EFFECTS OF SYNTHETIC ANALOGUES OF PHOSPHOENOLPYRUVATE ON MUSCLE AND LIVER PYRUVATE KINASE, MUSCLE ENOLASE, LIVER PHOSPHOENOLPYRUVATE CARBOXYKINASE AND ON THE INTRA-/EXTRA-MITOCHONDRIAL TRICARBOXYLIC ACID CARRIER TRANSPORT SYSTEM

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Received 25 September 1971

1. Introduction

Recently several groups [1–3] have reported experiments with synthetic analogues of phosphoenolpyruvate (PEP). So far only the effect of these compounds on muscle pyruvate kinase has been measured with at least partially contradictory results. During our studies on the regulation of gluconeogenesis we were interested in PEP analogues in order to get more information on the different reactions in which PEP participates.

The present paper describes the effects of phosphoenol-α-ketobutyrate (PEB) and phosphoenol-α-ketovalerate (PEV) on muscle and liver pyruvate kinase (EC 2.7.1.40), muscle enolase (EC 4.2.1.11), liver phosphoenolpyruvate carboxykinase (EC 4.1.1.32) and on the tricarboxylic acid anion carrier system in rat liver mitochondria.

Both analogues are strong inhibitors of all enzymes tested, with characteristic differences for the various enzymes. Both analogues were transported in exchange for citrate through the inner mitochondrial membrane, but again with characteristic quantitative differences. PEB inhibits gluconeogenesis and lactate utilization in pigeon liver homogenates.

2. Experimental

PEB and PEV were synthetized from the corresponding α-keto acids according to Clark and Kirby [4]. The products were characterized by their melting points, their phosphate content, and by IR and NMR spectroscopy. The preparations were completely free of PEP. The cyclohexyl ammonium salts of the analogues were converted to their corresponding sodium salts by treatment with Dowex-WX-50 (Na^+-form).

Pyruvate kinase from rabbit muscle, enolase from rabbit muscle, auxillary enzymes used for the coupled enzyme essays, and all coenzymes and PEP were obtained from Boehringer Mannheim Corp. Liver pyruvate kinase was partially purified from rat liver according to Schoner et al. [5] including the ammonium sulfate precipitation step. For test conditions for pyruvate kinase and enolase see fig. 1. For the preparation of phosphoenolpyruvate carboxykinase a pigeon liver homogenate was sonified and centrifuged at 100,000 g for 1 hr. The supernatant was freed from monovalent cations and bicarbonate on a Sephadex G-25 column. The enzyme-containing fractions were pooled and freeze-dried. Under the test conditions used (see fig. 2) pyruvate kinase activity did not interfere with the determination of phosphoenolpyruvate carboxykinase activity. All enzyme essays were performed at 30°.

Anion transport was studied in rat liver mitochondria, isolated according to Myers and Slater [6]. The mitochondria were loaded with 1,5-^{14}C-citrate at 0° according to Meijer [7] and the efflux of citrate was

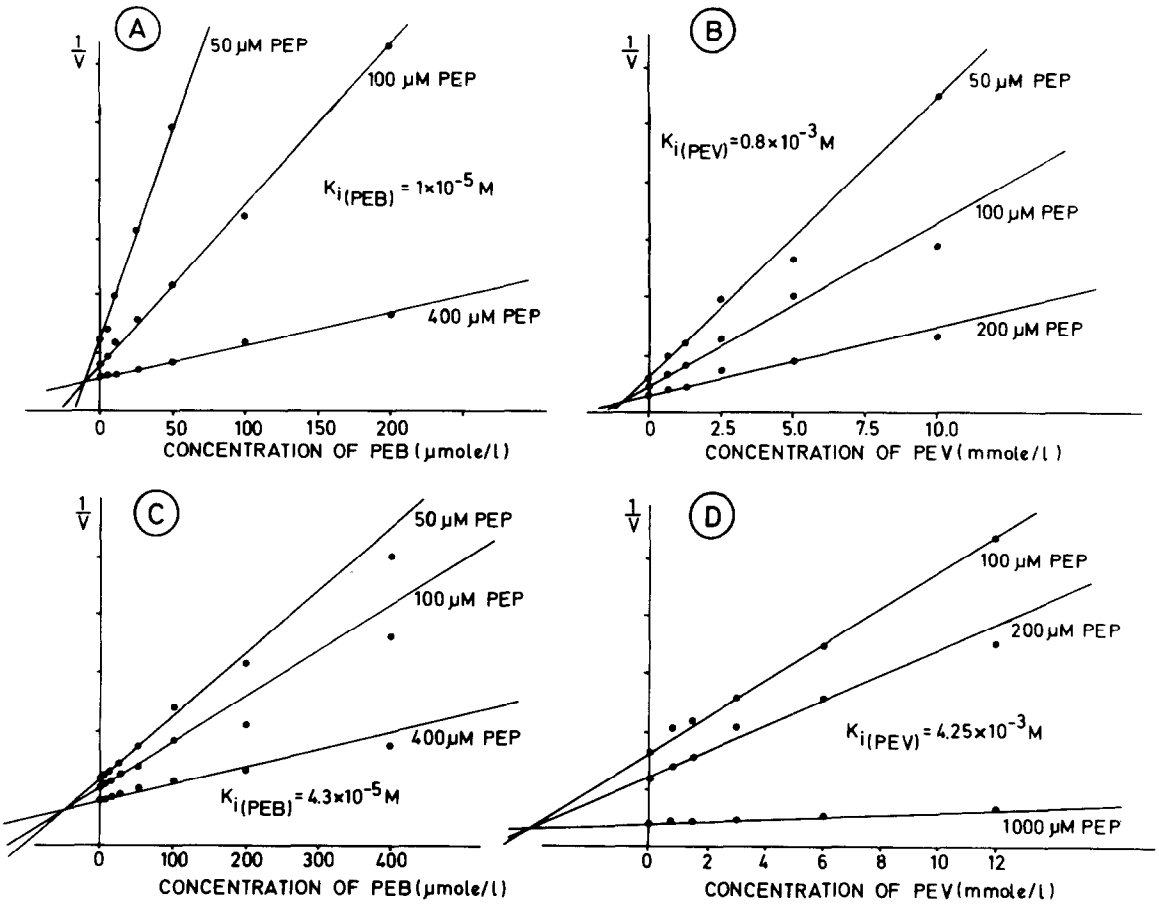

Fig. 1. (A) and (B): Dixon plots for the determination of K_i values for PEB (fig. 1A) and PEV (fig. 1B) with rabbit muscle pyruvate kinase. The test conditions were as follows (final concentrations given): Triethanolamine (pH 7.6) 80 mM; ADP 1.33 mM; NADH 0.27 mM; $MgSO_4$ 16 mM; KCl 66.0 mM; lactate dehydrogenase (pig heart) 13.2 μg/ml; pyruvate kinase (rabbit muscle) 0.07 μg/ml. The concentrations of PEP, PEB and PEV were varied as indicated in the graphs. (C) and (D): Dixon plots for the determination of K_i values for PEB (fig. 1C) and PEV (fig. 1D) with rabbit muscle enolase. The test conditions were as follows (final concentrations given): Triethanolamine (pH 7.6) 66 mM; ADP 1.1 mM; NADH 0.20 mM; 2,3-diphosphoglycerate 0.12 mM, EDTA 0.45 mM; $MgSO_4$ 8.4 mM; glyceraldehyde-3-phosphate dehydrogenase (yeast) 83 μg/ml; phosphoglycerate kinase (yeast) 16.7 μg/ml; phosphoglycerate mutase (rabbit muscle) 8.33 μg/ml; enolase (rabbit muscle) 0.67 μg/ml. The concentrations of PEP, PEB and PEV were varied as indicated in the graphs.

was measured at 25° in the presence of rotenone (2 μg/ml) using PEP [8] or the PEP analogues as counter anions (for details see table 1). The inhibitor benzene-1,2,3-tricarboxylate [8] was obtained from Schuchardt and Co., Munich, Germany.

3. Results

3.1. *Pyruvate kinase from rabbit muscle*

PEB as well as PEV are both converted to the corresponding α-keto acids as can be seen from the fact that the reaction products led to an oxidation of NADH in the presence of pig heart lactate dehydrogenase. At saturating concentrations (10 mM) PEP was 900 times more rapidly converted to the

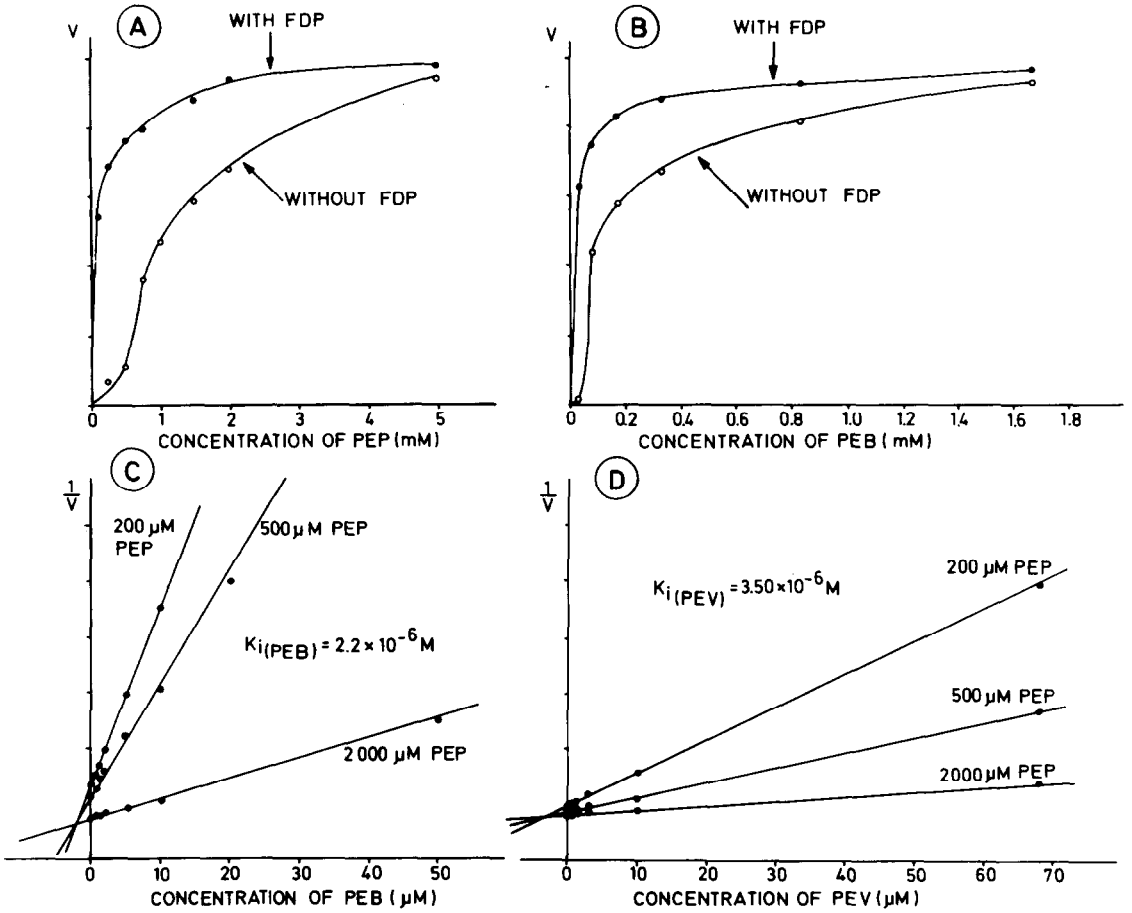

Fig. 2. (A) and (B): Comparison of the homotropic cooperativity with PEP (fig. 2A) and PEB (fig. 2B) as substrates in the pyruvate kinase reaction (pyruvate kinase from rat liver), and the effect of 7 μM fructose-1,6-diphosphate (FDP). For test conditions see legend to fig. 1. The activity of partially purified liver pyruvate kinase was about 20 munits/ml with PEP as substrate and about 120 munits/ml with PEB as substrate. The concentrations of PEP and PEB were varied as indicated on the abscissa. (C) and (D): Dixon plots for the determination of K_i values for PEB (fig. 2C) and PEV (fig. 2D) with pigeon liver phosphoenolpyruvate carboxykinase. The test conditions were as follows (final concentrations given): Tris-HCl (pH 7.4) 57 mM; IDP 1.17 mM; $NaHCO_3$ 20 mM; NADH 0.63 mM; $MnCl_2$ 1.17 mM; malate dehydrogenase (pig heart, free of NH_4^+ ions) 38 μg/ml; phosphoenolpyruvate carboxykinase equivalent to 25 munits/ml. The concentrations of PEP, PEB and PEV were varied as indicated in the graphs.

corresponding α-keto acid than PEB, and 850 times more rapidly than PEV. It had been excluded before that the lactate dehydrogenase reaction was rate limiting. Both analogues inhibited competitively the conversion of PEP to pyruvate (fig. 1A, B), PEB being about 100 times more inhibitory than PEV.

3.2. *Pyruvate kinase from rat liver*

In contrast to muscle pyruvate kinase, the L-type liver pyruvate kinase exhibits a strong cooperativity with its substrate PEP [5, 9–11]. In the presence of fructose-1,6-diphosphate (FDP), however, a Michaelis-Menten type kinetics can be obtained [9–11].

When PEB instead of PEP was used as a substrate for liver pyruvate kinase, again a sigmoidal shaped concentration–velocity curve was obtained which could also be converted to a hyperbolicly shaped curve in the presence of FDP (fig. 2C and D).

Table 1

Conditions	Exchange of intramitochondrial citrate (Percent of internal citrate exchanged)
Control	0
PEP	73.9
PEB	41.4
PEV	27.4
PEP + PEB	72.4
PEP + PEV	74.0
PEB + BTC	2.9
PEB + BTC	− 6.4
PEV + BTC	− 2.4
PEP + PEB + BTC	± 0
PEP + PEV + BTC	+ 0.2

Exchange of intramitochondrial 1,5-^{14}C-citrate with extramitochondrial PEP or PEP analogues in absence and presence of 50 mM benzene 1,2,3-tricarboxylate (BTC). The concentration of PEP or PEP analogues was always 1 mM in the single as well as in the combined experiments. ^{14}C-citrate loaded mitochondria (equivalent to 5 to 6 mg mitochondrial protein) were incubated in the presence of PEP or PEP analogues, 2 μg/ml of rotenone, 0.5 μg/ml antimycin, and 5 mM n-butylmalonate at 25° for 1 min. The mitochondria were rapidly separated by centrifugation and the radioactivity measured in the supernatant and the rinsed pellet. The results are corrected for time dependent spontaneous leakage of citrate. The percentage of exchange was calculated according to Robinson and Williams [13]. For further details see [12].

Table 2
Inhibition by PEB (1 mM) of glucose formation and L-lactate utilization with pigeon liver homogenate.

	Net formation of glucose	Net utilization of L-lactate
	(μmoles/g wet liver/60 min)	
Controls	64.15 ± 2.04	376.39 ± 12.93
With 1 mM PEB	33.60 ± 1.11	212.68 ± 8.27
p-value	< 0.001	< 0.001

The pigeon liver homogenate and the incubation medium were prepared according to Krebs et al. [15]. The homogenates were incubated at 40° for 60 min. The reaction was stopped by addition of $HClO_4$. Values given are mean values ± S.E.M.

3.3. *Enolase from rabbit muscle*

Both PEP-analogues are strong competitive inhibitors of enolase, PEB again being about 100 times more inhibitory than PEV (fig. 1C and D). The K_i values for both analogues are in the same range as for muscle pyruvate kinase. It had been excluded by control experiments that the PEP-analogues might inhibit one of the auxillary enzymes.

3.4. *Phosphoenolpyruvate carboxykinase from pigeon liver*

Both PEP-analogues are very strong competitive inhibitors of this enzyme (fig. 2C and D). In contrast to pyruvate kinase and enolase, phosphoenolpyruvate carboxykinase was nearly as strongly inhibited by PEV as by PEB. It was excluded that the PEP analogues might inhibit malate dehydrogenase.

3.5. *Exchange with citrate through the inner mitochondrial membrane*

As can be seen from table 1, both analogues exchange with mitochondrial citrate. PEB exchanges slightly, PEV considerably less in comparison to PEP. Neither PEB nor PEV inhibit the exchange of PEP with citrate. Similarly to the PEP/citrate exchange, the PEB/citrate exchange as well as the PEV/citrate exchange are strongly inhibited by benzene-1,2,3-tricarboxylate (table 1).

4. Discussion

In accordance with recent reports of Bondinell and Sprinson [1], and Stubbe and Kenyon [3], but in contrast to Woods et al. [2], PEB and PEV were shown to be not only inhibitors but also substrates for the pyruvate kinase reaction.

The K_i value with muscle pyruvate kinase for PEB corresponds to the value measured by Woods et al. [2], who found a value of 6.5×10^{-5} M. Our K_i value for PEV, however, is higher than their value of 1.07×10^{-4} M. The fact that with pyruvate kinase and enolase the K_i values for PEB were about 100 times lower than for PEV, but that no great difference could be found with phosphoenolpyruvate carboxykinase, points to a large difference in the prop

erties of the PEP binding site in the latter enzyme as compared with pyruvate kinase and enolase.

The difference between PEB and PEV became again visible when the mitochondrial PEP analogue/citrate exchange was measured.

From the results reported one could expect an inhibition of gluconeogenesis by PEP analogues. When the net formation of glucose from 10 mM L-lactate by a pigeon liver homogenate was measured according to Krebs et al. [15], we found indeed a significant inhibition of glucose formation and lactate utilization (table 2).

Further experiments with more lipophilic PEP-analogues are under way. Moreover, the question has to be answered whether PEB and/or PEV are converted by enolase or phosphoenolpyruvate carboxykinase to the corresponding reaction products.

Acknowledgement

This work was supported by a grant from the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg, Germany.

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