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1-Methoxy-5-Methylphenazinium Methyl Sulfate

A Photochemically Stable Electron Mediator between NADH and Various Electron Acceptors[1]

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This paper describes the properties and application of 1-methoxy-5-methylphenazinium methyl sulfate (1-methoxyPMS), which is a photochemically stable, versatile electron carrier. Like 5-methylphenazinium methyl sulfate (PMS), it mediates electron transfer between NADH and various electron acceptors such as tetrazolium dyes or the electrode of an enzymic electric cell, and yet it does not deteriorate upon storage under scattered light in normal laboratories. The rate of reduction of 1-methoxyPMS coupled to the reoxidation of NADH produced by the lactate dehydrogenase reaction, was even faster than that of PMS. It was also successfully employed as an electron mediator in the enzymic electric cell method for the assay of NAD-linked dehydrogenases. 1-MethoxyPMS solution is rosy pink, and its standard redox potential (E_0') is approximately +0.063 V. The use of 1-methoxyPMS will be beneficial in biochemistry as well as medical technology, where PMS has been used as an electron mediator in various electron transfer systems.

NAD^+, a ubiquitous electron carrier in nature, is reduced with various substrates in the presence of specific dehydrogenases. Although NADH is an active electron donor for many biological redox systems in the presence of appropriate enzymes, it resists non-enzymic oxidation in spite of its low redox potential ($E_0' = -0.34$ V). It is not autoxidizable, and does not transfer electrons to redox dyes or coenzymes such as methylene blue, tetrazolium dyes, FAD, FMN, or pteridine coenzymes in the absence of specific enzymes. Only hexacyanoferrate (III) and 5-methylphenazinium methyl sulfate (PMS) (*1*) are known to accept electrons non-enzymatically.

Although PMS has long been used as an electron carrier for the coupling of the reoxidation of NADH and the reductive coloration of tetrazolium dyes to visualize dehydrogenase activities (*2–4*), it undergoes photochemical oxidation, especially in aqueous solutions. Even under scattered light in normal laboratories, it deteriorates rapidly. Recently German chemists synthesized a new electron carrier, Meldora Blue (*5*) as a substitute for PMS. It was reported to be stable and not to deteriorate under scattered light. Insolubility of the reduced form thereof,

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Abbreviations: PMS, 5-methylphenazinium methyl sulfate (numbering system of phenazine, in accordance with that of *Chem. Abst.*); INT, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride.

however, restricts its uses; the rate of reduction of Meldora Blue cannot be followed photometrically.

In our recent research on the enzymic electric cell method for the assay of NAD-linked dehydrogenases (*6,7*), in which PMS has been used as an electron mediator between NADH and the electrode, it was necessary to find a soluble electron mediator of better stability. We have tested various phenazinium derivatives, and found that 1-methoxy-5-methylphenazinium methyl sulfate (1-methoxyPMS) is a very stable, non-enzymic electron mediator between NADH and electrodes. This paper describes some properties of 1-methoxyPMS and application of this dye in the assay of lactate dehydrogenase, as an example of an NAD-linked dehydrogenase.

MATERIALS AND METHODS

1-MethoxyPMS was prepared according to the method used for the synthesis of PMS by Surrey (*8*) from 1-methoxyphenazine; yield, 95.3 %. It was recrystallized from ethanol–ether; mp 171°C (uncol.), lit. (*9*) 171–172°; NMR (in d_6-dimethyl sulfoxide), δ3.32 (S, 3H), δ4.20 (S, 3H), and δ7.5–9.0 ppm (m, 7H). The starting material, 1-methoxyphenazine, was prepared according to the method of Yoshioka (*10*), and recrystallized from ligroin; mp 170–171°C (uncol.), lit. (*10*) 170–171°; NMR (in $CDCl_3$), δ4.17 (S, 3H), and δ6.9–8.5 ppm (m, 7H). 1-Hydroxy-5-methylphenazinium methyl sulfate (1-hydroxyPMS) and 2-amino-5-methylphenazinium methyl sulfate (2-aminoPMS) were prepared similarly from 1-hydroxyphenazine and 2-aminophenazine, respectively.

Lactate dehydrogenase [EC 1.1.1.27] was purchased from Sigma (Type I, Lot 72C-9510, from rabbit muscle). The activity assay and the definition of the activity unit were as described in Ref. *6*. Neutralized NAD^+ stock solution (0.010 M) was prepared (*11*) from NAD^+ (Wako Pure Chemicals, Lot. ELL 7802).

The enzymic electric cell for dehydrogenase assay, slightly modified from that in our previous papers (*6,7,12*) was used in this study. The details will be given later in this paper.

Cyclic voltammetry of 1-methoxyPMS was carried out with a Yanaco P-8 polarograph equipped with an X-Y recorder (KA–20MII, Rikadenki Kogyo Co.).

RESULTS

Rate of Reduction of Redox Dyes by the Lactate Dehydrogenase-NAD System—A mixture containing 0.75 ml of 0.2 M phosphate buffer (pH 7.0), 0.3 ml of 1.0 M sodium DL-lactate, 0.3 ml of 0.010 M NAD^+, and 0.1 ml of 0.010 M redox dye solution in 2.5 ml, was anaerobically incubated in an optical cell under nitrogen at 30°C for 5 min. Then 0.5 ml of lactate dehydrogenase (0.19 unit) was added to the mixture under nitrogen, the rate of decolorization was recorded at an appropriate wavelength with a Hitachi 124 spectrophotometer equipped with a recorder, and the rate of the reduction of the dye was calculated (Table I). As shown in this table, the rate of reduction of 1-methoxyPMS was the highest of all. The rate of reduction of 1-methoxyPMS was proportional to the amount of lactate dehydrogenase up to 0.3 unit (data not shown).

Efficiency of 1-MethoxyPMS as an Electron Mediator in the Enzymic Electric Cell Method—An improved enzymic electric cell used for the assay of NAD-linked dehydrogenases, shown in Fig. 1, was placed in a thermostat at 30°C. A mixture containing 0.6 ml of 1.0 M sodium DL-lactate, 0.6 ml of 0.01 M 1-methoxyPMS, 0.6 ml of 0.010 M NAD^+, 1.5 ml of 0.2 M phosphate buffer (pH 7.0), and water in 5.8 ml, was stirred with a rotating platinum electrode (surface area: 4.0 cm^2) (see footnote 2) at 360 rpm by means of a synchronous motor. Nitrogen

TABLE I. Rate of reduction of some PMS derivatives in the lactate dehydrogenase -NAD system.

Electron carrier	λ[a] (nm)	$\varepsilon^{\lambda}_{mM}$[b]	Rate of reduction (μmol·min^{-1})
PMS (freshly prepared)	430[c]	2.9	0.070
1-MethoxyPMS	505	2.84	0.111
1-HydroxyPMS	380	1.71	0.001
2-AminoPMS	420	4.3	0

[a] λ is the wavelength at which the rate of reduction was measured. [b] $\varepsilon^{\lambda}_{mM}$ is the millimolar absorbance coefficient at λ. [c] Determination of the rate of reduction of PMS at this shoulder was not disturbed by the intermediary formation of a small amount of the semiquinone form of this dye.

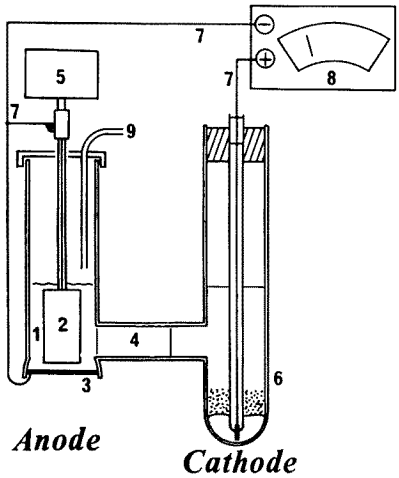

Fig. 1. An improved enzymic electric cell used for the assay of lactate dehydrogenase. 1, Reaction mixture containing lactate dehydrogenase, NAD^+, lactate, and an electron mediator such as 1-methoxyPMS in phosphate buffer; 2, rotating platinum electrode (10 × 20 mm); 3, gold electrode at the bottom of the anode (diameter, 20 mm); 4, salt bridge composed of polyacrylamide gel (diameter, 5 mm) (see Ref. *6* or *7* for its preparation); 5, synchronous motor (360 rpm); 6, saturated calomel electrode; 7, lead wire; 8, ammeter; 9, inlet tube for nitrogen gas (flow rate, 5 ml·s^{-1}).

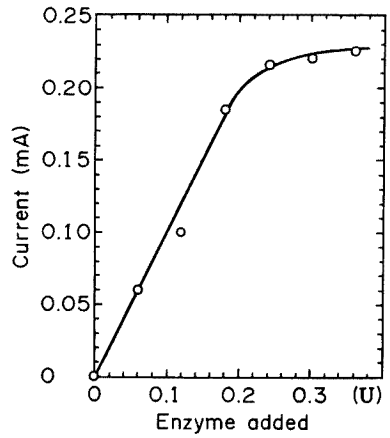

Fig. 2. Relation between the steady-state current and the amount of lactate dehydrogenase in the anode container of the cell. The reaction conditions are described in the text.

was bubbled through the mixture for about 10 s (flow rate, 5 ml·s^{-1}), and then a stream of nitrogen was swept over the mixture to prevent it from being exposed to atmospheric oxygen. The circuit of the cell was then closed and the background current was recorded. At zero time, 0.2 ml of lactate dehydrogenase solution (various activities) was added to the mixture, over which the nitrogen stream was passed continuously. The short circuit current increased gradually, reached a plateau in 7 min, and was stable for at least 30 min. Figure 2 shows that the steady-state current is proportional to the amount of enzyme up to 0.25 unit. With PMS as an electron mediator, considerable background current was observed, and the steady-state current upon addition of the enzyme was less stable. With Meldora Blue as an electron mediator, the current was not stable, probably because of the insolubility of the reduced form of the dye.

Stability of 1-MethoxyPMS during Storage—Stock solutions of 0.010 M PMS and 0.010 M 1-methoxyPMS were prepared, and stored in the dark, or under scattered light in our laboratory. At intervals, aliquots of the stock solution were withdrawn, diluted 30-fold, and the spectra, as well as their efficiencies as electron mediators, were recorded. The spectrum of PMS changed within a day under scattered light, or several days in the dark. On the other hand, the spectrum of 1-methoxyPMS did not change at all for as long as 100 days either in the dark or under scattered light. The efficiency of the stored 1-methoxyPMS as a non-enzymic electron acceptor for NADH as measured photometrically, or that as an electron mediator in the enzymic electric cell method, was the same as that of the freshly prepared solution.

Reactivity of 1-MethoxyPMS in Some Other Reactions—The activity of NAD-linked dehydrogenase can be visualized by coupling the reduction of NAD^+ to that of a tetrazolium dye such as INT in the presence of PMS as an electron mediator between NADH and INT (*4*). We attempted to visualize lactate dehydrogenase activity under the experimental conditions described by Bergmeyer (*4*), except that 1-methoxyPMS was used instead of PMS at the same concentration. As shown in

[2] In order to increase the surface area of the electrode, a rotating electrode was used in addition to the gold electrode (3.1 cm^2) placed at the bottom of the anode.

Fig. 3, 1-methoxyPMS works in this method as efficiently as PMS.

1-MethoxyPMS also successfully replaced PMS as an electron acceptor for formate dehydrogenase of *Desulfovibrio vulgaris*, Miyazaki. This enzyme was reported to reduce cytochrome *c*-553 of this bacterium as a natural electron acceptor; only hexacyanoferrate (III) and PMS have been reported, so far, to be effective as artificial electron acceptors (*13*).

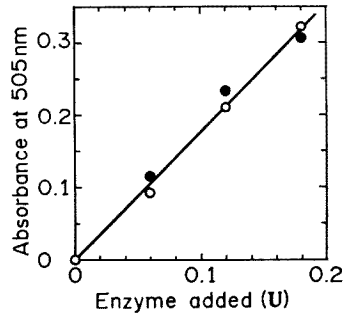

Fig. 3. Reductive coloration of INT coupled to re-oxidation of NADH *via* 1-methoxyPMS or PMS. A mixture containing 200 μmol of Tris-HCl (pH 8.5), 100 μmol of sodium DL-lactate, and various amounts of lactate dehydrogenase in 0.6 ml, was incubated at 37°C. To this mixture, a carrier mixture containing 0.32 μmol of 1-methoxyPMS or PMS, 1.5 μmol of NAD^+, and 0.8 μmol of INT in 0.4 ml was added and the mixture was incubated at 37°C. After 500 s, 5.0 ml of 0.1 M HCl was added to the reaction mixture to stop the reaction, and it was left to stand for 20 min. The intensity of red color caused by reduction of INT was measured at 525 nm, and was corrected for that of the background mixture without the enzyme. ○, With 1-methoxyPMS; ●, with PMS.

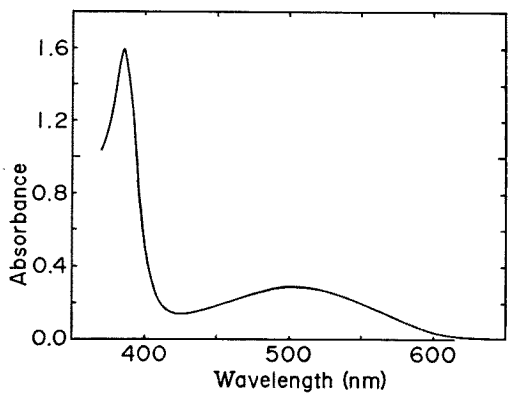

Fig. 4. Absorption spectrum of 10^{-4} M 1-methoxyPMS solution.

Some Properties of 1-MethoxyPMS—The color of 1-methoxyPMS solution is rosy pink. The absorption spectrum is shown in Fig. 4. This has two absorption maxima at 505 and 386 nm. The millimolar absorbance coefficients are 2.84 at 505 nm, and 15.9 at 386 nm.

Cyclic voltammetry of 1-methoxyPMS in 0.01 M phosphate buffer (pH 7.0; ionic strength, 0.022) at 25°C did not give a reversible wave because of adsorption of the reduced form of the dye on the surface of the platinum electrode. This technique (*14*), therefore, gave only a rough estimate of the standard redox potential ($E_0' = +0.08$ V). A more accurate value was obtained by potentiometric titration: E_0' was +0.063 V in 0.05 M phosphate buffer (pH 7.0; ionic strength, 0.12).

DISCUSSION

PMS is a useful non-enzymic electron mediator between reduced nicotinamide coenzymes and artificial electron acceptors such as tetrazolium dyes (*1–4*) or the electrode of an enzymic electric cell (*6*, *7*). PMS is also used in the study of electron transfer in photosystems and many other biological redox systems. PMS is, however, susceptible to photochemical oxidation, and in most cases, freshly prepared PMS solution is required to give reproducible results. In the course of our studies to improve the assay method of NAD-linked dehydrogenases by means of the enzymic electric cell (*6*, *7*), we have found that 1-methoxyPMS is a very efficient electron mediator between NADH and the electrode. The rate of reaction between 1-methoxyPMS and NADH is faster than that betweenPMS and NADH (Table I). 1-MethoxyPMS also successfully replaces PMS in other biochemical electron transfer systems. An aqueous solution of 1-methoxyPMS can be stored and used without any precautions against photochemical deterioration. The use of 1-methoxyPMS will be beneficial in biochemistry as well as medical technology, where PMS has been used as an electron mediator in various electron transfer systems. A theoretical basis to show why a 1-methoxy group endows PMS with photochemical stability while

maintaining the reactivity with NADH remains to be elucidated.

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