The Purification and Properties of Reduced Diphosphopyridine Nucleotide Oxidase from Uninfected and T2 Infected *Escherichia coli* B*

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Received January 10, 1962

Reduced diphosphopyridine nucleotide oxidase from *Escherichia coli* B was demonstrated after cell lysis by sonic treatment and after lysis by freezing and thawing of cells infected with T2 bacteriophage. In spite of the marked instability the enzyme was purified considerably. The purified preparations contained other components of the respiratory chain and were markedly stimulated by liver extracts and by catalase but not by cytochrome *c*. No evidence for oxidative phosphorylation by the preparations was obtained. The preparations oxidized DPNH (reduced diphosphopyridine nucleotide) optimally at *p*H 7.2 and showed rather low affinity for this substrate as demonstrated by a K_m value of 3 to 6 $\times 10^{-2}$ M DPNH. The effect of temperature, metals, KCN, and amytal on stability and activity are also described. There were no marked differences in the properties of DPNH oxidase obtained from uninfected and T2 infected *E. coli*.

A particulate DPNH oxidase from sonically treated *Escherichia coli* has been reported (Asnis *et al.*, 1956). However, data regarding activity and purification of the DPNH oxidase were not included. On the other hand it has been demonstrated that extracts from *E. coli* were devoid of DPNH oxidase activity in the absence of menadione (Wosilait and Nason, 1954).

During a study of enzyme patterns of intact *E. coli* B, marked DPNH oxidase was detected after T2 phage infection, most likely due to the increased permeability of infected cells (Amelunxen and Grisolia, in press). Little activity was detected in uninfected *E. coli* preparations after freezing and thawing or after mechanical disruption by high-frequency shaking with glass beads. However, ultrasonic disintegration yielded very active preparations. It was therefore of interest to purify and to compare the DPNH oxidase from uninfected and T2-infected *E. coli* preparations. These studies are presented in this paper.

MATERIALS AND METHODS

E. coli, strain B, was grown in nutrient broth (Difco) or in the synthetic medium M-9 (Adams, 1959) with vigorous aeration at 37°, and the cell concentration was determined by viable count. The medium employed in each experiment is specified in the text. Infection was carried out at 37° with shaking; virus titrations were determined by the plaque technique (Gratia, 1936). Catalase was obtained from Sigma Chemical Company.

Cell breakage was accomplished with either the Braun cell homogenizer (B. Braun, Melsungen, Germany) or the MSE ultrasonic disintegrator (Medtron Associates, St. Louis, Mo.). After centrifugation, the cell-free lysates were analyzed for protein (Rodwell *et al.*, 1957).

DPNH oxidase was measured routinely by mixing in a Beckman cuvet 100 μmoles potassium phosphate buffer *p*H 7.4, 0.40 μmoles DPNH, and the bacterial preparation, in a final volume of 3.0 ml. The decrease in absorbancy was followed at 340 mμ in a Beckman DU spectrophotometer at 30°. One unit is defined as that amount of enzyme which under the above conditions causes a decrease in optical density of 0.001 per minute; specific activity is defined as the enzyme units per mg of protein.

RESULTS

Influence of Growth Conditions on Yield of DPNH Oxidase.—Best yield and specific activity were obtained by growing the *E. coli* in nutrient broth (8% containing 5 g NaCl per liter) under vigorous aerobic conditions for 2 to 3 hours at 37°. When a preparation was incubated for 15 hours without shaking the yield and specific activity were reduced to about 10–15%. Addition of glucose (0.4%) to the media did not increase yield or specific activity.

Influence of Mechanical Treatment on Release and on Stability of DPNH Oxidase.—In all cases a concentrated cell suspension (10^{10} viable cells/ml) was used. Ten ml of the cell suspension (in a 1-liter flask) and 10 ml of nutrient broth–glucose were shaken vigorously for 25 minutes and then centrifuged at 3,400 $\times$ *g* for 20 minutes. The cells were then washed with phosphate buffer (0.2 M, *p*H 7) and resuspended in 5 ml buffer; one sample was treated in the MSE ultrasonic disintegrator for $2^1/_2$ minutes and another in the Braun cell homogenizer for 1 minute. The lysates were centrifuged at 25,000 $\times$ *g* for 15 minutes and the supernatant fluids were analyzed. In a

* Supported by Grants from the National Institutes of Health, the Kansas Heart Association, and the American Heart Association.

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second set, 10 ml of the cell suspension was mixed with 4 ml of nutrient broth–glucose plus 6 ml of the T2 suspension (10^{11} plaque-forming units/ml), and then treated in the same manner as the uninfected samples. A third set of samples were infected at an input ratio (plaque-forming units/viable cell) of 40:1, which resulted in cell lysis within a few minutes ("lysis from without": Delbrück, 1940). The results of these experiments are shown in Table I.

TABLE I

DPNH-OXIDASE IN UNINFECTED AND INFECTED CELLS GROWN IN NUTRIENT BROTH–GLUCOSE AND BROKEN BY DIFFERENT METHODS

Treatment	Cells	Specific Activity of Lysates (units/mg protein)
Ultrasonication (MSE) for $2^1/_2$ min.	Uninfected	607[a]
	Infected	656[a]
Mechanical shaking (Braun) for 1 min.	Uninfected	69
	Infected	80
"Lysis from without"	Infected	169

[a] The supernatant fluids before mechanical lysis showed no DPNH oxidase activity for the uninfected and about 24 units per ml for the infected preparation. No DPNH oxidase can be demonstrated with intact uninfected cells (Amelunxen and Grisolia, in press).

Many other experiments were carried out, including testing the effect of mechanical treatment by both procedures in sequence. The results corroborated those shown in Table I, which clearly indicate the superiority of the sonic treatment in rupturing cells and in preserving DPNH oxidase.

Purification Procedure.—All operations were carried out at 0° unless specified otherwise. Three ml of nutrient broth was added to a slant of *E. coli* B and the suspended cells were transferred to a 1-liter flask containing 250 ml of nutrient broth. This inoculum was shaken for 3 hours at 37° and then transferred to a 20-liter bottle containing 6 liters of nutrient broth, which was shaken for 4 hours at 37°; at the end of this time absorbancy measurements at 600 mμ indicated approximately 10^9 cells/ml.

The culture was then centrifuged at 3,400 × *g* for 15 minutes, washed once with phosphate buffer (0.2 M, *p*H 7), and resuspended in buffer to approximately 60–70 ml. This suspension was treated in the MSE ultrasonic disintegrator for $2^1/_2$ minutes, in 10-ml portions. The sonicated suspensions were centrifuged at 25,000 × *g* for 15 minutes. Sedimented debris was resuspended in 10 ml phosphate buffer, treated in the MSE ultrasonic disintegrator as above, and centrifuged at 25,000 × *g* for 15 minutes; the supernatant fluids were then combined. This is the cell-free lysate.

To each milliliter of cell-free lysate 0.6 ml of saturated ammonium sulfate at *p*H 7.5 was added and the mixture was centrifuged at 25,000 × *g* for 10 minutes. The supernatant fluid was discarded, and the precipitate (fraction 1) was taken up in phosphate buffer (0.2 M, *p*H 7) to one fifth the volume of the original lysate. To each ml of this fraction 0.55 ml of saturated ammonium sulfate at *p*H 8.5 was added. The mixture was then centrifuged at 25,000 × *g* for 10 minutes. The precipitate was taken up in phosphate buffer to half the volume of fraction I to yield fraction II.

No additional purification was achieved with bentonite, phosphate gel, alumina, kaolin, hydroxyapatite, acetone, or protamine. However, additional purification was obtained by gradient ultracentrifugation. One ml of 2 M, 1.5 M, 1 M, and 0.5 M sucrose were layered into ultracentrifuge tubes followed by 1 ml of the enzyme preparation containing about 20 mg protein per ml. Centrifugation was carried out at 100,000 × *g* for 1 hour in the Spinco Model L ultracentrifuge and then 1-ml fractions were collected. Fractions III, IV, and V correspond to the second, third, and fourth ml withdrawn from the top of the centrifuge tubes. A summary of the purification procedure can be seen in Table II.

TABLE II

PURIFICATION OF DPNH OXIDASE FROM *E. coli* B

Fraction	Volume (ml)	Protein (mg)	Units[a] (× 10^{-3})	Specific Activity	Yield (%)
Lysate	74	2072	1495	721	100
I	12	518	1071	2070	71
II	6	372	987	2650	65
III	8	38	95	2465	6
IV	8	39	312	8489	20
V	8	16	75	4700	5

[a] Assayed under the standard conditions.

Purification of DPNH oxidase from T2 infected *E. coli* was conducted also. Freshly grown cells were concentrated by centrifugation to 2 × 10^9 viable cells/ml. Into each of six 1-liter flasks were placed 80 ml of the suspension plus 6 ml of the T2 phage preparation (10^{11} plaque-forming units/ml). The flasks were shaken vigorously for 20 minutes and immediately cooled in an ice bath. After centrifugation at 3,400 × *g* for 20 minutes, the cells were washed once with phosphate buffer (0.2 M, *p*H 7) and resuspended in 12 ml of buffer. This preparation was treated in the MSE ultrasonic disintegrator for $2^1/_2$ minutes and centrifuged at 25,000 × *g* for 10 minutes, and the cell-free lysate was purified as described above. There was essentially the same purification and distribution of activity as found for the uninfected preparation.

Effect of Time and Enzyme Concentration.—Under the conditions of the standard assay, the

rate was essentially linear provided that less than half the amount of DPNH remained unused. There was also proportionality between enzyme concentration and rate over a 10-fold range of enzyme concentration.

Influence of Substrate Concentration on Enzyme Activity.—The effect of substrate concentration was studied under the standard assay conditions by varying the amounts of DPNH. An extensive series of measurements with crude and with purified preparations showed no marked differences. The Michaelis constant (K_m), calculated graphically (Lineweaver and Burk, 1934), yielded values from 3 to 6 $\times 10^{-2}$ M.

Influence of Metals and of EDTA on DPNH Oxidase.—As shown in Table III a number of metals[1] were tested for inhibition or stimulation of DPNH oxidase from uninfected and infected *E. coli*. There was extensive inhibition with Zn^{++}, Co^{++}, U^{+6}, and Fe^{+++} ions. It is of interest that Mg^{++} and particularly Mn^{++} were also inhibitory (at high concentrations), although there was little difference between uninfected and infected preparations.

TABLE III

INFLUENCE OF METALS ON DPNH OXIDASE

The standard assay conditions were used. After 7 minutes' incubation at 30° of the enzyme with the metal, the reaction was started by the addition of DPNH. About 250 units of enzyme were used.

DPNH Oxidase from *E. coli*	Reagent Added	% Activity and μmoles Metal Added		
		1	5	10
Uninfected	$MgCl_2$	108	94	81
Infected	$MgCl_2$	122	74	62
Uninfected	$MnCl_2$	89	65	49
Infected	$MnCl_2$	81	—	78
Uninfected	$ZnSO_4$	1[a]	—	—
Infected	$ZnSO_4$	1	—	—
Uninfected	Pb acetate	50	—	—
Uninfected	$CuCl_2$	14	—	—
Infected	$CuCl_2$	6	—	—
Uninfected	U acetate	5	—	—
Uninfected	Na_2HASO_4	90–100	90–100	90–100
Infected	Na_2HASO_4	90–100	90–100	90–100
Uninfected	$FeCl_3$	86	0	0
Infected	$FeCl_3$	93	0	0

[a] Partially reversible with EDTA (10 μmoles).

Ethylenediaminetetraacetate up to 10 μmoles per ml had no effect on activity. However, at 3 μmoles/ml it partially reversed the inhibitory action of Zn^{++} (from almost complete inhibition to 90% inhibition).

Influence of KCN.—As shown in Table IV, KCN inhibited both uninfected and infected preparations, and the preparations did not differ markedly in susceptibility to KCN. Although not shown in Table IV, KCN at 5 $\times 10^{-3}$ M and 8.5 $\times 10^{-3}$ M inhibited the oxidase 93% and 95% respectively.

TABLE IV

THE ACTION OF KCN ON DPNH OXIDASE FROM UNINFECTED AND T2-INFECTED *E. coli*

The standard assay conditions were used. KCN was added to yield the indicated final molarities. The experiments were conducted as described for those of Table III.

KCN Molarity	% Inhibition	
	Uninfected	Infected
0	0	0
8.5 $\times 10^{-4}$	20	40
1.7 $\times 10^{-3}$	54	60
3.4 $\times 10^{-3}$	80	75

Effects of Amytal.—As shown in Table V, both uninfected and infected preparations were inhibited by amytal but the infected preparations appeared to be more susceptible than the uninfected ones.

TABLE V

THE EFFECT OF SODIUM AMYTAL ON DPNH OXIDASE FROM UNINFECTED AND T2-INFECTED *E. coli*

The conditions were as for the experiments of Table IV except that sodium amytal was added as indicated.

μmoles Amytal Added	% Inhibition	
	Uninfected	Infected
0	0	0
10	12	0
20	28	5
40	50	30
60	63	45

Diaphorase Activity.—Since the DPNH oxidase preparations were inhibited by KCN and by amytal, the presence of other components of the respiratory chain was indicated. Ammonium sulfate fractions from uninfected and infected *E. coli* and preparations further purified by sucrose gradient had considerable diaphorase activity. They showed, however, about 10-fold higher turnover for DPNH oxidation than for dye reduction and the dye reoxidation was carried out at about one third the velocity of dye reduction. Preliminary evidence indicated that the infected preparations had somewhat lower DPNH oxidase–diaphorase ratios than uninfected ones. It should be noted that addition of up to 1 mg of cytochrome *c* in the assay system did not affect the DPNH oxidase.

Effect of H_2O_2.—There was the possibility that the DPNH oxidase was a peroxidase. Addition of H_2O_2 (from 5 to 50 μmoles) to the assay system was essentially without effect. In fact there was little change in activity when 200 μmoles were

[1] Although Hg^{++} is highly inhibitory, the inhibition by this metal is complicated since it mimics DPNH oxidase. For example, 3 $\times 10^{-3}$ Hg^{++} in the assay system caused the disappearance of 0.1 μmole DPNH per minute.

added although there was some 75% inhibition with 400 μmoles.

Effect of Pigeon Liver and of Catalase.—Since H_2O_2 had little if any effect on DPNH oxidase it was of interest to test the effect of catalase addition. When a crude supernatant fraction from pigeon liver was used as a source of catalase and when a commercial catalase preparation was used, very extensive stimulation was noted, as shown in Table VI. It is noteworthy that the maximum stimulation was about twofold and that *the crude pigeon liver preparation was as effective as the catalase;* thus it is unlikely that the stimulation noted was due mainly to H_2O_2 removal.

TABLE VI

EFFECT OF PIGEON LIVER AND CATALASE ON DPNH OXIDASE

The standard assay conditions were used except that when indicated pigeon liver (P.L.) (12 mg protein/ml) or catalase (C.) (10 mg/ml) were added as shown. When experiments 2 and 3 were carried out the original DPNH oxidase activity had decreased considerably as shown. The figures in parentheses have been corrected for the small amount of DPNH oxidase present in the pigeon liver preparation.

Exper.	DPNH Oxidase Added (μl)	Supplementation (μl)	Activity (units)	Stimulation (%)
1	10	—	73	—
	50	—	394	—
	100	—	790	—
	10	50 P.L.	102 (99)	35
	10	100 P.L.	138 (132)	80
	10	150 P.L.	156 (147)	102
2	10	—	46	—
	10	5 C.	46	0
	10	10 C.	54	17
	10	50 C.	68	58
	10	100 C.	93	101
	10	200 C.	76	65
3	4	100 P.L.	(40)	
	6	100 P.L.	(64)	
	8	100 P.L.	(88)	
	10	100 P.L.	(103)	
	25	100 P.L.	(270)	

Manometric Experiments.—Attempts were carried out to test for O_2 uptake and for oxidative phosphorylation. There was excellent correlation between DPNH oxidation measured spectrophotometrically and O_2 uptake measured manometrically. Although O_2 uptake ceased when the theoretical DPNH present was oxidized, no evidence for oxidative phosphorylation with ADP (adenosine diphosphate) as an acceptor could be obtained. Whether or not this was due to ATPase interference was not investigated.

Stability.—In general the enzyme was more stable in concentrated solution and in the presence of salt or sucrose. Greater stability was noted when the preparations were kept frozen rather than at 4°. For example, after 4 days' storage, a concentrated preparation kept at −20° retained 80% of its activity versus 70% at 4°; a one-tenth dilution of this concentrated preparation kept at −20° retained 45% activity compared to only 18% at 4°. Differences in stability between uninfected and infected preparations could not be detected.

There was rapid enzyme inactivation above 50°. For example, 5 minutes of heating at this temperature reduced the activity of a sample containing 1.2 mg protein per ml from 2150 to 335 units per ml. The preparations were unstable below *p*H 5; at *p*H 4.5 there was immediate loss of 80% of the activity. There was marked instability of the enzyme to dialysis.

As indicated, little difference has been noted between the many studied properties of DPNH oxidase from uninfected and infected *E. coli*, and in all cases the preparations were only moderately stable. The DPNH oxidase used for the experiments thus far presented was prepared in all cases by sonic disintegration, since mechanical breakage was of little value, as demonstrated above. It was of interest to conduct additional studies on the DPNH oxidase liberated by T2 infection, and a résumé of these studies is shown below.

Procedure for the Preparation of Particulate DPNH Oxidase.—*E. coli* was grown in nutrient broth to 10^9 viable cells/ml, centrifuged at 3,500 × *g* for 30 minutes, and resuspended in M-9 medium to half the original volume. The cells were infected at an input ratio of 2 to 3 for 25 minutes. After centrifugation at 3,500 × *g* for 30 minutes and two washings with M-9, the packed cells were suspended to one tenth the original volume with M-9.

A typical preparation, for example, contained 260 units per ml. Less than 10 units remained in the supernatant fluid after centrifugation (30 minutes at 2,500 × *g*). Rapid freezing (−70°) and thawing resulted in lysis, yielding 2100 units[2] per ml; under these conditions 1350 units per ml remained in the supernatant fluid after centrifugation as above. Additional centrifugation at 25,000 × *g* for 30 minutes yielded a clear supernatant fluid devoid of activity and a gelatinous precipitate containing the bulk of activity which was taken up in water (approximately 2.2 mg protein per ml containing 7% nucleic acid). Unless otherwise specified this gelatinous pellet was used in the experiments. Little variation has been found among preparations, none being too stable either frozen or at 4°. In practice, the preparations were divided in equal portions, frozen and thawed as needed. Even with these precautions, there was some 10% loss of activity per week.

[2] It should be noted that the yield of DPNH oxidase (about 1 unit per 5 viable cells) is about equal to that obtained from uninfected cells after sonic disruption (see Table II).

TABLE VII

DPNH AND TPNH OXIDASES, DIAPHORASE, AND CYTOCHROME c REDUCTASE ACTIVITY OF T2-INFECTED E. coli PREPARATIONS

About 0.18 μmole of DPNH and TPNH was used per assay of oxidases and about 0.6 μmole per assay of diaphorase and reductase; 0.12 μmoles of 2,6-dichlorophenol indophenol was used in testing for diaphorase and 0.07 μmole of cytochrome c in testing for the reductase. The figures in the table are μmoles of coenzyme, dye, or cytochrome c turnover per minute per ml of the preparation. When deoxycholate was used the final concentration in the assay was 0.002%.

Preparation	Oxidase		Diaphorase		Reductase	
	DPNH	TPNH	DPNH	TPNH	DPNH	TPNH
Pellet in H_2O	0.22	0.002	0.026	0.017	0.0024	0.0
Pellet in 0.05% deoxycholate	0.01	0.0	0.017	0.0	0.0014	0.0
Supernatant fluid after deoxycholate treatment[a]	0.0	0.0	0.007	0.0	0.0024	0.0

[a] Centrifuged at 25,000 × g for 20 minutes.

It is of interest that the velocity of oxidation was the same in the presence of tris(hydroxymethylaminomethane) or phosphate buffer. Good correlation was found between DPNH oxidation measured spectrophotometrically and O_2 uptake measured manometrically.

Some Properties of the DPNH Oxidase.—Optimum activity was at *p*H 7.2 to 7.4, and the relative activity at other *p*H values was 56% at *p*H 6.0, 82% at *p*H 6.4, 96% at *p*H 6.8, 89% at *p*H 7.6, and 80% at *p*H 8.0. The K_m value for DPNH oxidation was calculated to be 6×10^{-2} M.

Treatment of pellets with ammonium sulfate resulted in marked inactivation of the enzyme without solubilization. Resuspension of the pellets in deoxycholate also resulted in marked inactivation and no solubilization. As little as 0.002% deoxycholate in the assay system resulted in 38% reduction in activity, and 0.02% deoxycholate decreased activity by 90%. The addition of trypsin to the DPNH oxidase preparation (1:10 ratio on the basis of protein content) resulted in about 68% inactivation of the enzyme and no solubilization. Lyophilization brought about 62% inactivation without solubilization. Incubation of the enzyme with $NaHCO_3$ (40 to 80 μmoles per ml) at 4° for several hours resulted in 86% inactivation.

Activity of Other Enzymes of the Electron Transport System.—Table VII shows comparative activities for DPNH and TPNH oxidases, cytochrome c reductase, and diaphorase. While kinetics for DPNH oxidase were linear over a long time period, the other enzymes showed an almost immediate decrease in velocity. The fact that 5.5 μmoles of H_2O_2 in the assay system for oxidase had no effect on the activity indicated the absence of DPNH peroxidase (Dolin, 1957). Preliminary attempts to test for oxidative phosphorylation were negative.

DISCUSSION

As shown in this paper, *E. coli* B possesses a very active DPNH oxidase, which can be demonstrated after cell lysis by sonic treatment or by T2 infection. No DPNH oxidase can be demonstrated in intact uninfected *E. coli* (Amelunxen and Grisolia, in press), since such cells are impermeable to DPNH.

In spite of marked instability of the DPNH oxidase, this enzyme can be purified considerably to yield very active preparations. While it is known that *E. coli* contains menadione reductase (Wosilait and Nason, 1954) and DPNH cytochrome c reductase (Brody, 1952), it has also been reported that it does not contain cytochrome c (Smith, 1954). It is therefore of interest that the preparations contain other components of the respiratory chain, and are not stimulated by the addition of cytochrome c. The marked stimulation by pigeon liver and catalase preparations is of particular interest in view of the fact that the enzyme is not a DPNH peroxidase as demonstrated by the lack of effect of H_2O_2.

As shown, there were no marked differences between the DPNH oxidases obtained from sonically treated uninfected and infected *E. coli* with regard to properties, response to a number of treatments, or change in total activity. It is of interest that the particulate DPNH oxidase obtained subsequent to infection and lysis by freezing and thawing could not be purified by ammonium sulfate treatment, in contrast to the soluble enzyme from sonically treated preparations.

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