NON-ENZYMIC CONVERSION OF PYRUVATE IN AQUEOUS SOLUTION TO 2,4-DIHYDROXY-2-METHYLGLUTARIC ACID

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1. Introduction

Pyruvate oxidation is frequently studied as a system for investigations of metabolic capacity and regulation. The purity of radioactive substrates such as [1-^{14}C]pyruvate and [2-^{14}C]pyruvate is a crucial factor for correlating the trapped $^{14}CO_2$ with the total radioactivity originally present in these substrates. Moreover, if the impurity influences metabolism, the results obtained may be misleading [1].

The presence of the dimer of pyruvate in aqueous solutions is described [2,3]. The oxidative decarboxylation of 2-oxoglutarate in rat heart mitochondria is inhibited by this compound. Subsequently it was demonstrated that this compound produces a block in the citric acid cycle so that also pyruvate cannot be oxidized at normal rate [4]. Pyruvate can also decompose by a nonenzymatic decarboxylation. In aqueous solutions this may result from radicals arising from water in the presence of the radiation which attacks the dissolved ^{14}C compounds [5]. Metal ions, especially Zn^{2+}, present in solutions of pyruvate, catalyze not only the decarboxylation of pyruvate [6] but also the enhancement of pyruvate aldolization [7].

Here, the conversion of pyruvate into 2,4-dihydroxy-2-methylglutaric acid, the reduced form of parapyruvate, is demonstrated. The structure of this compound was established by mass spectrometry. We could not detect any inhibition by this compound of pyruvate and 2-oxoglutarate oxidation in rat liver homogenate.

2. Materials and methods

High-voltage electrophoresis was performed with a Camag apparatus on Whatmann (3 MM) for 40 min at 2000 V in formic acid/acetic acid/water (1.7:1.7:100, by vol.) adjusted with pyridine to pH 3.1. The paper strips were scanned with a Packard paper scanner. Radioactivity was quantitatively determined with liquid scintillation counting. Pyruvate was determined enzymatically as in [8].

Chemical-ionization mass spectra were recorded with a Hewlett-Packard 5982A mass spectrometer equipped with a dual EI/CI source. CH_4 at a pressure of 2×10^{-4} torr was used as CI reagent. Source temperature was 55°C and probe temperature 260°C. Electron emission current was 300 μA and electron energy 300 eV. The solution of the unknown compound in ethylacetate was introduced into the source.

A 5% rat liver homogenate was prepared in 10 mM Tris–HCl buffer (pH 7.4), containing 0.25 M sucrose and 2 mM EDTA, with a Potter-Elvejhem homogenizer. The supernatant, obtained after centrifugation at $600 \times g$ for 10 min at 4°C, was used in the assay. The basic incubation medium contained 50 mM potassium phosphate buffer, 0.5 mM EDTA, 6 mM $MgCl_2$, 2 mM ADP and 75 mM KCl adjusted to pH 7.4. The final substrate concentration of [1-^{14}C]pyruvate or 2-oxo-[1-^{14}C]glutarate (0.5 μCi/mmol) amounted to 1 mM in final vol. 0.5 ml. In the experiments with pyruvate as substrate 1 mM malate was added to the incubation medium. Incubation with 0.1–0.5 mg protein was performed in 15 ml glass scintillation

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vials, sealed by rubber stoppers, containing two small glass tubes, one fitting inside the other [9]. The enzyme assay was stopped with 0.2 ml 3 M perchloric acid and the $^{14}CO_2$ was trapped in Hyamine hydroxide (Packard). Radioactivity was measured after addition of 10 ml scintillation fluid (4 g Omnifluor/liter toluene). Radioactive substrates were purchased from the Radiochemical Centre, Amersham.

3. Results

An electropherogram of a 4-days old aqueous solution of [1-^{14}C]pyruvate stored at −20°C is presented in fig.1. The peak with the highest electrophoretic mobility represents the authentic [1-^{14}C]-pyruvate as judged by its convertibility to [1-^{14}C]-lactate under conditions for quantitative conversion with lactate dehydrogenase. The peak with the lower electrophoretic mobility could not be converted under the same conditions, even after incubation for 22 h. The migration rate of the impurity was about the same as that for 2-oxo-[1-^{14}C]glutarate under the conditions used.

A pure solution of the unknown compound was obtained after storing a 1 mM pyruvate solution (pH 5.5) at −20°C for 2 months. In this solution no pyruvate could be detected enzymatically. The formation of the impurity appeared to be irreversible. The sample was adjusted to pH 1, then prepared for mass spectrometry by freeze drying and dissolving in ethylacetate.

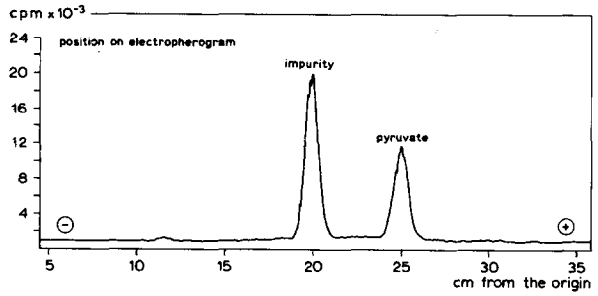

Fig.1. High voltage electrophoresis of radioactive [1-^{14}C]-pyruvate, stored at −20°C as an aqueous solution for 4 days. Electrophoresis was performed as described in section 2. Radioactivity was 25 nCi, concentration of pyruvate 300 μM and sample size 5 μl.

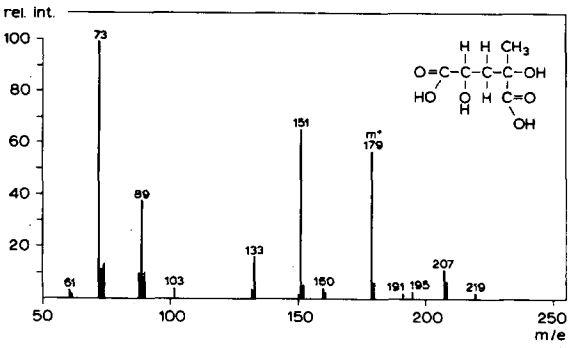

Fig.2. Mass spectrum of 2,4-dihydroxy-2-methyl glutaric acid

The presence of parapyruvate in stored solutions of pyruvate was revealed [2,5,7]. Therefore our solution was tested as to the presence of a compound, which reacts with 2,4-dinitrophenylhydrazine. Formation of a hydrazone was not observed at 483 nm.

The compound could be identified by mass spectrometry as 2,4-dihidroxy-2-methylglutaric acid (fig.2, table 1).

Addition of this substance up to 0.25 mM caused no inhibition of the oxidation of [1-^{14}C]pyruvate and 2-oxo-[1-^{14}C]glutarate with rat liver homogenate (table 2). About 20% inhibition occurred in the presence of 0.5 mM of this substance.

Table 1
Mass spectrum of 2,4-dihydroxy-2-methyl glutaric acid (m = 178)

m/e	Fragment	Relative intensity (%)
73	$\frac{1}{2}m - 16$	100
89	$\frac{1}{2}m$	38
133	$m + 1 - 46$	17
151	$m + 1 - 28$	65
179	$m + 1$	57
207	$m + 29$	11
219	$m + 41$	3

The peak at m/e 179 represents the protonated molecule (m + 1). The loss of a carbonylgroup from m/e 179 yields the peak of m/e 151. The peak at m/e 133 is due to loss of the terminal protonated carboxyl group. The peak at m/e 89 is due to the cleavage of the molecule between C-3 and C-4. The base peak (m/e 73) is formed by loss of an oxygen atom from m/e 89

Table 2
Influence of 2,4-dihydroxy-2-methyl glutarate on pyruvate and 2-oxoglutarate oxidation in rat-liver homogenate

Concentration (μM)	Pyruvate (% control)	2-Oxoglutarate (% control)
0	100	100
25	104	98
50	99	99
100	95	102
150	103	95
200	98	96
250	95	94
500	81	85

Incubation during 1 h was performed as described in section 2

4. Discussion

Artificial decreases in apparent activity of pyruvate dehydrogenase resulted with [1-^{14}C]pyruvate when the substrate was not stored with meticulous care [1]. The marked formation of the impurity, that may occur in aqueous solutions of pyruvate, necessitates a control of the purity of the radioactive substrate. After storage for several months multiple impurities appeared to be formed [2]. Data presented [2,5,7] suggested that parapyruvate (2-hydroxy-2-methyl-4-oxoglutarate) was formed in aqueous solutions of pyruvate. We found an irreversible conversion of pyruvate into the reduced form of parapyruvate. The mechanism of this conversion is not known. The discrepancy between our results and [2,5,7] cannot be explained. Our compound does not react with 2,4-dinitrophenylhydrazine which indicates that the compound contains no carbonyl group. Identification of this substance by means of mass spectrometry has not been reported until now.

That pyruvate and 2-oxo-glutarate could not be oxidized in the normal manner in the presence of the impurity [4,10–13] are in contrast with our findings. This disagreement might be due to the fact that the reduced form of parapyruvate was present in our incubation medium. Preliminary experiments indicated that aqueous solutions of 2-oxoglutarate are unstable, too, probably as a consequence of a similar conversion as in the case of pyruvate.

Batches of radioactive pyruvate and 2-oxoglutarate should, therefore, be freeze dried immediately after dilution and stored at −20°C in small aliquots as dry sodium salt sealed under nitrogen.

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