STUDIES WITH PHENAZINE METHOSULFATE: EFFECT ON MITOCHONDRIA — AN ASSAY FOR REDUCED PYRIDINE NUCLEOTIDE COENZYMES*

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(Received March 28th, 1960)

SUMMARY

1. Phenazine methosulfate rapidly oxidizes DPNH and TPNH to DPN and TPN, respectively.

2. Phenazine methosulfate can be used in an assay system for microgram quantities of total reduced pyridine nucleotide coenzymes.

3. The results of this assay method in animal tissues agree closely with values previously reported for reduced pyridine nucleotides using enzymic assay methods.

4. Phenazine methosulfate oxidizes the reduced pyridine nucleotides within mitochondria, causes swelling of the mitochondria, and leakage of the coenzymes from mitochondria.

5. Ethylenediaminetetraacetate while inhibiting the swelling and leakage does not inhibit the oxidation. The reduced coenzymes do not appear necessary for the prevention of swelling.

6. Ethanol strongly inhibits the rate of oxidation of DPNH by phenazine methosulfate.

INTRODUCTION

Using appropriate enzymic procedures, it is possible to measure, in microgram quantities, the total reduced and total oxidized pyridine nucleotide coenzymes. Also, the amounts of the individual diphospho- and triphosphopyridine nucleotides may be determined[1,2].

These methods are based on the fact that the coenzymes, in their oxidized states only, form strongly fluorescent products after the addition of certain compounds. By noting, therefore, the increase in fluorescence upon oxidation of the reduced

Abbreviations: DPN and DPNH, the oxidized and reduced forms of diphosphopyridine nucleotide; TPN and TPNH, the oxidized and reduced forms of triphosphopyridine nucleotide; TCA, trichloroacetic acid; PMS, phenazine methosulfate; ADH, alcohol dehydrogenase; EDTA, ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; IDH, isocitric dehydrogenase.

* This is publication number 65 of the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts.

coenzyme, and by reference to a standard, one can determine the amount of reduced coenzyme in a sample.

Using the principles just outlined, a method is reported for the measurement of total reduced pyridine nucleotides utilizing nonenzymic oxidation. The oxidizing agent employed is phenazine methosulfate.

This communication demonstrates the oxidation of microgram quantities of both DPNH and TPNH by PMS. Also, several animal tissues are assayed for total reduced pyridine nucleotides and the results compared with other reported values. This method eliminates the necessity of having to prepare a bacterial enzyme (*Cl. kluyveri*) for the assay.

Effects of phenazine methosulfate on intact mitochondria are also reported.

MATERIALS AND METHODS

DPNH was obtained from Pabst Laboratories and TPNH from Sigma Chemical Company. Yeast alcohol dehydrogenase was purchased from Worthington Biochemical Company. TPN-specific isocitric dehydrogenase was prepared by the method of OCHOA[3]. Phenazine methosulfate was obtained from Sigma Laboratories, and it appears quite stable when kept cold and in the dark.

Spectrophotometric measurements were done in either a Beckman DU or Zeiss spectrophotometer. The fluorometric determinations were performed in a Farrand model A fluorometer or a Coleman electronic photofluorometer using filters as prescribed for thiamine determinations.

Fluorescence of the oxidized pyridine nucleotides was developed by the methyl-ethyl ketone method[4].

Tissues for analysis were homogenized in 5 volumes of sucrose (0.25 *M*)–nicotinamide (0.05 *M*) at 0°. This homogenate was then added to an equal volume of preheated 0.2 *M* Na_2CO_3, and heated in a boiling water bath for 3 min. After centrifugation the supernatant solution was analyzed for total reduced pyridine nucleotides.

Mitochondria were isolated and then suspended in sucrose (0.25 *M*)–nicotinamide (0.05 *M*) at 0°. In these studies, 2.00 ml of mitochondria (1 g of original liver tissue) were added to 0.5 ml 0.25 *M* sucrose and incubated at 28° with shaking. The following substances when used were present in the 0.5 ml sucrose in the following concentrations: potassium phosphate buffer, pH 7.5, 0.1 *M*; phenazine methosulfate, $2 \cdot 10^{-3}$ *M*; EDTA, pH 7.4, $2 \cdot 10^{-2}$ *M*.

Swelling of mitochondria and leakage of pyridine nucleotides were followed as described by KAUFMAN AND KAPLAN[5]. Oxidation of pyridine nucleotides was determined by adding trichloroacetic acid to the whole incubation mixture and developing the fluorescence of an aliquot from the supernatant solution.

RESULTS

Phenazine methosulfate (10^{-5} *M*) in aqueous solution rapidly oxidized DPNH and TPNH to DPN and TPN (Fig. 1). The products of oxidation were identified by their reactions with yeast ADH and isocitric dehydrogenase, respectively. The chemical oxidation and subsequent enzymic reduction were followed by recording the absorption at 340 mμ of the reduced compounds. Absorption due to PMS and to the

enzymes was subtracted from the initial figures to give the values recorded here.

Known solutions of DPNH and TPNH in microgram amounts (2–12 μg) were oxidized by PMS, and it was shown that the fluorometric readings were directly proportional to the initial concentration of reduced pyridine nucleotide (Fig. 2).

The effect of increasing ethanol concentrations on DPNH oxidation by PMS was also studied. The reaction mixture contained 0.35 μmole DPNH, 2.85 μmoles phosphate buffer (pH 7.5), and varying amounts of ethanol in a total volume of 3.0 ml. The oxidation was begun by the addition of 0.15 ml$\cdot 10^{-4}$ *M* PMS and was followed by the decrease in absorption at 340 mμ.

It was found that as the ethanol concentration was increased there was a marked decrease in the observed first order rate constant from 0.43 min^{-1} at 19 % ethanol to 0.19 at 38 %, and to 0.014 at 76 % ethanol.

A number of animal tissues were assayed for total reduced pyridine nucleotides by the PMS method (Table I). From the results it appears that the values agree fairly closely with previously reported values[2]. As shown in Table II, phenazine

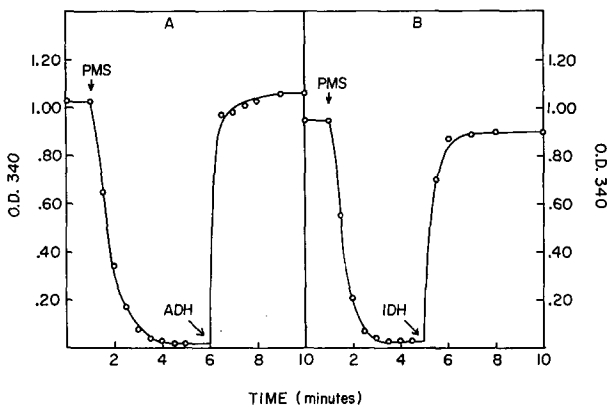

Fig. 1. Oxidation of DPNH and TPNH by phenazine methosulfate and subsequent enzymic reduction. Reactions were run in 1-ml cuvettes. A. Solution contained 0.02 ml DPNH (approx. 6 mg/ml) and 0.98 ml Tris–ethanol (0.5 *M* with respect to each). At 1 min 0.01 ml of 10^{-3} *M* PMS was added and at 6 min 0.05 ml of yeast ADH (0.1 dilution) was added. B. Solution contained approx. 0.15 μmole TPNH, 2.5 μmoles sodium isocitrate, 20 μmoles $MgCl_2$, and 0.1 ml 1.0 *M* phosphate buffer, pH 7.5, in a total volume of 1.0 ml. At 1 min 0.01 ml of 10^{-3} *M* PMS was added. At 5 min 0.1 ml of isocitric dehydrogenase was added.

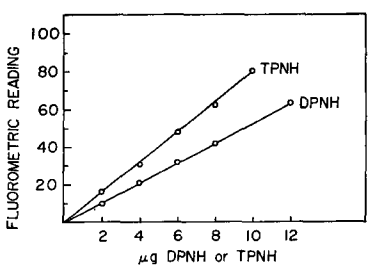

Fig. 2. Oxidation of DPNH and TPNH by phenazine methosulfate. DPNH: Each reaction mixture contained 0.25 ml of 0.1 *M* Na_2CO_3, 0.20 ml of 1.0 *M* phosphate buffer, pH 7.5, DPNH at various concentrations, and 0.02 ml$\cdot 10^{-3}$ *M* PMS in a total volume of 0.9 ml. After 5 min at room temperature, 0.1 ml of 100 % TCA was added. TPNH: Each reaction mixture contained 0.25 ml of 0.1 *M* Na_2CO_3, 0.20 ml of 1.0 *M* phosphate buffer, pH 7.5, TPNH at various concentrations, and 0.02 ml$\cdot 10^{-3}$ *M* PMS in a total volume of 0.9 ml. After 5 min at room temperature 0.1 ml of 100 % TCA was added. For each concentration of DPNH or TPNH two tubes were prepared. In one tube the PMS preceded the TCA, and in the other the TCA was added first. After incubation the fluorescence was developed by the methylethyl ketone method. The fluorometer readings in the accompanying figure represent the differences between the two samples. The fluorometer settings were arbitrary and were not the same for DPNH as for TPNH.

TABLE I

TOTAL REDUCED PYRIDINE NUCLEOTIDE COENZYMES IN SEVERAL RAT ORGANS, EXPRESSED AS mμmoles/g TISSUE

The values reported by the other workers have been converted to mμmoles using 1 μmole, 790 μg. The alkaline extract of the tissue was prepared as described in METHODS. Each assay tube contained 0.25 ml of the alkaline extract, 0.20 ml of 1.0 *M* potassium phosphate buffer pH 7.5 and 0.02 ml·10^{-3} *M* PMS in a total volume of 0.9 ml. After incubation for 10 min 0.1 ml of 100% TCA was added. For each sample, a similar tube was prepared in which the addition of TCA preceded PMS. After centrifugation the fluorescence was developed using an 0.5-ml aliquot of the supernatant solution. The difference in fluorometric readings between the 2 tubes, when compared with the readings given by a known amount of DPNH similarly treated, gave a value for reduced pyridine nucleotide.

	No. of samples	*PMS method*	*S d*	*Values reported by* JACOBSON AND KAPLAN[2]	*Values reported by* GLOCK AND MC LEAN[6]
Rat liver	4	532	± 36	455	516
Rat heart	4	298	± 20	234	274
Rat kidney	4	241	± 26	210	337
Rat spleen	4	49	± 19	—	92

TABLE II

OXIDATION OF INTRAMITOCHONDRIAL PYRIDINE NUCLEOTIDES BY PHENAZINE METHOSULFATE

Relative amounts of oxidized intramitochondrial pyridine nucleotides, as shown by fluorometric readings. 2.00 ml of the mitochondrial suspension were added to 0.5 ml of 0.25 *M* sucrose containing the other compounds in concentrations as stated in METHODS. Incubation was at 28° with shaking. At the times indicated, 0.5 ml of 50% TCA was added to the sample and the volume brought to 5 ml with H_2O. After centrifugation a 1.0-ml aliquot was taken, and the fluorescence was developed by the methylethyl ketone method. For the zero time readings with PMS, it was necessary to add the TCA before the PMS. An increase in fluorescence indicates the appearance of oxidized pyridine nucleotides.

	Fluorometer readings after		
	0 min	*10 min*	*20 min*
Mitochondria in sucrose	20	33	54
In sucrose and EDTA	16	39	46
In sucrose and phosphate	22	91	95
In sucrose, phosphate, and EDTA	17	55	54
In sucrose and PMS	22	82	80
In sucrose, PMS and EDTA	21	78	80

TABLE III

EFFECT OF PHENAZINE METHOSULFATE AND ETHYLENEDIAMINETETRAACETATE ON LEAKAGE OF PYRIDINE NUCLEOTIDES FROM MITOCHONDRIA

Relative amount of leakage is indicated by fluorometric readings. 2.00 ml of the mitochondrial suspension were added to 0.5 ml of 0.25 *M* sucrose containing the other compounds in concentrations as stated in METHODS. Incubation was at 28° with shaking. At the times indicated the contents of each tube were centrifuged at 0° for 5 min at 12,000 × *g*. To 1.0 ml of clear supernatant solution was added 0.1 ml 100% TCA. After centrifuging this, 0.5 ml was used for the development of fluorescence by the methylethyl ketone method. Increased fluorescence in the supernatant solution indicates leakage of oxidized pyridine nucleotides from mitochondria.

	Fluorometer readings after		
	0 min	*10 min*	*20 min*
Mitochondria in sucrose	3	9	11
In sucrose and EDTA	3	4	6
In sucrose and phosphate	4	69	80
In sucrose, phosphate, and EDTA	4	9	12
In sucrose and PMS	5	16	65
In sucrose, PMS, and EDTA	4	6	8

methosulfate in the concentration used, oxidized the intramitochondrial reduced pyridine nucleotide coenzymes. EDTA, while inhibiting the oxidation in a phosphate medium, did not inhibit the oxidation by the phenazine compound.

Phenazine methosulfate addition also led to swelling of mitochondria (Fig. 3) and to leakage of the oxidized coenzymes from the mitochondria (Table III). However, both the swelling and leakage were inhibited by EDTA.

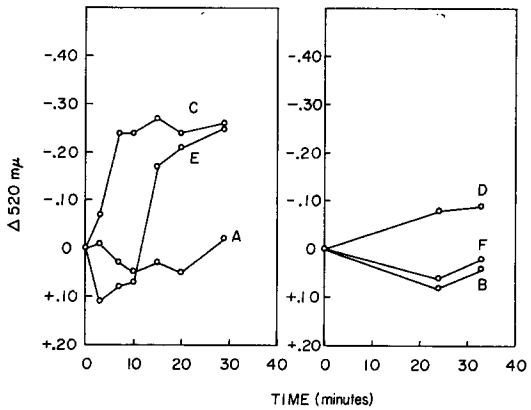

Fig. 3. Effect of phenazine methosulfate and ethylenediaminetetraacetate on swelling of mitochondria. A, in sucrose alone; B, in sucrose and EDTA; C, in sucrose and phosphate; D, in sucrose, phosphate, and EDTA; E, in sucrose and PMS; F, in sucrose, PMS, and EDTA. 2.00 ml of the mitochondrial suspension were added to 0.50 ml 0.25 *M* sucrose containing the other compounds in the concentrations as stated in METHODS. Incubation was at 28° with shaking. At various times 0.05 ml of this suspension was added to 2.95 ml of 0.25 *M* sucrose containing 0.05 *M* EDTA, and the O.D. at 520 mμ was determined. A decrease in O.D. at 520 mμ signified mitochondrial swelling.

DISCUSSION

Phenazine methosulfate (10^{-5} *M*) rapidly oxidized DPNH and TPNH to DPN and TPN. The fact that addition of the appropriate enzyme caused a reappearance of the reduced coenzyme shows that the oxidation products with PMS are DPN and TPN, rather than some further oxidation products such as pyridone compounds.

Fig. 2 shows that the fluorescence developed varied directly with the initial concentration of DPNH or TPNH in the sample being oxidized.

As already observed, when the polarity of the solvent was decreased, by increasing the concentration of ethanol, the rate of oxidation of DPNH by PMS was quite markedly slowed. This would suggest that the transition state in this reaction is more highly charged than the starting compounds.

Since the values in animal tissues by this method of assay compared closely with previously reported values, it would appear that this method is an acceptable and useful one for the oxidation of reduced coenzymes.

Although the mechanisms of mitochondrial swelling are not yet fully understood, there appears to be a relationship to the oxidation–reduction state of the electron transport system. This is shown in the suppression of swelling caused either by anaerobiosis or by a number of inhibitors of electron transport[7–9].

More recently it has been shown that a number of substances stimulating mitochondrial swelling also cause oxidation of pyridine nucleotides and leakage of these

coenzymes from mitochondria[10]. It was thought that something further might be learned about these three interrelated phenomena by the use of PMS, which is a potent oxidant of succinic dehydrogenase and of both of the reduced pyridine nucleotide coenzymes.

From the studies with intact mitochondria it appears that phenazine methosulfate is able to oxidize the reduced pyridine nucleotides within mitochondria. It is also of interest that EDTA inhibited the PMS-induced swelling and leakage, without affecting the oxidation of the pyridine nucleotides by PMS. This would suggest that the presence of the reduced coenzymes is not necessary to preserve the structural integrity of the mitochondrion, in agreement with the work of HUNTER *et al.*[9]. However, the means by which EDTA inhibits swelling and leakage remains poorly understood.

Inspection of Fig. 3 and Tables II and III also suggests that the oxidation of the pyridine nucleotides occurs both before the swelling of the mitochondria and before leakage of the pyridine nucleotides from the mitochondria.

ACKNOWLEDGEMENTS

The author would like to thank Drs. N. O. KAPLAN and A. M. STEIN for their interest and suggestions in this work. These investigations were supported in part by a training grant from the National Cancer Institute of the National Institutes of Health (CRT-5033-C1) and also Grant No. CY-3611-C2 of the National Cancer Institute.

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