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The Involvemnt of Phosphoenolpyruvate Carboxykinase in Succinate Formation in *Phascolosoma arcuatum* (Sipuncula) Exposed to Environmental Anoxia

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ABSTRACT—Succinate, propionate and alanine were accumulated in the sipunculid worm, *Phascolosoma arcuatum*, with a decrease in aspartate content upon anoxic exposure. Aspartate degradation could not account for all the succinate accumulated during the 48 hr anoxic period and no stoichiometric relationship between alanine accumulation and aspartate degradation was observed. The glutamate content in the worm remained constant throughout the 48 hr period of anoxia. Adenylosuccinate synthetase and adenylosuccinate lyase were not detected in *P. arcuatum*, rendering the formation of succinate through the purine nucleotide cycle in this organism improbable. Results indicate that succinate synthesis in *P. arcuatum* involved phosphoenolpyruvate carboxykinase at the phosphoenolpyruvate branch point of glycolysis. The activity of phosphoenolpyruvate carboxykinase from *P. arcuatum* exposed to anoxia remained unchanged throughout the 48 hr period, but the activity of pyruvate kinase measured at a subsaturating concentration of phosphoenolpyruvate decreased, possibly due to deactivation by phosphorylation. After 36 hr of anoxic exposure, the activity ratio of pyruvate kinase: phosphoenolpyruvate carboxykinase, obtained at 0.1 mM of phosphoenlopyruvate, decreased to half of the control value. Furthermore, the formation of oxaloacetate, and hence succinate, through phosphoenolpyruvate carboxykinase was favoured by the combined effects of lower pH and increased bicarbonate and alanine contents in the worm exposed to anoxia.

INTRODUCTION

Members of the phylum Sipuncula provide some interesting features of biochemical studies of the anaerobic metabolism of intertidal invertebrates. The local sipunuclid worm, *P. arcuatum*, can usually be found 20–30 cm deep in the mud at the higher regions of the mangrove swamp. To survive in such a hypoxic environment, *P. arcuatum* must be capable of oxygen-independent energy metabolism. Indeed, the sipunculid worm, *Sipunculus nudus*, has been demonstrated to be hightly tolerant to environmetal anoxia [1].

Portner *et al.* [1] demonstrated that *S. nudus* supplied its energy requirements mainly from anaerobic glycolysis during the first 2 to 6 hr of environmental hypoxia, leading to the accumulation of strombine and octopine. However, after the sixth hour, the opine concentrations levelled off in *S. nudus* and succinate production predominated.

At the beginning of anaerobiosis, aspartate was degraded in *S. nudus*, leading Portner *et al.* [1] to suggest that the carbon chain of aspartate might be channelled into succinate and its amino group into alanine. However, an accumulation of alanine was not shown in the same study due to large individual variations [1]. Portner *et al.* [1] further concluded that carboxylation of phosphoenolpyruvate (PEP) to oxaloacetate by phosphoenolpyruvate carboxykinase (PEPCK), leading to the formation of succinate, in *S. nudus* was extremely low as the PEPCK activity in *S. nudus* could hardly be measured.

Kreutzer *et al.* [2] verified the degradation of aspartate and accumulation of succinate in *S. nudus*. They also confirmed that an accumulation of alanine could not be shown. Based on such results and without any experimental support on

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the presence of PEPCK in *S. nudus,* Kreutzer *et al.* [2] concluded that this organism possessed the PEP branch-point for succinate formation. However, in the later study by Portner [3], the accumulation of alanine in *S. nudus* exposed to anoxia was verified.

In order to shed light on the above-mentioned enigma, the present study was undertaken to elucidate the pathway of succinate production in the local sipunculid, *P. arcuatum,* under environmental anoxia, with a special emphasis on demonstrating the presence of the PEP branch-point. Theoretically, succinate formation can also involve the purine nucleotide cycle as described by Lowenstein [4] and Waarde *et al.* [5]. Aspartate combines with inosine monophosphate (IMP) to form adenylosuccinate (AMPS), which is broken down to adenosine monophosphate (AMP) and fumarate. Fumarate is reduced to succinate along with the generation of adenosine triphosphate (ATP). Hence, the possible involvement of this biochemical pathway in succinate formation in *P. arcuatum* was also examined.

MATERIALS AND METHODS

Collection and maintenance of specimen

P. arcuatum were collected from the mudflats of the mangrove swamp at Mandai, Singapore and maintained at 25°C in the laboratory in plastic aquaria with aerated 50% seawater (15‰ salinity). No effort was made to feed the worms and experiments were performed after 10 days of acclimatization.

Anaerobic incubation

Groups of 12 worms were placed in darkened 250 ml flasks containing 150 ml of 50% seawater which had been flushed with 100% N_2 for 1 hr prior to the start of the experiment. Flushing was continued for another 1 hr after the introduction of the worms before the flasks were sealed and left undisturbed at 25°C throughout the incubation period of either 12, 24, 36 or 48 hr. Worms kept in 50% seawater saturated with air were used as controls for comparison.

Sample preparation for metabolite analyses

At the end of the incubation period, the worms were taken out of the flask and quickly blotted dry. A small incision was made on the body wall and the coelomic fluid was collected in ice-cold test-tubes. The worm tissues were immediately freeze-clamped in liquid nitrogen and stored at −80°C until further analyses. The time taken to process a group of 12 worms was approximately 3 min.

Samples of coelomic fluid were immediately centrifuged at $1,000 \times g$ and 4°C for 10 min in a Kokusan H-103N refrigerated centrifuge to obtain the coelomic plasma. Coelomic plasma from three worms were pooled together to constitute a sample. The sample was mixed with an equal volume of ice-cold 0.6 N perchloric acid followed by centrifugation to remove the precipitated protein. The supernatant fluid obtained was used for carboxylic acid analyses.

The frozen tissue sample was ground to a powder under liquid nitrogen using a mortar and pestle. The powdered sample was weighed and suspended in seven volumes of ice-cold 0.6 N perchloric acid (w/v) and homogenized thrice using an Ultra-Turrex homogenizer (Janke and Kunkel GMBH and Co.) at maximum speed (20,500 rpm) for 15 sec each with 10 sec off intervals. The homogenized sample was centrifuged at $10,000 \times g$ for 15 min in a Sigma 202MK refrigerated centrifuge. The supernatant fluid obtained was divided into two portions. One portion was used for carboxylic acid analyses while another portion was titrated with potassium carbonate (5 M) to pH 6.5–7.0 for the quantitative analyses of alanine, aspartate and glutamate.

Metabolite analyses

Succinate and propionate were analysed by using an Eyela Carboxylic Acid Analyser S-14 (Tokyo Rikakikai Co., Japan) with chemicals obtained from Sigma Chemical Co. (Mo, USA) as references for comparison.

Aspartate was assayed according to the method of Bergmeyer *et al.* [6]. Alanine was assayed following the procedure of Williamson [7]. Glutamate was assayed following the procedure of Bernt and Bergmeyer [8]. The absorbance at 340 nm was

recorded using a Shimadzu UV-120 spectrophotometer. Metabolite content was expressed as μmol per g tissue.

Determination of pH and bicarbonate content in the coelomic plasma

Coelomic fluid was drawn throught a small incision at the posterior end of the worm into a Intramedic PE 60 tubing (Clay Adams, USA) (inner diameter 0.76 mm) connected to a 5 ml syringe prefilled with 0.5 ml of mineral oil. The fluid was quickly transfered to a cold test-tube containing 0.5 ml of mineral oil. Coelomic plasma was obtained by centrifugation as mentioned before. The bicarbonate content in the plasma was determined enzymatically as total carbon dioxide using the Sigma Diagnostic Kit 130-A, following the procedure No. 130-UV. The pH of the coelomic plasma was measured with an Orion 501 digital ionalyzer and a combination electrode.

Sample preparation for enzyme assays

Worms were dissected, drained of coelomic fluid, blotted dry, weighed and homogenized as stated before in 5 volume (w/v) of freshly prepared ice-cold buffer containing 50 mM imidazole-HCl (pH 7.5), 50 mM NaF, 3 mM EGTA, 1 mM DTT and 0.5 mM PMSF. The sample was centrifuged as mentioned previously and a portion of the a supernatant fluid obtained was dialysed in a similar buffer but without PMSF for 3 hr and then used for the determination of pyruvate kinase and PEPCK activities. For the determination of adenylate deaminase (AMP deaminase), adenylosuccinate lyase (AMPS lyase) and adenylosuccinate synthetase (AMPS synthetase) activities, the sample was further centrifuged at $100{,}000 \times g$ by a Beckman L8-80M ultracentrifuge for 1 hr and the supernatant fluid obtained was used.

Enzyme assays

Pyruvate kinase (PK) (ATP: pyruvate 2-O-phosphotransferase, EC 2.7.1.40) was assayed according to the method of Gutmann and Bernt [9] in a medium (2.75 ml) containing 120 mM triethanolamine-HCl (pH 7.5), 16 mM $MgSO_4$, 90 mM KCl, 1 mM EDTA, 5 mM (unless stated otherwise) PEP, 5 mM ADP 0.15 mM NADH, 7 iu lactate dehydrogenase (Sigma, MO) and 100 μl of sample.

PEPCK (ITP: oxaloacetate carboxylase, EC 4.1.1.31) was measured in the direction of oxaloacetate formation based on a modification of the method of Colombo *et al.* [10]. The assay medium (2.75) contained 65 mM PIPES (pH 6.6), 1.5 mM GDP, 5 mM (unless stated otherwise) PEP, 20 mM $NaHCO_3$, 3 mM $MnCl_2$, 3 mM $MgCl_2$, 0.15 mM NADH, 15 mM NaF, 5 iu malate dehydrogenase (Sigma, MO) and 100 μl of sample. A blank was conducted with $NaHCO_3$ being substituted by NaCl, correcting for PK which might show some activity under the assay conditions.

Activities of the above enzymes were determined at 340 nm and 25°C using a Shimadzu UV-260 spectrophotometer. Specific enzyme activities were presented as μmol NADH oxidized per mg protein per min.

AMP deaminase (EC 3.5.4.6) was assayed according to the method of Gibbs and Bishop [11]. AMPS lyase (EC 4.3.2.2) was determined according to the procedure of Casey *et al.* [1] by monitoring at 280 nm and 25°C the decrease in absorbance due to the disappearance of AMPS. AMPS synthetase (EC 6.3.4.4) was also determined according to the method of Casey *et al.* [12] with samples which had been dialysed over night in the extraction buffer.

The protein content in the samples were determined according to the method of Bradford [13]. Bovine serum globulin dissoved in 25% glycerol was used as a standard for reference.

Statistical analyses

Results were presented as means$\pm$SE. The Student's *t*-test and the analysis of variance test (one-way) were used to compare differences between means wherever appropriate. Difference with $p<0.05$ were regarded as statistically significant.

RESULTS

At the end of the 48 hr anoxic exposure, *P. arcuatum* was still capable of introvert invagination and muscular contraction upon being mechanically stimulated. No mortality was recorded for worms

kept in the experimental anoxic conditions for the various periods.

Succinate was not detected in the tissue (detection limit=0.05 μmol/g) and coelomic plasma (detection limit=0.01 μmol/ml) of *P. arcuatum* kept in normoxia. However, they were present in the tissues and coelomic plasma in the worms exposed to various periods of anoxia in significant quantities (Table 1). The alanine contents of the worm tissues also increased after anoxic exposure, with the greatest increment observed to be within the first 24 hr of anoxia. However, the aspartate content decreased significantly only after the 24th hr of exposure to anoxia (Table 1). No significant change in the glutamate level was observed throughout the 48 hr of anoxic period.

Using the methods adopted in the present studies, AMPS synthetase and AMPS lyase were not detected in the tissues of *P. arcuatum* although the assay methods gave positive results when samples of rat muscle were examined. The specific activity of adenylate deaminase in the tissue extracts from *P. arcuatum* was found to be 9.67±2.74 (n=3) μmol ammonia produced/30 min per mg protein.

After 48 hr of anoxic exposure, the PK activity (V_{max}) in the tissue extracts of *P. arcuatum* assayed at 5 mM PEP was not significantly lower than that of the control. However, when assayed in the presence of 0.1 mM PEP, the PK activity decreased significantly after 36 hr of anoxic exposure. In contrast, the PEPCK activities at both saturating (V_{max}) and subsaturating concentrations

TABLE 1. Concentrations (μmol/gww) of succinate, alanine, aspartate and glutamate in the body of *P. arcuatum* (without coelomic fluid), and the concentration (mM) of succinate in the coelomic fluid of *P. arcuatum* exposed to environmental anoxia for various periods. Data represent means±S.E. with the numbers of determinations on separate preparations of extracts from different animals in parentheses

Anoxia duration (hr)	Worm body (without coelomic fluid)				Coelomic fluid
	Succinate	Alanine	Aspartate	Glutamate	Succinate
0	N.D. (7)	0.64±0.07 (5)	2.57±0.28 (7)	1.22±0.04 (4)	N.D. (2)
12	3.62±0.52 (7)*	0.92±0.09 (5)*	2.50±0.37 (7)	1.21±0.09 (4)	0.34±0.02 (5)*
24	6.65±0.36 (7)*	2.03±0.21 (5)*	2.07±0.22 (5)	1.33±0.05 (4)	0.58±0.05 (4)*
36	7.84±0.38 (7)*	2.04±0.22 (5)*	1.11±0.25 (7)*	1.50±0.16 (4)	0.96±0.10 (5)*
48	8.69±0.89 (7)*	2.25±0.15 (5)*	1.15±0.18 (7)*	1.13±0.17 (4)	0.81±0.12 (3)*

* significantly different from the control value at 0 hr of anoxic exposure; N.D.=not detectable

TABLE 2. Specific activities (μmol NADH oxidized/min per mg protein) of pyruvate kinase (PK) and phosphoenolpyruvate carboxykinase (PEPCK) assayed at saturating (5 mM) and subsaturating (0.1 mM) PEP concentrations, and their ratios at the respective PEP concentrations, in the extracts from *P. arcuatum* (without coelmic fluid) exposed to environmental anoxia for various periods. Data represent menas±S.E. of 5 determinations on separate preparations of enzyme extracts from different animals

Anoxia duration (hr)	Protein content of worm body extract (mg/g ww)	Specific enzyme activity at 5 mM PEP		PK/PEPCK at 5 mM PEP	Specific enzyme activity at 0.1 mM PEP		PK/PEPCK at 0.1 mM PEP
		PK	PEPCK		PK	PEPCK ($\times 10^{-3}$)	
0	2.84±0.16	0.26±0.07	0.013±0.002	21.4±2.8	0.18±0.03	0.93±0.13	195.5±13.2
12	2.85±0.10	0.31±0.08	0.013±0.004	25.8±2.4	0.13±0.02	0.65±0.08	194.4±19.7
24	2.83±0.06	0.24±0.02	0.012±0.002	21.0±1.6	0.13±0.02	0.76±0.13	170.2±17.5
36	2.80±0.11	0.27±0.04	0.014±0.001	19.8±2.0	0.061±0.004*	0.70±0.09	95.1±14.3*
48	2.71±0.16	0.29±0.05	0.013±0.001	21.5±1.9	0.040±0.008*	0.89±0.11	44.9± 5.5*

* significantly different from the corresponding control value at 0 hr of anoxic exposure

of PEP, were unaltered (Table 2). When the ratios of PK to PEPCK obtained at subsaturating PEP concentration were calculated, it was found that the results obtained for worms exposed to 36 hr and 48 hr of anoxia were significantly lower than that of the normoxic control (Table 2).

The pH optima for PK and PEPCK activities were found to be 7.5 and 6.7, respectively (Fig. 1). The activity of PK was not affected by the presence of up to 70 mM bicarbonate (Fig. 2). However, the PEPCK activity increased drastically between 0 and 10 mM of bicarbonate (Fig. 2). In addition, the PK of the control normoxic worm was less sensitive to inhibition by alanine compared to that of the anoxic one (Fig. 3).

After 24 hr of anoxic exposure, the pH of the coelomic plasma of *P. arcuatum* decreased significantly, while the bicarbonate concentration increased significantly, as compared to those of the control (Table 3).

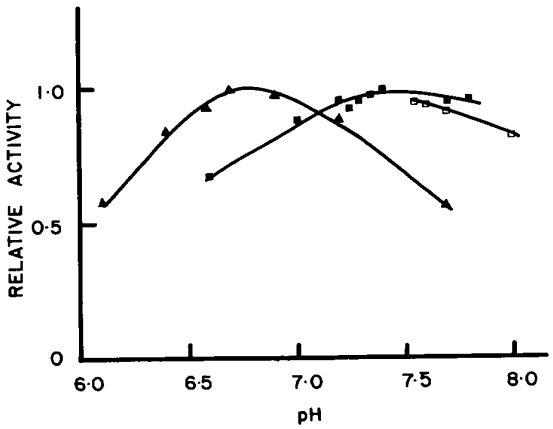

FIG. 1. Effects of pH on the activities of pyruvate kinase (PK) and phosphoenolpyruvate carboxykinase (PEPCK) extracted from normoxic *P. arcuatum*. Each point represents the average of 5 determinations. ▲=PEPCK (PIPES buffer); ■=PK (imidazole buffer); □=PK (triethanolamine-HCl buffer).

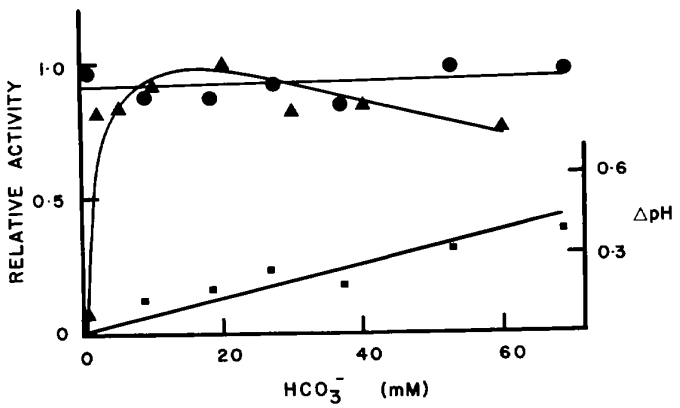

FIG. 2. Effects of increasing bicarbonate (HCO_3^-) concentrations on the activities of pyruvate kinase (PK) and phosphoenolpyruvate carboxykinase (PEPCK) extracted from normoxic *P. arcuatum*. Each point represents the average of 7 determinations. ●=PK; ▲=PEPCK, ■=changes in pH (△pH) in the assay medium.

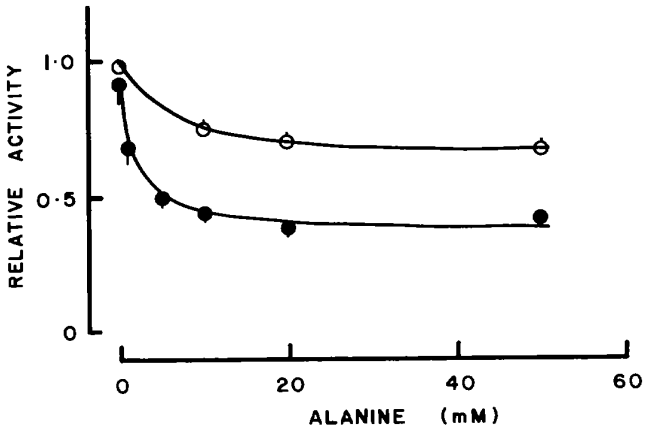

FIG. 3. Effects of increasing alanine concentrations on the activity of pyruvate kinase (PK) extracted from normoxic *P. arcuatum* and *P. arcuatum* exposed to 48 hr of environmental anoxia. Each point represents the average of 3 determinations. ●=PK from anoxic worm; ○=PK from normoxic worm.

TABLE 3. pH and the total carbon dioxide concentration (mM) in the coelomic plasma of *P. arcuatum* exposed to environmental anoxia for various periods. Data represent means±S.E.

Anoxia duration (hr)	n	pH	Total carbon dioxide
0	5	7.42±0.04	1.04±0.06
12	5	7.25±0.04	1.09±0.13
24	4	7.07±0.06*	1.60±0.23*
36	4	7.04±0.04*	1.66±0.11*
48	5	6.69±0.12*	1.73±0.14*

* significantly different from the corresponding control value at 0 hr of anoxic exposure

DISCUSSION

P. arcuatum could survive 48 hr anoxic exposure. During this period, significant amounts of succinate was accumulated in the tissue and coelomic plasma.

Different from the observations of Portner *et al.* [1] and Kreutzer *et al.* [2] on *S. nudus,* results reported herein confirmed the accumulation of alanine in *P. arcuatum* exposed to environmental anoxia. However, there was no indication of stoichiometric relationship between alanine accumulation and aspartate degradation during various periods of anoxic incubation. Moreover, the concentrations of glutamate, which is involved in the conversion between alanine and aspartate, remained constant throughout the experimental period. Hence, the amino group of aspartate is not transferred to alanine in *P. arcuatum* as suggested by Portner *et al.* [1] for *S. nudus.*

The carbon skeleton resulted from the degradation of aspartate may be channelled to the formation of succinate. However, the rate of aspartate degradation could not account for all the succinate accumulated in *P. arcuatum* throughout the 48 hr anoxic period. Twelve hours after incubation in anoxia, tissue succinate content rose to 3.62 μmol/g ww with no corresponding disappearance of tissue aspartate and, between the 36th and 48th hr of anoxic exposure, aspartate degradation in *P. arcuatum* ceased while accumulation of succinate continued. Hence, there must be another pathway involved in succinate fromation in *P. arcuatum.*

In the snail, *Helix aspersa* [14], and the goldfish, *Carassius auratus* [5], succinate can be formed through the operation of the purine nucleotide cycle. However, two enzymes involved in the cycle, AMPS synthetase and AMPS lyase were not detected in *P. arcuatum* making it improbable for the purine nucleotide cycle to operate in this organism.

At the PEP branch point of glycolysis, PEP could either form pyruvate through PK or oxaloacetate through PEPCK. Oxaloacetate can be further metabolized to form succinate and propionate. Contrary to the report on the presence of almost undetectable PEPCK activity in *S. nudus* [1], both PK and PEPCK were present in *P. arcuatum.*

After 48 hr of anoxic exposure, the PK activity in the tissue extracts from *P. arcuatum* measured at subsaturating PEP concentration was significantly lower than that of the control value obtained under similar assay conditions. It is possible that during anoxic exposure PK in *P. arcuatum* was deactivated by covalent modification as suggested by Storey [15]. Anoxia-induced phosphorylation of PK resulting in a much less active enzyme form is probably the key mechanism reducing the PK activity in the anoxia state. Compared with the aerobic form, the anoxic variant of PK from the radular retractor of the whelk showed lower maximal activity, greatly reduced affinity for PEP, and enhanced inhibition by alanine [16]. In the whelk, changes in the levels of substrates and effectors of PEPCK, coupled with the strong depression of PK activity as a result of enzyme phosphorylation, are probably sufficient to promote re-routing of PEP via PEPCK into the succinate pathway [17].

The formation of oxaloacetate through PEPCK in *P. arcuatum* exposed to anoxia would be further favoured by the combined effects of lower pH and increased bicarbonate content on the activities of PK and PEPCK *in vivo.* After 24 hr of anoxic exposure, the pH of the coelomic plasma was significantly lowered. Since there may be a delay in the proton exchange between the intracellular and extracellular compartments [18], it is possible that the lowering of intracellular pH was even greater. Such a change in pH would enhance the PEPCK activity as its pH optimum was 6.7 and suppress the PK activity, which has a pH optimum of 7.5, *in situ.* In addition, the accumulation of bicarbonate in the worm upon anoxic exposure, as reflected by the increased bicarbonate concentration in the coelomic plasma, would enhance the activity of PEPCK as it is a substrate for the carboxylation reaction.

The increased alanine content in *P. arcuatum* exposed to anoxia would further inhibit the PK activity *in situ.* Alanine can increase the Michaelis constant $Km_{(PEP)}$ of PK and reduce its maximum catalytic rate. At the same time, it reverses the ITP inhibition on PEPCK, enabling PEPCK to work faster [19].

Hence, it would appear that the formation of succinate in *P. arcuatum* involves the PEPCK route at the PEP branch point of glycolysis. As to why Portner *et al.* [1] were unable to detect PEPCK in *S. nudus,* it is possible that the conditions of the assay system adopted [20] in their

studies were not optimal for the enzyme of the sipunculid worm. The pH of the assay medium recommended by Utter and Kurahashi [20] was 7.4–7.6 which deviates significantly from the pH optimum of 6.7 obtained for the PEPCK of *P. arcuatum* in the present studies. At this pH range, the PEPCK activity obtained would be lowered by 50–60% as compared to that determined at the optimal pH.

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