Studies on the Glyoxylate Reductase and Glycollate Oxidizing Enzyme System. (I)

Enzymic Oxidation of Glycollate and Lactate by Flavoproteins of Moulds

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The enzymic oxidation of glycollate and lactate was investigated with various moulds including *Aspergillus oryzae*, *Aspergillus niger*, *Penicillium purpurogenum*, *Rhizopus nigricans*, *Rhizopus oryzae*, *Rhizopus javanicus*, *Mucor javanicus*, *Neurospora sitophila*, *Fusarium lini* and *Fusarium oxysporum*. Glycollate was converted to glyoxylate, pyruvate and α-ketoglutarate when incubations were aerobically carried out with the dried cells mentioned above or the dialyzed cell-free extracts. The addition of semicarbazide to the glycollate-media brought about a striking increase in the yield of glyoxylate against decreasing yields of pyruvate and α-ketoglutarate. The oxidation of glycollate to glyoxylate was not activated by the addition of DPN**, TPN, cocarboxylase or ATP, but stimulated extremely by the presence of methylene blue.

It was demonstrated that the glycollate or lactate dehydrogenase-apoenzymes inactivated by the acid-ammonium sulfate procedure, were reactivated by the addition of FMN, FAD or riboflavin. Based on the data presented here, the prosthetic group of glycollate and lactate dehydrogenases of moulds were shown to be FMN. Occurrence of isocitritase was also demonstrated in the glucose-grown cells of various moulds such as: *Aspergillus*, *Penicillium*, *Neurospora*, *Rhizopus*, *Mucor* and *Fusarium* species.

Finally, the function of the isocitritase and glyoxylate reductase-glycollate oxidizing enzyme system was discussed in microbial respiration.

INTRODUCTION

Glyoxylate has been known as a metabolite of mould for many years. Isocitritase catalyzing the cleavage of isocitrate to glyoxylate and succinate, has recently been found in various microorganisms including bacteria[1,4-15], yeasts[2,3,23,29] and moulds[2,3].

On the other hand, the oxidation of glycollate to glyoxylate has been investigated with the biological preparations of higher plant, animal and microorganisms[18-21] and it has been demonstrated that glycollic oxidase of both higher plant and animal is a flavoprotein with FMN as prosthetic group by Zelitch *et al.*[16] and Kun *et al.*[17], respectively.

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** The following abbreviations are used: DPN (TPN)=di (tri) phosphopyridine nucleotides; DPN$^+$(TPN$^+$) and DPNH (TPNH)=oxidized and reduced di (tri) phosphopyridine nucleotides; ATP=adenosine triphosphate; FMN=riboflavin-5′-phosphate; FAD=flavin adenine dinucleotide.

The authors have investigated the mechanisms of microbial respiration and it has recently been indicated that the combined reaction of isocitritase, TPN-linked glyoxylate reductase and FMN-linked glycollate oxidase (dehydrogenase) functions as a TPNH-oxidizing system in microorganisms[11-15,22-29]. The present paper deals with the enzymic oxidation of glycollate by various moulds, the properties of the glycollate or lactate dehydrogenase, and distribution of isocitritase in moulds.

EXPERIMENTAL

Chemicals and Methods

All of the materials used were the commercial products. Analytical procedures were performed by the methods described previously[14,22,23].

Growth of Organisms

Microorganisms used in this work were: *Aspergillus oryzae, Aspergillus niger, Penicillium Purpurogenum, Rhizopus nigricans, Rhizopus oryzae, Rhizopus javanicus, Mucor javanicus, Neurospora sitophila, Fusarium lini* and *Fusarium oxysporum*. The organisms were grown on a shaker at 30°C for 24-72 hours with a medium (pH 5.6) containing 3% glucose, 0.1% $(NH_4)_2SO_4$, 0.1% each KH_2PO_4 and Na_2HPO_4, 0.03% $MgSO_4 \cdot 7H_2O$, 0.3% yeast extract, 0.5% peptone, 500γ% each $FeSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$ and NaCl, and 1.5% $CaCO_3$.

Enzyme preparations

Dried cells: After cultivation, the organisms were harvested by a centrifuge and washed with distilled water, and then dried by an electric fan at room temperature for 4-8 hours.

Ground cells and cell-free preparations: The dried cells were ground with powdered glass at 0°C for 30 minutes, extracted with 0.1 M phosphate buffer, pH 7.3. The insoluble residue was removed by centrifuge and the resulting supernatant fluids were dialyzed against distilled water at 5°C for 20-40 hours. Ammonium sulfate precipitated enzymes were prepared according to the methods mentioned in the previous papers.

RESULTS

Oxidation of Glycollate by Ground Dry cells and Cell-Free Preparations of Moulds

Tables 1-3 show the oxidation of glycollate by the ground dry cells of various moulds including *Aspergillus oryzae, Aspergillus niger, Penicillium Purpurogenum, Rhizopus nigricans, Rhizopus javanicus, Rhizopus oryzae, Mucor javanicus, Neurospora sitophila, Fusarium lini* and *Fusarium oxysporum*. It will be seen that when the cell preparations are incubated with glycollate, they are able to produce three kinds of ketonic acids including glyoxylic, pyruvic and α-ketoglutaric acids. The yields of ketonic acids were remarkablely influenced by the presence of a trapping agent and methylene blue. The presence of semicarbazide resulted in a high yield

Table 1. Formation of glyoxylate, pyruvate and α-ketoglutarate from glycollate by ground cells of various moulds.

Species used	Additions, pH 7.4							
	Minus Methylene blue Plus Semicarbazide			Minus Methylene blue Minus Semicarbozide				
	Ketonic Acid found (μ moles)							
	Pyruvate	Glyoxylate	α-Keto-glutarate	Pyruvate[a)]	Glyoxylate[b)]	α-Keto-glutarate[c)]	a/b	c/b
Aspergillus oryzae	0.6	2.3	trace	1.1	0.2	0.4	5.5	2.0
Aspergillus niger	1.0	7.6	nil	3.5	0.9	3.9	3.9	4.3
Penicillium purpurogenum	2.2	4.0	1.2	4.1	0.6	3.9	6.8	6.5
Neurospora sitophila I	3.4	15.0	0.6	8.9	3.3	1.5	2.7	0.5
Neurospora sitophila II*	0.6	0.9	trace	1.3	0.3	0.5	4.3	1.7
Mucor javanicus	1.7	2.0	0.5	1.8	0.4	0.8	4.5	2.0
Rhizopus nigricans	1.7	1.4	0.6	2.0	0.4	0.9	5.0	2.3
Rhizopus javanicus	2.6	3.5	trace	4.0	0.5	0.8	8.0	1.6
Rhizopus oryzae	1.6	5.1	trace	5.5	0.7	0.9	7.9	1.3

* Experiment carried out with dialyzed cell-free extracts containing 18 mg protein.

Table 2. Formation of glyoxylate, pyruvate and α-Ketoglutarate from glycollate by ground cells of various moulds.

Species used	Additions, pH 7.4							
	Plus Methylene blue Plus Semicarbazide			Plus Methylene blue Minus Semicarbazide				
	Ketonic Acids found (μ moles)							
	Pyruvate	Glyoxylate	α-Keto-glutarate	Pyruvate[a)]	Glyoxylate[b)]	α-Keto-glutarate[c)]	a/b	c/b
Aspergillus oryzae	1.0	14.3	trace	4.3	0.3	1.5	14.3	5.0
Aspergillus niger	1.4	36.5	trace	9.0	10.3	7.6	0.9	0.7
Penicillium purpurogenum	2.7	11.0	1.3	7.9	1.1	6.8	7.2	6.2
Neurospora sitophila I	3.6	38.0	0.5	6.4	9.6	2.0	0.7	0.2
Neurospora sitophila II*	1.6	21.5	trace	3.5	17.5	1.6	0.2	0.9
Mucor javanicus	2.1	16.2	0.8	9.4	0.7	2.0	13.4	2.9
Fusarium lini	2.1	12.9	0.6	7.1	0.4	2.5	17.7	6.1
Fusarium oxysporum	3.9	14.3	0.7	18.5	0.5	2.0	37.0	4.0
Rhizopus nigricans	1.7	4.0	0.6	3.3	0.4	0.9	8.3	2.3
Rhizopus javanicus	2.7	7.8	trace	5.7	0.6	0.8	9.5	1.3
Rhizopus oryzae	2.5	50.5	trace	5.2	14.9	0.8	0.4	0.1

* Experiment carried out with dialyzed cell-free extracts containing 18mg protein.

Table 3. Formation of glyoxylate, pyruvate and α-ketoglutarate from glycollate by ground cells of various moulds.

Species used	Additions, pH 5.9							
	Plus Methylene blue Plus Semicarbazide			Plus Methylebe blue Minus Semicarbazide				
	Ketonic Acids found (μ moles)							
	Pyruvate	Glyoxylate	α-Keto-glutarate	Pyruvate[a]	Glyoxylate[b]	α-Keto-glutarate[c]	a/b	c/b
Aspergillus oryzae	0.8	0.9	trace	4.0	0.3	1.2	13.3	4.0
Neurospora sitophila I	3.2	40.5	0.3	3.6	18.0	1.2	0.2	0.7
Neurospora sitophila II*	1.5	16.9	nil	1.7	10.4	0.7	0.2	0.1
Fusarium lini	1.4	8.0	trace	6.5	0.6	0.5	10.8	0.8
Fusarium oxysporum	4.1	16.3	0.7	11.0	2.6	1.1	4.2	0.4

Species used	Additions							
	Minus Methylene blue Plus Semicarbazide			Minus Methylene blue Minus Semicarbazide				
	Ketonic Acids found (μ moles)							
	Pyruvate	Glyoxylate	α-Keto-glutarate	Pyruvate[a]	Glyoxylate[b]	α-Keto-glutarate[c]	a/b	c/b
Aspergillus oryzae	0.5	2.4	trace	—	—	—	—	—
Neurospora sitophila I	2.6	16.8	0.3	4.4	2.0	0.5	2.2	0.3
Neurospora sitophila II*	0.5	1.0	trace	0.4	0.2	0.3	2.0	1.5

* Experiment carried out with dialyzed cell-free extracts containing 18mg protein.

Experiments shown in Tables 1-3 were carried out with reaction mixtures containing: 750 μ moles phosphate buffer, pH 5.9 or pH 7.4, 400 μ moles Na-glycollate, 500 mg ground cells in the presence and absence of 400 μ moles semicarbazide and 1 μ mole FMN; total volume, 15ml; 3 hours' incubation on a shaker at 30°C.

of glyoxylate, whereas, in the case of incubations without the trapping agent, there were the increasing yields of both pyruvate and α-ketoglutarate against extremely decreasing glyoxylate. The highest yield of glyoxylate was obtained in the presence of both semicarbazide and methylene blue. It may here be pointed out that the oxidation of glycollate occurring under the absence of methylene blue, proceeds by way of glycollic dehydrogenase and cytochrome system. Similar results were obtained with the dialyzed cell-free extracts of *Neurospora sitophila*, *Aspergillus oryzae* and *Aspergillus niger*.

Table 4 shows the results of experiments carried out with the ammonium sulfate precipitated enzyme of *Neurospora sitophila*. It will be seen that the salt 0.4-

Table 4. Oxidation of glycollate by ammonium sulfate precipitated protein of *Neurospora sitophila*.

Ammonium sulfate saturation fraction	0-0.2	0.2-0.4	0.4-0.6	0.6-0.8
Glyoxylate formed (μ moles/10 mg protein)	0.62	1.13	2.16	0.44

0.6 saturation fraction possesses the highest enzyme activity. The dehydrogenase activity was scarcely stimulated by the addition of DPN, TPN, ATP or cocarboxylase (Table 5).

Table 5. Effect of cofactors on oxidation of glycollate by dialyzed cell-free extract of *Aspergillus niger*.

Vessel No.	I	II	III	IV	V	VI
Cofactors added						
Cocarboxylase (2 μ moles)	−	+	+	+	+	+
DPN (0.5 μ mole)	−	−	+	+	+	+
ATP (1 μ mole)	−	−	−	−	−	+
TPN (0.3 μ mole)	−	−	−	−	+	+
$MgSO_4$ (20 μ moles)	−	−	+	−	+	+
Products (μ moles) Pyruvate	3.0	2.4	2.6	2.5	2.8	2.8
Glyoxylate	7.8	5.7	4.4	4.2	5.4	4.8
α-Ketoglutarate	3.1	1.8	2.4	3.0	2.3	2.6

Reaction mixture: 750 μ moles phosphate buffer, pH 7.4, 400 μ moles Na-glycollate, 3 μ moles methylene blue, 1 μ mole FMN, indicated additions, dialyzed cell-free extract (33.7 mg as protein); 15 ml; 3 hours' incubation on shaker at 30°C.

Oxidation of Various Substrates by Cell-free preparations

The oxidizing activity of the dialyzed cell-free extract from *Neurospora sitophila* was examineds toward various substrates such as: L-alanine, glycine, succinate, glutamate, DL-lactate, acetate, ethyl alcohol and glycollate. The results are shown in Table 6. The preparation showed activity only towards glycollate and lactate. Table 7 shows the oxidation of glycollate and lactate by cell-free extracts of several moulds. The affinities of enzymes towards glycollate and lactate were observed to vary with the kinds of moulds and also with the pH of reaction mixtures. From the data presented in Table 7, it will be indicated that lactate was oxidized much more rapidly than glycollate, and the relative values (μ moles of pyruvate formed from lactate/μ moles of glyoxylate formed from glycollate) of the substrate specificity varied from approximately 1.7 to 5.2, using *Aspergillus niger*, *Penicillium*

Table 6. Oxidation of various substrates by dialyzed cell-free extract of *Neurospora sitophila*.

Substrates	Ketonic acids formed (μ moles)
Glycine	0
DL-Alanine	trace
Succinate	0
DL-Lactate	8.5 (pyruvate)
Ethyl alcohol	0
Glycollate	4.1 (glyoxylate)
Glutamate	0

Reaction mixture: 750 μ moles phosphate buffer, pH 7.4, 400 μ moles substrates, 400 μ moles semicarbazide, cell-free extract (26.8 mg as protein), 3 μ moles methylene blue, 1 μ mole FMN; total volume 15 ml; 3 hours' incubation on a shaker at 30°C.

Table 7. Oxidation of glycollate and lactate by dialyzed cell-free extracts of several moulds.

Cell-free extracts		Substrates, pH 5.6			Substrates, pH 7.4		
		Glycollate	Lactate		Glycollate	Lactate	
Organisms	Protein (mg)	Glyoxylate[a] formed (μ moles)	Pyruvate[b] formed (μ moles)	b/a	Glyoxylate[c] formed (μ moles)	Pyruvate[d] formed (μ moles)	d/c
Aspergillus niger	37.2	6.4	29.6	4.6	7.6	39.2	5.2
Penicillium Purpurogenum	20.5	9.1	14.4	1.7	5.5	11.7	2.1
Mucor javanicus	45.7	5.1	14.6	2.9	8.0	27.8	3.5
Neurospora sitophila	26.8				4.1	8.5	2.1
Fusarium lini	64.7	25.8	44.8	1.7	24.3	60.0	2.5

Reaction mixture: 750 μ moles phosphate buffer, 400 μ moles Na-glycollate or Na-DL-lactate, 400 μ moles semicarbazide, 3 μ moles methylene blue, 1 μ mole FMN; total volume 15ml; 2 hours' incubation on a shaker at 30°C.

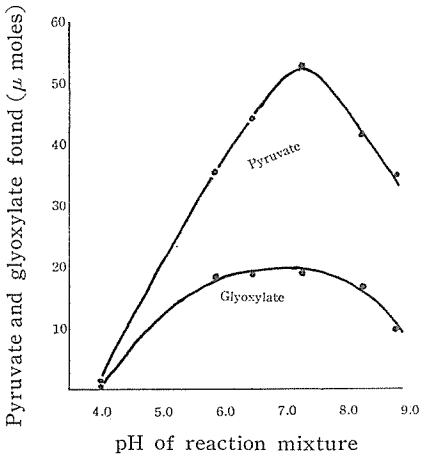


Fig. 1. Influence of pH on oxidation of glycollate and lactate by ammonium sulfate precipitates from *Neurospora* species.

Reaction mixture: 750 μ moles phosphate buffer, 400 μ moles Na-glycollate or Na-DL-lactate, 400 μ moles semicarbazide, 1 μ mole FMN, 3 μ moles methylene blue, 52mg ammonium sulfate 0.2–0.6 saturation fraction of *Neurospora sitophila*; total volume 15 ml; 1 hour's incubation on a shaker at 30°C.

purpurogenum, *Mucor javanicus* and *Neurospora sitophila*. The pH activity curves of both glycollic and lactic dehydrogenases are shown in Fig. 1. Glycollic dehydrogenase of *Neurospora sitophila* exhibits a broad pH optimum between pH 5.9 and 8.2, whereas the lactic dehydrogenase exhibits a rather sharp optimum at pH 7.2.

Prosthetic Group of Glycollic and Lactic Dehydrogenase

It has been demonstrated that according to the acid-ammonium sulfate procedure of Warburg and Christian[30], the glycollic dehydrogenase preparations of various moulds is resolved to yield an inactive apoenzyme which can be reactivated by

FMN, FAD or riboflavin. The ammonium sulfate (0.8 saturation) precipitated enzymes were again suspended in a 0.7 saturated solution of ammonium sulfate at 0°C. The suspensions were acidified with cold 2 *N* HCl to pH 2.2-2.4. After being kept for one hour at 0°C, the precipitated proteins were centrifuged and the supernatant fluids discarded. The precipitates were washed with a saturated ammonium sulfate and dissolved in 0.1 *M* phosphate buffer, pH 7.4.

Table 8 shows the reactivation of the apoenzymes of several moulds by riboflavin, FMN or FAD. The highest reactivation of apoenzymes was observed with FMN. There was less effect with either FAD or riboflavin than with FMN. Reactivating effect of riboflavin also indicates that enzyme preparations used here contain flavokinase catalyzing the phosphorylation of riboflavin to form FMN by ATP. The addition of riboflavin produces only slight inhibition to the reactivation of

Table 8. Reactivation of glycollic dehydrogenase apoenzyme of moulds by FMN, FAD or riboflavin.

Organisms	Apoenzyme (mg as protein)	Riboflavin and the nucleotides added				
		None	FMN (2μ moles)	FAD (2μ moles)	Riboflavin (4μ moles)	FMN (2μ moles) plus Riboflavin (4μ moles)
		Glyoxylate formed (μ moles)				
Aspergillus niger	37.8	0.3	21.2	8.2	10.5	21.0
Neurospora sitophila	31.8	0.3	11.0	7.4	5.6	10.1
Penicillium purpurogenum	27.3	0.2	5.2	1.8	1.6	5.5
Fusarium lini	36.9	0.5	14.6	4.9	5.8	10.9
Mucor javanicus	28.7	0.2	1.4	0.5	0.9	1.0
Rhizopus oryzae	17.5	0.2	0.5	0.3	0.4	0.5

Reaction mixture: 800 μ moles phosphate buffer, pH 7.5, 400 μ moles Na-glycollate, 400 μ moles semicarbazide, 3 μ moles methylene blue; total volume 15 ml; 3 hours' incubation on a shaker at 30°C.

Table 9. Reactivation of lactic dehydrogenase apoenzyme of moulds by FMN, FAD or riboflavin.

Organisms	Apoenzyme (mg as protein)	Riboflavin and the nucleotides added (2μ moles)			
		None	FMN	FAD	Riboflavin
		Pyruvate formed (μ moles)			
Aspergillus niger	32.4	0.8	23.2	2.1	2.4
Penicillium purpurogenum	24.1	1.2	22.5	8.2	8.2
Mucor javanicus	42.2	0.8	7.9	2.7	9.5
Fusarium lini	70.6	4.6	35.2	17.3	21.1

Reaction mixture: 1130 μ moles phosphate buffer, pH 7.4, 400 μ moles Na-DL-lactate, 400 μ moles semicarbazide, 3 μ moles methylene blue; total volume 15ml; 2 hours' incubation on a shaker at 30°C.

apoenzyme by FMN (Table 8). Results of experiments carried out with the glycollic dehydrogenase apoenzyme of *Neurospora sitophila* in the presence of varying amounts of FMN, indicated that the Michaelis constant for FMN was approximately $5\times10^{-6}M$.

Similar experiments were carried out with lactate as substrate. The results are shown in Table 9. It will be seen that FMN serves as the prosthetic group of lactic dehydrogenase of moulds. In the case of lactic dehydrogenase, the Michaelis constant for FMN was approximately $7\times10^{-6}M$.

Distribution of Isocitritase in Moulds

Isocitritase has already been demonstrated in moulds including *Penicillium chrysogenum*, *Aspergillus niger* and *Rhizopus* sp. It has also been reported by some workers that higher activity of microbial isocitritase is found when the organisms are grown on simple acetate-media than on complex nutrient media.

Table 10 shows the occurrence of isocitritase in various moulds including *Aspergillus oryzae*, *Aspergillus niger*, *Penicillium purpurogenum*, *Neurospora sitophila*, *Rhizopus nigricans*, *Mucor javanicus*, *Fusarium lini* and *Fusarium oxysporum*. High activities of the enzyme were observed in *Penicillum*, *Neurospora*, *Mucor* and *Fusarium* species.

Table 10. Distribution of isocitritase in moulds.

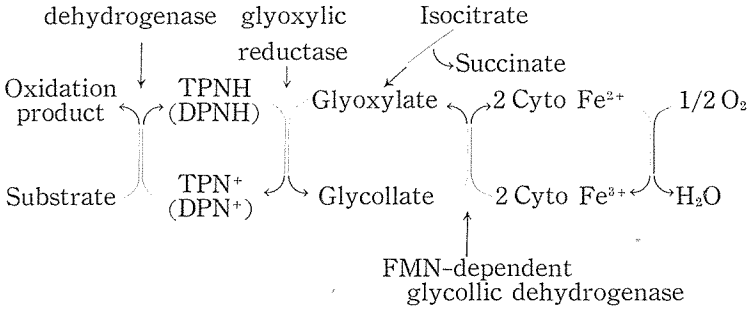
	Ketonic acid formed (μ moles)					
	pH 5.9			pH 7.5		
	Pyruvate	Glyoxylate	α-Keto-glutarate	Pyruvate	Glyoxylate	α-Keto-glutarate
Aspergillus oryzae	0.5	0.5	trace	0.7	0.9	trace
Aspergillus niger	1.1	1.8	nil	1.4	1.9	nil
Penicillium purpurogenum	1.7	8.8	2.5	1.9	9.5	2.7
Neurospora sitophila	1.1	25.5	0.6	1.4	31.5	1.0
Rhizopus nigricans	1.0	1.4	1.3	1.6	3.2	1.2
Mucor javanicus	2.6	10.7	1.2	2.5	20.4	1.2
Fusarium lini	1.4	28.1	trace	1.7	33.2	trace
Fusarium oxysporum	1.8	30.0	trace	5.3	30.5	trace

Reaction mixture: 1500 μ moles phosphate buffer, 1000 μ moles Na-citrate, 800 μ moles semicarbazide, 500 mg ground cells, 40 μ moles $MgSO_4$; total volume 15 ml; 4 hours' incubation at 37°C under static conditions.

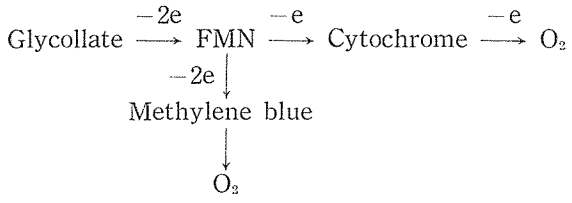
Function of Glyoxylate ⇄ Glycollate System

The occurrence of isocitritase, glyoxylic reductase and glycollic dehydrogenase in the glucose-grown cells implies that these enzymes may play an important role in the carbon metabolisms of microorganisms. The biological function of isocitritase

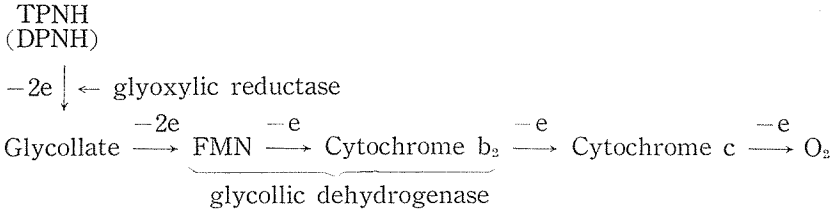
and glyoxylate reductase-glycollate oxidizing enzyme system may be explained according to the following scheme:



Oxidation of glycollate to glyoxylate in microbial system:



It has already been demonstrated that both cytochrome b_2 and cytochrome c are instantly reduced by glycollate in the presence of FMN-linked glycollate dehydrogenase of yeast. Thus, a new electron transport system would be possible as follows[26,27]:



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