

GLUTAMIC ACID DEHYDROGENASE OF *PASTEURELLA TULARENSIS*¹

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Received for publication April 16, 1957

As part of a general program of research on the metabolism of *Pasteurella tularensis*, Kann and Mills (1955) reported that glutamic acid was oxidized rapidly by resting cells. α -Keto-glutaric acid was produced, and further oxidation of the carbon chain proceeded via the citric acid cycle. The glutamic acid dehydrogenase involved was not studied intensively.

In the present studies the electron transfer system operating between glutamic acid and oxygen was examined. This paper reports some experiments on the triphosphopyridine nucleotide (TPN)-dependent glutamic acid dehydrogenase, and the conditions necessary for the transfer of electrons from glutamate to TPN to oxygen in a crude sonic extract during the uncomplicated oxidation of glutamate to α -ketoglutarate. The subsequent paper (Rendina and Mills, 1957) describes some of the properties of the reduced triphosphopyridine nucleotide (TPNH) oxidase system of this organism.

MATERIALS AND METHODS

Preparation of cell free extracts. Highly virulent *Pasteurella tularensis* strain Sm, received from Dr. Cora Downs, Department of Bacteriology, University of Kansas, was used. Stock cultures were maintained on glucose-cysteine-blood-agar slants (Downs *et al.*, 1947). Broth cultures were started from a 24 hr blood agar slant, and transferred daily in casein hydrolyzate-decamino medium (Hill and Mills, 1954). Incubation was at 37 C for 18 to 24 hr on a reciprocating platform shaker. New broth series were started monthly from blood agar slants.

¹ This report is based upon work supported in part by the Chemical Corps, Fort Detrick, Frederick, Maryland, and in part by research grant number E-806, from the National Institute of Allergy and Infectious Diseases, U. S. Public Health Service.

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Cell free extracts were prepared as follows: 750 ml of fresh casein hydrolyzate-decamino liquid medium in Fernbach flasks was inoculated with 10 ml of a 24 hr broth culture, and incubated for 18 to 24 hr at 37 C, with shaking. The cells were centrifuged, washed once with 50 ml of 0.9 per cent NaCl, and resuspended in 25 to 37.5 ml of 0.1 M potassium phosphate buffer, pH 7.4. The cell suspension was disintegrated for 20 to 45 min at 5 C in a Raytheon magneto-constriction oscillator at 9 kc and 100 v. The broken-cell suspension was centrifuged at 3000 \times G for 30 min, and the supernatant dialyzed 24 hr at 5 C against two changes of 1 L of distilled water. The dialyzed extracts contained 3.5 to 5.0 mg N/ml.

Oxygen uptake was measured with the standard manometric procedures of Umbreit *et al.* (1951).

Assay of glutamic acid dehydrogenase. Dehydrogenase activity was determined by several methods:

(A) The reduction of 2,6-dichlorophenolindophenol (Haas, 1944). Reactions were followed at 600 m μ in a Coleman Junior spectrophotometer until equilibrium was reached, with readings at 15 sec intervals for the first 2 min and at 1 to 2 min intervals thereafter. Endogenous controls were always run.

(B) The reduction of triphosphopyridine nucleotide (TPN). Reactions were followed at room temperature at 340 m μ in the Beckman DU spectrophotometer, using quartz cuvettes with 1.0 cm light path.

(C) The ferricyanide-bicarbonate manometric method of Alivisatos and Denstedt (1951). Flasks were equilibrated with a gas mixture of 95 per cent N₂ and 5 per cent CO₂ for 10 min, the stopcocks were closed, enzyme tipped in, and CO₂ production measured after 5 min.

Materials. Diphosphopyridine nucleotide (DPN) (90 per cent), TPN (6, 10, 90, and 95 per cent), flavin mononucleotide (synthetic), TPNH (chemically reduced), *p*-chloromercuribenzoate,

and *p*-chloromercuriphenyl sulfonate were obtained from Sigma Chemical Company, reduced glutathione from Nutritional Biochemicals Corporation, coenzyme A and TPN (100 per cent) from Pabst Laboratories, and riboflavin and 2,6-dichlorophenolindophenol from General Biochemicals, Inc.

RESULTS

Conditions for glutamate oxidation. Impure TPN (10 per cent), containing appreciable amounts of DPN and coenzyme A, markedly stimulated oxygen uptake by a dialyzed sonic extract, while DPN (90 per cent) alone caused only slight stimulation (table 1). Addition of glutathione to the system containing the impure TPN almost doubled the corrected oxygen uptake. Other sulfhydryl compounds such as cysteine, thioglycolate, and British Anti-Lewisite did not appreciably increase glutamate oxidation (table 2). Oxidation of glutathione itself was fairly rapid, and it also increased glutamate oxidation. Cysteine, on the other hand, increased endogenous oxygen uptake without influencing glutamate oxidation. Forty μ moles of glutathione per flask allowed maximal glutamate oxidation under the experimental conditions. Maximum oxidation of glutathione occurred at pH 6.5, while maximum oxidation of glutamate occurred at pH 8.0.

Under the conditions indicated in table 3 rapid oxidation of glutamate occurred with TPN

TABLE 1
Effect of diphosphopyridine (DPN) and triphosphopyridine (TPN) on oxidation of glutamate by sonic extracts

Cofactor Added	Oxygen Uptake		
	Endog-enous	Glutamate	Net
	μ L/hr	μ L/hr	μ L/hr
None.....	21	19	0
DPN.....	24	35	11
TPN.....	59	117	58

Each flask contained: glutathione, 35 μ moles; K phosphate, pH 8.0, 90 μ moles; 10 per cent KOH in center well, 0.2 ml; sonic extract in side arm, 0.5 ml, tipped at zero time; total volume, 3.2 ml. Additions (where indicated): K glutamate, 50 μ moles; DPN (90 per cent), 0.055 mg; TPN (10 per cent), 0.5 mg.

(95 per cent) in the absence of glutathione, DPN, and coenzyme A. Oxygen uptake was increased by 0.13 M KCN. It was also increased by glutathione, DPN, and coenzyme A, which allowed subsequent oxidation of the α -ketoglutarate produced. Riboflavin and flavin mononucleotide were included in the flasks for reasons discussed in the subsequent paper (Rendina and Mills, 1957).

Oxidation of α -ketoglutarate, on the other

TABLE 2
Effects of various sulfhydryl compounds on oxidation of glutamate

Addition to Flask	Oxygen Uptake		
	Endog-enous	Gluta-mate	Net
	μ L/hr	μ L/hr	μ L/hr
None.....	3	41	38
Glutathione, 40 μ moles....	84	151	67
Cysteine, 40 μ moles.....	81	117	36
Thioglycolate, 40 μ moles..	3	34	31
British Anti-Lewisite, 40 μ moles.....	430	400	0

Each flask contained: TPN (10 per cent), 0.5 mg; sonic extract, 0.5 ml; K phosphate, pH 8.0, 90 μ moles; 10 per cent KOH in center well, 0.2 ml. Total volume, 3.2 ml. K glutamate, 50 μ moles, also present in indicated flasks.

TABLE 3
Cofactor requirements for oxidation of glutamate

Additions to Flasks	Oxygen Uptake*
	μ L/hr
None.....	0
TPN.....	95
TPN + KCN.....	111
TPN + DPN + glutathione + coenzyme A.....	160

* Corrected for endogenous uptake.
Each flask contained: K phosphate, pH 8.0, 60 μ moles; riboflavin, 1.5 μ moles; flavin mononucleotide, 7.5 μ moles; MgSO₄, 25 μ moles; sonic extract in side arm, 0.5 ml, tipped at zero time; 20 per cent KOH in center well, 0.2 ml. Total volume, 3.2 ml. Additions (where indicated): K glutamate, 50 μ moles; TPN (95 per cent), 0.25 mg; glutathione, 40 μ moles; DPN (90 per cent), 0.2 mg; coenzyme A, 0.2 mg; and KCN 400 μ moles.

hand, depended on DPN, glutathione, and coenzyme A, and was inhibited by cyanide. It must be concluded, therefore, that the oxygen uptake obtained in table 3 with TPN alone resulted almost entirely from the oxidation of glutamate to α -ketoglutarate, and not from the subsequent oxidation of the α -ketoglutarate produced. The glutathione in table 2 stimulated oxidation of the α -ketoglutarate formed, but not the oxidation of glutamate.

All subsequent work, therefore, was done with glutamate and TPN (95 per cent), in the absence of added glutathione, DPN, and coenzyme A. The system allowed study of both glutamic acid dehydrogenase, reported below, and of the TPNH oxidase system, reported in a subsequent paper.

Glutamic acid dehydrogenase. Coenzyme specificity is indicated by the rapid reduction of TPN by a dialyzed cell free extract; DPN was not appreciably reduced in the same time interval (figure 1). Similar results were obtained in 2,6-dichlorophenolindophenol and methylene blue reduction experiments, and with the manometric ferri-cyanide-bicarbonate assay.

Aspartic acid, glycine, L-lysine, L-proline, L-

arginine, DL-threonine, DL-serine, and D-glutamic acid were inactive as substrates for 2,6-dichlorophenolindophenol reduction by the dialyzed extract.

Figures 2 and 3 illustrate the reversibility of the dehydrogenase. Reduced 2,6-dichlorophenolindophenol was reoxidized slowly but defi-

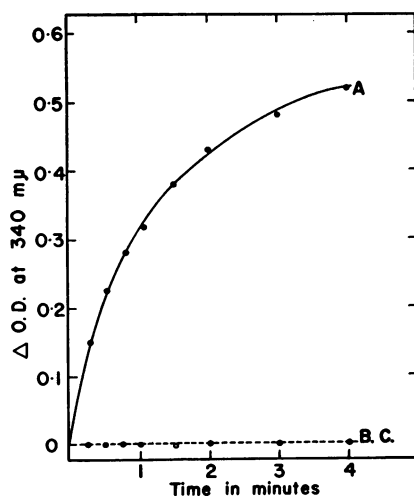


Figure 1. Reduction of TPN and DPN by glutamic acid dehydrogenase of *Pasteurella tularensis*. Each cuvette contained: sonic extract, 0.1 ml; K phosphate, pH 8.0, 40 μ moles; K glutamate, 25 μ moles, added at zero time. Total volume 3.0 ml. Coenzymes in cuvettes as follows: A. TPN (95 per cent), 0.4 mg; B. DPN (90 per cent), 0.4 mg; C. None. Each cuvette read against blank containing no substrate.

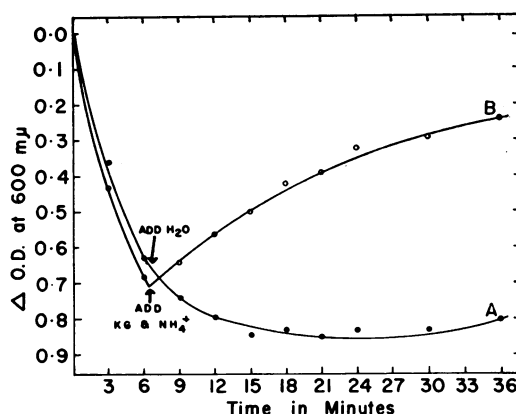


Figure 2. Reversal of reduction of 2,6-dichlorophenolindophenol by glutamic acid dehydrogenase. Each tube contained: K glutamate, 10 μ moles; TPN (95 per cent), 0.1 mg; 2,6-dichlorophenolindophenol, 0.5 μ mole; K phosphate, pH 8.5, 200 μ moles; sonic extract, 1.0 ml. Total volume before additions, 6.0 ml. At 7 min, added 0.5 ml H_2O to A, and 60 μ moles α -ketoglutarate plus 60 μ moles NH_4Cl (in 0.5 ml) to B.

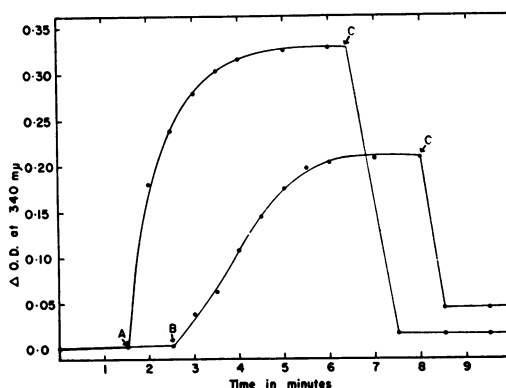


Figure 3. Reversal of TPN reduction by glutamic dehydrogenase. Each cuvette contained: sonic extract, 0.1 ml; K phosphate, pH 8.0, 90 μ moles; TPN (100 per cent), 0.15 mg. Total volume, 2.5 ml. At A added 0.5 ml 0.05 M glutamate. At B added 0.5 ml 0.05 M isocitrate. At C added 0.2 ml containing 10 μ moles α -ketoglutarate and 10 μ moles NH_4Cl . Total final volume, 3.2 ml.

nately after the addition of NH_4^+ and α -keto-glutarate. In contrast, reoxidation of TPNH was very rapid after similar additions. Both glutamate (curve A) and isocitrate (curve B) were used as substrates for the reduction of TPN.

The effects of *p*-chloromercuribenzoate, *p*-chloromercuriphenyl sulfonate, CuSO_4 , HgCl_2 ,

AgNO_3 , basic lead acetate, and ZnSO_4 on glutamic acid dehydrogenase were determined, using both TPNH production (340 $\text{m}\mu$) and 2,6-dichlorophenolindophenol reduction (600 $\text{m}\mu$) as assays. All were inhibitory except ZnSO_4 . Results for the first 4 inhibitors are summarized in table 5. Per cent inhibition, as compared to a run in the absence of inhibitor, was calculated from the total optical density change at a time when the reaction was essentially complete. It should be noted that in all cases the sensitivity to the inhibitor was greatest when the dye reduction assay was used.

TABLE 4
Cofactor requirements for oxidation of ketoglutarate

Additions to Flasks	Oxygen Uptake*
	$\mu\text{L/hr}$
1. TPN.....	28
2. TPN + DPN + coenzyme A.....	22
3. TPN + coenzyme A + glutathione.	19
4. TPN + glutathione + coenzyme A + DPN.....	143
5. As 4 + KCN.....	51

* Corrected for endogenous.
Flask contents and additions as in table 3, except substrate was 50 μ moles α -ketoglutarate instead of glutamate.

DISCUSSION

The glutamic acid dehydrogenase of *P. tularensis* is apparently typical, as indicated by its TPN specificity, substrate specificity, and reversibility. The dependence on TPN fits into the generalization that the bacterial enzyme is TPN-dependent, the plant enzymes are DPN-dependent, and those in mammalian tissues can use either coenzyme. Effective concentrations of inhibitors were in the range of those reported by

TABLE 5
Inhibition of reduction of 2,6-dichlorophenolindophenol and formation of TPNH by various compounds

Final Conc of Inhibitor	Inhibition with <i>p</i> -Chloromercuribenzoate		Final Conc of Inhibitor	Inhibition with <i>p</i> -Chloromercuriphenyl Sulfonate	
	2,6-Dichlorophenolindophenol reduction*	TPNH formation†		2,6-Dichlorophenolindophenol reduction*	TPNH formation†
<i>M</i>	%	%	<i>M</i>	%	%
6×10^{-3}	94	21	1.7×10^{-3}	78	33
1×10^{-3}	77	6	4.2×10^{-4}	63	15
6×10^{-4}	75	0	1.7×10^{-4}	57	3
1×10^{-4}	70	0	8.5×10^{-5}	53	0
6×10^{-5}	65	0	4.2×10^{-5}	49	0
Inhibition with CuSO_4			Inhibition with HgCl_2		
1×10^{-3}	87	45	5×10^{-5}	56	24
5×10^{-4}	79	17	1×10^{-5}	32	0
1×10^{-4}	66	15	5×10^{-6}	28	0
5×10^{-5}	56	6			
1×10^{-5}	27	0			

* Each tube contained: K phosphate, pH 8.0, 200 μ moles; K glutamate, 25 μ moles; TPN (95 per cent), 0.05 mg; 2,6-dichlorophenolindophenol, 0.04 mg; sonic extract, 0.2 ml. Endogenous control for each tube contained no glutamate. Inhibitors at concentrations listed. Total volume, 6.0 ml. Optical densities read at 600 $\text{m}\mu$.
† Each cuvette contained: sonic extract, 0.1 ml; TPN (95 per cent), 0.3 mg; K glutamate, 25 μ moles; K phosphate, pH 8.0, 90 μ moles; inhibitors at concentrations indicated; endogenous control run for each tube. Total volume, 3.5 ml. Optical density read at 340 $\text{m}\mu$.

Olson and Anfinsen (1953). ZnSO_4 did not inhibit the glutamic acid dehydrogenase reported here, while it did inhibit the mammalian dehydrogenase. On the other hand, basic lead acetate and CuSO_4 inhibited TPN reduction, whereas Olson and Anfinsen found that they did not inhibit crystalline glutamic acid dehydrogenase.

The reoxidation of TPNH by α -ketoglutarate and NH_4^+ is much more rapid than the reduction of TPN by glutamate. Olson and Anfinsen (1952) reported that DPNH is oxidized about 10 times as rapidly as DPN is reduced by the mammalian enzyme. Reduced 2,6-dichlorophenolindophenol, on the other hand, is much more slowly reoxidized; the enzymatic reaction transferring electrons from TPNH to 2,6-dichlorophenolindophenol is apparently not so rapidly reversible.

Nonenzymatic reduction of the 2,6-dichlorophenolindophenol by TPNH did not occur under the conditions used in these experiments. The enzyme catalyzing reduction of the dye by TPNH is considered part of the TPNH oxidase system, and is discussed in a further paper.

SUMMARY

Oxidation of glutamate to α -ketoglutarate by dialyzed sonic extracts of *Pasteurella tularensis* required added triphosphopyridine nucleotide (TPN); glutathione had no effect. The oxygen uptake of this reaction was not inhibited by cyanide.

Oxidation of α -ketoglutarate required added glutathione, diphosphopyridine nucleotide (DPN), and coenzyme A. TPN had no effect. Oxygen uptake was inhibited by cyanide.

Glutamic acid dehydrogenase of this organism is TPN dependent and specific, and is specific for L-glutamic acid.

The reductions of TPN and 2,6-dichlorophenolindophenol were reversed by addition of α -ketoglutarate and ammonium salts.

Certain sulfhydryl inhibitors and heavy metal salts inhibited the glutamic acid dehydrogenase.

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