2-oxoglutarate dehydrogenase complex. Activity in the presence of Ca²⁺ (5 mM-EGTA+5 mM-CaCl₂) at pH 7.6 (▲) and 7.0 (△); activity in the absence of Ca²⁺ (5 mM-EGTA) at pH 7.6 (●) and 7.0 (○). Insert: the Hill plot.
Table 2

Effect of pH and Ca\(^{2+}\) on kinetic constants of the 2-oxoglutarate dehydrogenase complex from bovine kidney cortex

<table>
<thead>
<tr>
<th>pH</th>
<th>(V_{\text{max}})</th>
<th>(h)</th>
<th>(S_{0.5})</th>
<th>(V_{\text{max}})</th>
<th>(h)</th>
<th>(S_{0.5})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>((\text{\mu mol} \times \text{min}^{-1} \times \text{mg}^{-1}))</td>
<td></td>
<td>(mm)</td>
<td>((\text{\mu mol} \times \text{min}^{-1} \times \text{mg}^{-1}))</td>
<td></td>
<td>(mm)</td>
</tr>
<tr>
<td>6.8</td>
<td>8.40</td>
<td>0.90</td>
<td>0.03</td>
<td>10.08</td>
<td>1.00</td>
<td>0.02</td>
</tr>
<tr>
<td>7.0</td>
<td>9.24</td>
<td>0.90</td>
<td>0.07</td>
<td>10.92</td>
<td>1.00</td>
<td>0.05</td>
</tr>
<tr>
<td>7.2</td>
<td>10.08</td>
<td>0.83</td>
<td>0.20</td>
<td>12.18</td>
<td>1.00</td>
<td>0.10</td>
</tr>
<tr>
<td>7.4</td>
<td>10.92</td>
<td>0.77</td>
<td>0.80</td>
<td>13.44</td>
<td>0.98</td>
<td>0.20</td>
</tr>
<tr>
<td>7.6</td>
<td>10.10</td>
<td>0.78</td>
<td>2.50</td>
<td>13.44</td>
<td>1.00</td>
<td>0.30</td>
</tr>
<tr>
<td>8.4</td>
<td>5.6</td>
<td>0.75</td>
<td>4.50</td>
<td>10.50</td>
<td>0.98</td>
<td>0.70</td>
</tr>
</tbody>
</table>

(Table 2). The maximum reaction rate was also increased by Ca\(^{2+}\): \(V_{\text{max}}\) at pH 7.6 in presence of Ca\(^{2+}\) was 13.44 and in the absence of Ca\(^{2+}\) was 10.10 \(\mu\text{mol} \times \text{min}^{-1} \times \text{mg}^{-1}\) (Fig. 3). Ca\(^{2+}\) also affected the Hill coefficient \((h)\). At pH 7.6 the values of \(h\) were 1.00 and 0.78, in presence and absence of Ca\(^{2+}\), respectively (Table 2), which could point to a negative cooperativity. Thus, Ca\(^{2+}\) regulates the activity of 2-oxoglutarate dehydrogenase complex by changing the \(S_{0.5}\), the initial rate of the reaction and its maximum rate. The regulatory role of Ca\(^{2+}\) was also manifested in the release of negative cooperativity, which was observed in the absence of Ca\(^{2+}\), as can be concluded from the Hill coefficient lower than unity and from the hyperbolic shape of the Lineweaver-Burk plot (Fig. 4).

![Fig. 4. Effect of Ca\(^{2+}\) on the activity of 2-oxoglutarate dehydrogenase complex at pH 7.6: Lineweaver-Burk plot. Activity in the presence of Ca\(^{2+}\) (5 mm-EGTA+5 mm-CaCl\(_2\)), (○); and in the absence of Ca\(^{2+}\) (5 mm-EGTA), (●).](image-url)
Effect of pH on the activity of the 2-oxoglutarate dehydrogenase complex. It was found that pH optimum of the reaction catalysed by the complex was at the range of 7.4 - 7.6 both in the presence and absence of Ca\(^{2+}\) (Fig. 5). All changes of pH beyond this range were accompanied by decreased \(V_{\text{max}}\) (Fig. 5). In the absence of Ca\(^{2+}\) lowering of pH from 7.6 to 6.8 caused a decrease in \(V_{\text{max}}\) by 17% (Table 2), whereas an increase from pH 7.6 to 8.4 led to a decrease of \(V_{\text{max}}\) by as much as 45% (Table 2). The same changes of pH in the presence of Ca\(^{2+}\) caused a lowering of the activity.
of $V_{\text{max}}$ by about 20%, both at higher and lower pH values (Table 2). $S_{0.5}$ of the 2-oxoglutarate dehydrogenase complex for 2-oxoglutarate was also pH-dependent. An increase in pH from 6.8 to 8.6 caused a 150-fold increase of $S_{0.5}$ in the absence of Ca$^{2+}$ and a 35-fold increase in its presence (Table 2). The Hill coefficient was lower than unity in the absence of Ca$^{2+}$; with an increase of pH from 6.8 to 8.4 its value decreased from 0.9 to 0.75 (Table 2). On the other hand, in the presence of Ca$^{2+}$, the Hill coefficient was close to unity and was pH-independent (Table 2).

**Effect of pH and Ca$^{2+}$ on the activity of 2-oxoglutarate decarboxylase.** As can be seen in Fig. 6, Ca$^{2+}$ at pH 6.9 caused an increase in the initial rate of the reaction catalysed by this enzyme assayed by measuring reduction of potassium ferricyanide. The initial reaction rate for 0.01 mm 2-oxoglutarate in the absence of Ca$^{2+}$ and in its presence was 0.10 and 0.29 $\mu$mol $\times$ min$^{-1}$ $\times$ mg$^{-1}$, respectively. Lawlis & Roche (1981b) observed a similar effect of Ca$^{2+}$ on the activity of resolved 2-oxoglutarate decarboxylase from bovine kidney. The maximum reaction rate which could be obtained at 2-oxoglutarate concentration as low as 0.2 mm was 0.44 $\mu$mol $\times$ min$^{-1}$ $\times$ mg$^{-1}$ in the presence of Ca$^{2+}$; in the absence of Ca$^{2+}$ it was by almost 50% lower.

![Graph](image_url)

**Fig. 7.** Effect of Ca$^{2+}$ on the activity of 2-oxoglutarate decarboxylase at pH 6.9. Lineweaver-Burk plot. Activity in the presence of Ca$^{2+}$ (5 mm-EGTA + 5 mm-CaCl$_2$), (○); in the absence of Ca$^{2+}$ (5 mm-EGTA), (●).
(0.23 μmol×min⁻¹×mg⁻¹). In the plot of the dependence of the initial reaction rate on 2-oxoglutarate concentration, three distinct ranges can be distinguished which permit to calculate low \( K_m^1 \), medium \( K_m^2 \) and high \( K_m^3 \) partial Michaelis constants (Fig. 7). This is at variance with the results reported for the enzyme from pigeon breast muscle (Gomazkowa & Krasovskaya, 1979), which shows two \( K_m \) values.

**Table 3**

*Effect of pH and Ca²⁺ on kinetic constants of 2-oxoglutarate decarboxylase*

<table>
<thead>
<tr>
<th>pH</th>
<th>( K_m^1 )  (mm)</th>
<th>( K_m^2 )  (mm)</th>
<th>( K_m^3 )  (mm)</th>
<th>( V_{max} ) ((μmol×min⁻¹×mg⁻¹))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-Ca²⁺</td>
<td>+Ca²⁺</td>
<td>-Ca²⁺</td>
<td>+Ca²⁺</td>
</tr>
<tr>
<td>6.9</td>
<td>0.005</td>
<td>0.005</td>
<td>0.035</td>
<td>0.020</td>
</tr>
<tr>
<td>7.6</td>
<td>0.010</td>
<td>0.010</td>
<td>0.036</td>
<td>0.020</td>
</tr>
</tbody>
</table>

As shown in Table 3, \( K_m^1 \) was independent of Ca²⁺ both at pH 6.9 and 7.6, whereas \( K_m^2 \) and \( K_m^3 \) were lowered by Ca²⁺. It is also evident that \( K_m^1 \) and \( K_m^2 \) were increased by increasing pH from 6.9 to 7.6 both in the presence and absence of Ca²⁺. The optimum pH for 2-oxoglutarate decarboxylase was 6.9 (Fig. 8), similarly as it was observed for the enzyme from pig heart (Koike et al., 1974).

![Graph](image)

**Fig. 8.** Effect of pH and Ca²⁺ on \( V_{max} \) of 2-oxoglutarate decarboxylase. Saturating concentrations of 2-oxoglutarate were used. In the presence of Ca²⁺ (5 mM-EGTA+5 mM-CaCl₂), (○); and in the absence of Ca²⁺ (5 mM-EGTA), (●). The results are mean values from 4 determinations I S.E.M.

**Effect of pH and Ca²⁺ on the activity of lipoamide succinyltransferase.** This was measured with succinyl-S-lipoamide-SH as substrate. At pH 7.6 \( K_m \) for this substrate was 0.083 mM both in the presence and absence of Ca²⁺ (Fig. 9). pH optimum was 8.0 irrespective of the presence of Ca²⁺ (Fig. 10). Over the pH range from 7.0 to 8.6, Ca²⁺ had no effect on the initial rate, \( K_m \) and \( V_{max} \). Thus, it can be concluded that the activity of lipoamide succinyltransferase is Ca²⁺-independent.
Effect of pH and Ca\textsuperscript{2+} on the activity of lipoamide dehydrogenase. The activity of this enzyme was determined with lipoamide and NADH, or dihydrolipoamide and NAD as substrates. At pH 7.6 the Michaelis constant for dihydrolipoamide

![Graph](image_url)

**Fig. 9.** Effect of Ca\textsuperscript{2+} on the activity of lipoamide succinyltransferase at pH 7.6. Activity in the presence of Ca\textsuperscript{2+} (5 mm-EGTA+5 mm-CaCl\textsubscript{2}), (○); in the absence of Ca\textsuperscript{2+} (5 mm-EGTA), (●).

Insert: Lineweaver-Burk plot.

![Graph](image_url)

**Fig. 10.** Effect of pH and Ca\textsuperscript{2+} on $V_{max}$ of lipoamide succinyltransferase. Saturating concentrations of succinyl-S-lipoamide-SH were used. In the presence of Ca\textsuperscript{2+} (5 mm-EGTA+5 mm-CaCl\textsubscript{2}), (○); and in the absence of Ca\textsuperscript{2+} (5 mm-EGTA), (●).
was 0.6 mM both in the presence and absence of Ca\(^{2+}\) (Fig. 11). For lipoamide at pH 7.3 \(K_m\) was 0.8 mM, and was also Ca\(^{2+}\)-independent. The enzyme showed the highest activity with dihydrolipoamide as a substrate at pH 8.0, irrespective of the presence of Ca\(^{2+}\) (Fig. 12), similarly as it has been reported for the enzyme

![Graph](image1)

**Fig. 11.** Effect of Ca\(^{2+}\) on the activity of lipoamide dehydrogenase at pH 7.6. Activity in the absence of Ca\(^{2+}\) (5 mM-EGTA), (●); and in the presence of Ca\(^{2+}\) (5 mM-EGTA + 5 mM-CaCl\(_2\)), (○). Insert: Lineweaver-Burk plot.

![Graph](image2)

**Fig. 12.** Effect of pH and Ca\(^{2+}\) on \(V_{\text{max}}\) of lipoamide dehydrogenase. \(V_{\text{max}}\) in the presence of Ca\(^{2+}\) (5 mM-EGTA + 5 mM-CaCl\(_2\)), (○); in the absence of Ca\(^{2+}\) (5 mM-EGTA), (●). Saturating concentration of dihydrolipoamide was used.

from rat liver (Reed, 1973). Over the whole pH range studied, from 7.0 to 8.6, Ca\(^{2+}\) had no effect on the kinetics of this enzyme, either with lipoamide or dihydrolipoamide as substrate. It can be thus concluded that Ca\(^{2+}\) has no effect on the activity of lipoamide dehydrogenase. This is in contrast to the stimulation by Ca\(^{2+}\) of lipoamide dehydrogenase from bovine kidney observed by Lawlis & Roche (1981b) who studied this enzyme as a component of the 2-oxoglutarate dehydrogenase complex.
DISCUSSION

It has been previously shown that bovine kidney 2-oxoglutarate dehydrogenase complex is inactivated by a specific protease which was released from mitochondria exposed to a hypotonic solution and that this inactivation is not accompanied by the loss of activity of the three component enzymes (Linn, 1974). It has been also shown (Wieland, 1974) that the activity of the dehydrogenase complex in bovine kidney mitochondria is stabilized in the presence of rat serum. Similar results were obtained on addition of rabbit (Roche & Cate, 1977) or horse serum, probably due to binding of the proteolytic activity by $\alpha_2$-macroglobulin (Salvesen & Barret, 1980: Gustavsson et al., 1980: Laskowski & Kato, 1980). Further information is needed to explain the mechanism of inactivation of the dehydrogenase complex by this protease and whether it is a contaminant or an intrinsic mitochondrial enzyme.

On studying the effect of $Ca^{2+}$ (1 - 40 $\mu$m) on the activity of the purified 2-oxoglutarate dehydrogenase complex from bovine kidney cortex, a considerable dependence of the enzymatic activity on $Ca^{2+}$ reported by other workers (Furuta et al., 1977: Denton et al., 1978: McCormack & Denton, 1979: Lawlis & Roche, 1980, 1981a,b) was confirmed. Moreover, it was demonstrated that $Ca^{2+}$ not only lowers the $S_{0.5}$ value for 2-oxoglutarate but also raises the maximum rate of the reaction catalysed by the 2-oxoglutarate dehydrogenase complex. On the other hand, no dependence of $V_{max}$ on $Ca^{2+}$ was observed by McCormack & Denton (1979) for the 2-oxoglutarate dehydrogenase complex from pig heart, and by Lawlis & Roche (1981a) for the complex from bovine kidney. This could be due to the presence in their incubation medium of $Mg^{2+}$ which in our experiments was not added. The degree of regulation by $Ca^{2+}$ of the activity of 2-oxoglutarate dehydrogenase complex from bovine kidney cortex is pH-dependent. The sensitivity of the enzyme complex to $Ca^{2+}$ decreases with decreasing pH value, which is manifested by the decreasing response to $Ca^{2+}$ of $S_{0.5}$ and $V_{max}$, observed at lower pH values (Table 2). From these observations it appears that the degree of protonation of the protein of the 2-oxoglutarate dehydrogenase complex is responsible for the enzyme sensitivity to $Ca^{2+}$. Over the whole pH range studied, the Hill coefficient in the presence of $Ca^{2+}$ corresponded to unity or was close to this value, whereas in the absence of $Ca^{2+}$ it was lower than unity (Table 2). The latter observation could point to negative cooperativity in the absence of $Ca^{2+}$. The Hill coefficient determined by Lawlis & Roche (1981a) for 2-oxoglutarate dehydrogenase complex from bovine kidney and pig heart was lower than unity, both in the presence and absence of $Ca^{2+}$. The data obtained on the activity of the 2-oxoglutarate dehydrogenase complex at different pH values (Table 2) indicate that pH affects the maximum rate of the reaction, $S_{0.5}$ constant and the Hill coefficient. On studying the effect of $Ca^{2+}$ on the activity of particular enzymes of the 2-oxoglutarate dehydrogenase complex, dependence of 2-oxoglutarate decarboxylase activity on $Ca^{2+}$ (6 - 33 $\mu$m) was demonstrated, which is in agreement with the results of Lawlis & Roche (1981b), whereas the activities of lipoamide dehydrogenase and lipoamide succinyltransferase were not affected by $Ca^{2+}$ (1 - 27 $\mu$m). Lawlis & Roche (1981b), studying the effect
of Ca\(^{2+}\) on inhibition by NADH of lipoamide dehydrogenase activity, found that Ca\(^{2+}\) raised the specific activity of this enzyme with 1.0 mm-dihydrolipoamide as substrate.

Three partial Michaelis constants were found for 2-oxoglutarate decarboxylase from bovine kidney cortex with 2-oxoglutarate as substrate, whereas for the enzyme from pigeon breast muscle two \(K_m\) values were obtained (Gomazkova & Krasovskaya, 1979). The results presented in Table 3 indicate that Ca\(^{2+}\) regulates the activity of 2-oxoglutarate decarboxylase by changing the second and the third partial Michaelis constants as well as the maximum reaction rate.

The changes in the activity of the 2-oxoglutarate dehydrogenase complex are not a simple reflection of changes in the activity of 2-oxoglutarate decarboxylase resulting from the action of Ca\(^{2+}\) on this enzyme. With the pH increasing from 6.9 to 7.6 the effect of Ca\(^{2+}\) on the activity of the latter enzyme became weaker (Table 3), whereas its effect on the activity of the whole enzymatic complex became stronger (Table 2). Thus, it can be assumed that in the pH range 6.5 - 7.2, Ca\(^{2+}\) could affect the activity of 2-oxoglutarate dehydrogenase complex by changing the kinetic parameters of 2-oxoglutarate decarboxylase, whereas over the pH 7.2 - 8.4 Ca\(^{2+}\) seems to regulate the activity of 2-oxoglutarate dehydrogenase complex by affecting the whole structure of this complex rather than by changing only the activity of 2-oxoglutarate decarboxylase.

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WSPÓLDZIAŁANIE POMIĘDZY $\text{Ca}^{2+}$ I pH W REGULACJI AKTYWNOŚCI KOMPLEKSU DEHYDROGENAZY 2-OXOGLUTARANOWEJ I JEJ KOMPONENT Z KORY NERKI WOŁOWEJ

Streszczenie

Przedstawiono zmodyfikowaną procedurę oczyszczania kompleksu dehydrogenazy 2-oksoglutaranowej z kory nerki wołowej. Otrzymany preparat enzymatyczny wykazał aktywność specyficzną 18.5 $\text{µmol} \times \text{min}^{-1} \times \text{mg}^{-1}$. Aktywność enzymu była zależna od $\text{Ca}^{2+}$ (1 - 40 $\mu$), i stężenia jonów wodorowych. W pH 7.6 w nieobecności $\text{Ca}^{2+}$ (<$10^{-1}$ m), $S_{0.5}$ dla 2-oksoglutaranu wynosiła 2.5 mm, natomiast w obecności $\text{Ca}^{2+}$ ulegała obniżeniu do 0.3 mm. Maksymalna szybkość reakcji w tym pH ulegała podwyższeniu przez $\text{Ca}^{2+}$ o 33%. Podwyższenie pH z 7.0 do 8.4 powodowało 150-krotnie zwiększenie $S_{0.5}$.

Aktywność dekarboksylazy 2-oksoglutaranowej również zależała od $\text{Ca}^{2+}$ i pH.

Oznaczając aktywność dekarboksylazy 2-oksoglutaranowej wobec żelazicyjanu potasu jako akceptora elektronów wykazano istnienie trzech cząstkowych stałych Michaelisa dla 2-oksoglutaranu, niskiej ($K_{m}^{a}$), średniej ($K_{m}^{b}$) i wysokiej ($K_{m}^{c}$). W pH 6.9 $K_{m}^{c}$ wynosiła 0.11 mm w nieobecności i 0.005 mm w obecności $\text{Ca}^{2+}$. Szybkość maksymalna reakcji w pH 6.9 w obecności $\text{Ca}^{2+}$ była o 72% wyższa niż w nieobecności $\text{Ca}^{2+}$. Zmiana pH z 6.9 do 7.6 powodowała podwyższenie $K_{m}^{c}$ z 0.005 do 0.01 mm i $K_{m}^{b}$ z 0.11 do 0.60 mm.

Aktywność dehydrogenazy lipoamidowej i sukcinylotransferazy lipoamidowej nie zależała od $\text{Ca}^{2+}$. Przedstawione dane wskazują, że w pH 6.5 - 7.2 jony wapnia regulują aktywność kompleksu poprzez zmianę aktywności dekarboksylazy 2-oksoglutaranowej, natomiast w zakresie pH 7.2 - 8.4 jony wapnia zmieniają aktywność kompleksu w większym stopniu przez oddziaływanie na strukturę całego kompleksu niż przez zmiany aktywności dekarboksylazy 2-oksoglutaranowej.

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