SOME LIMITATIONS IN THE USE OF METHYLENE BLUE AS ELECTRON ACCEPTOR

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Dedicated to Professor A. Okáč, on the occasion of his 70th birthday.

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The rate of oxygen consumption during oxidation of methylene blue is at first directly propotional, later indirectly proportional and finally without proportion to the amount of reducing agent, either enzyme or ascorbate. The indirect relationship is due to precipitation of reduced methylene blue from the reaction mixture. The stimulating effect of ostruthin on reoxidation of methylene blue is observed only in the region of indirect relationship of oxygen consumption on the amount of reducer and its basis consists in a partial inhibition of methylene blue reduction. The stimulating effect of menaquinone is of a different character.

When examining the effects of natural coumarin derivatives on succinate dehydrogenase in a Keilin–Hartree preparation we used artificial electron acceptors, of which the phenazine methosulfate (PMS)* and MB yielded results somewhat different from the others[1]. With PMS the inhibition by ostruthin (6-geranyl-7-hydroxycoumarin) was dependent on the temperature of the incubation mixture, with MB a stimulation of oxygen consumption under certain experimental conditions was observed. In both cases we are dealing with auto-oxidizable dyes where the enzyme activity was estimated from the oxygen consumption for reoxidation of the enzyme-reduced dye. Whereas the anomalous course of inhibition when using PMS could be accounted for by decreasing oxygen solubility in the reaction mixture at higher temperatures, no satisfactory explanation was found for the stimulation of the enzyme reaction by ostruthin when using MB. An explanation of this effect is presented here.

EXPERIMENTAL

Material. Coumarin and aesculin (6-glucoxy-7-hydroxycoumarin) were from Lachema, Brno. Ostruthin was a kind gift from Prof. Wessely, University of Vienna. Daphnetin (7,8-dihydroxycoumarin) was from the Department of Organic Chemistry, Purkyně University, Brno, geraniol was from the Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Scien-

* Abbreviations used: MB and MBH_2 oxidized and reduced forms of methylene blue, PMS and $PMSH_2$ oxidized and reduced forms of phenazine methosulfate.

ces, Prague. Synthetic menaquinone (MK-6), phylloquinone, menadione and α-tocopherol were gifts from Dr J. Weichet of the Research Institute of Pharmacy and Biochemistry, Prague. The derivatives of ubiquinone were kindly provided by Prof. O. Wiss, Hoffmann-LaRoche, Basel. The Keilin–Hartree heart muscle preparation was prepared as described elsewhere[2] and was stored at a concentration of 25–40 mg protein/ml for a week at +3°C. Proteins were assayed by the biuret method in the presence of 2% sodium deoxycholate to remove turbidity.

Methods. Oxygen consumption during reoxidation of MBH_2 was measured by the Warburg method, reduction of MB was estimated anaerobically in Thunberg tubes.

Estimation of solubility of MBH_2. A solution of 5 mg MB in 10 ml water or phosphate buffer of pH 7·4 at the concentration shown was combined with a catalytic amount of 5% palladium on barium sulfate. The air in the vessel was replaced with hydrogen and the mixture was stirred in a thermostat at 40°C to complete loss of colour (2 h). The solution was then filtered in absence of air, the filtrate was diluted 5–50 times and vigorously aerated to constant colour (1·5 h). The intensity of the colour was read on a Pulfrich photometer with an ELPHO attachment at 668 nm. Concentration of MB was determined with the aid of a calibration curve.

RESULTS

Effect of Rate of Methylene Blue Reduction on the Rate of Oxygen Consumption for Reoxidation of the Reduced Form

Fig. 1 shows that the rate of oxygen consumption by succinate-MB reductase first rises with the amount of the enzyme preparation but when the level of 5 mg protein is exceeded the rate starts to decrease. In the presence of more than 10 mg enzyme protein it is practically independent of the enzyme amount. When MB was reduced nonenzymically with ascorbate a similar dependence of the rate of oxygen consumption on the concentration of ascorbate was observed (Fig. 2). The drop of the rate of oxygen consumption sets in at 40–50 μmol and ceases at 80–120 μmol ascorbate.

The effect of previous reduction of MB on the rate of oxygen consumption by succinate-MB reductase and by the nonenzymic system of ascorbate–MB is shown in Table I. Reduction of MB was achieved by its preincubation with the enzyme and succinate or with ascorbate prior to measurement. This reduction brings about a substantial decrease of oxygen consumption estimated under otherwise identical conditions.

MBH_2 is much less soluble than MB. It was found that a saturated solution of MBH_2 in water at 40°C has 255 μg/ml. This value is close to the 220 μg/ml found by Wieland and Bertho[3] at room temperature. The solubility of MBH_2 decreases with increasing concentration of phosphate and attains 25 μg/ml in 2 mM and 18 μg/ml in 0·1 M phosphate buffer of pH 7·4. The dependence of oxygen consumption by a system of ascorbate–MB changes in the presence of lower concentrations of MB. It may be seen in Fig. 3 that a decrease of MB concentration to 25 μg/ml is responsible for the fact that the consumption of oxygen does not decrease at higher concentrations of ascorbate.

Mechanism of Stimulating Effect of Ostruthin and Menaquinone

The effect of ostruthin on oxygen consumption by succinate-MB reductase is shown in Table II. In the presence of 2 mg enzyme protein ostruthin inhibited the rate of oxygen consumption but it stimulated it in the presence of 10 mg protein. Similarly, the effect of ostruthin on the rate of oxygen consumption by a nonenzymic system

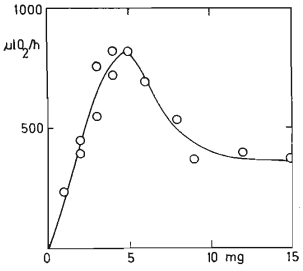

FIG. 1

Dependence of Oxygen Consumption by Succinate–Methylene Blue Reductase on the Amount of Enzyme (mg)

Manometry at 40°C. The reaction mixture contained in 3 ml 0·15M phosphate buffer of pH 7·3, 25 mM sodium succinate, 9 mM KCN, 1 mg MB and the enzyme as shown.

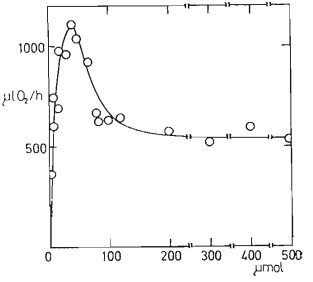

FIG. 2

Dependence of Oxygen Consumption by Ascorbate–Methylene Blue on the Amount of Ascorbate (μmol)

Manometry at 40°C. The reaction mixture contained in 2 ml 0·15M phosphate buffer of pH 7·3, 1 mg MB and ascorbic acid neutralized with sodium hydroxide.

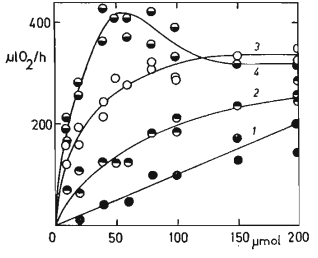

Fig. 3

Effect of Concentration of Methylene Blue on Oxidation of Ascorbate (μmol) Catalyzed by Methylene Blue

The reaction conditions were as in Fig. 2. Concentration of MB was (μg/ml): *1* 0, *2* 5, *3* 25, and *4* 50.

of ascorbate–MB was different at different amounts of ascorbate. As will be seen from Table III, stimulation is found at 40 μmol ascorbate, maximum stimulation being observed at 100 μmol ascorbate. In the presence of more than 200 μmol ascorbate ostruthin had practically no effect.

The effect of some compounds, many of which are frequently used for studying oxidation-reduction enzymic systems, on the nonenzymic system of ascorbate–MB is shown in Table IV. The greatest stimulation of oxygen consumption was exhibited by menaquinone, the others being less effective or having no effect. Table V compares the effect of ostruthin and menaquinone, two of the most effective stimulators of oxygen consumption, on ascorbate oxidation. Their effects are different. Anaerobic

TABLE I

Effect of Previous Reduction of Methylene Blue on Its Catalytic Power

The reaction conditions were as in Fig. 1 (enzymic reduction, 10 mg protein) and in Fig. 2 (reduction with ascorbate, 50 μmol ascorbate). The reaction mixture was incubated before measurement at 40°C (with the enzyme for 11 min, with ascorbate for 3 h) without agitation. In series 1 methylene blue was placed separately from the reaction medium and added as oxidized form just before measurement. In series 2 methylene blue was reduced by incubation in the reaction medium before measurement.

Series	Oxygen consumption, μl/h	
	enzyme	ascorbate
1	660	150
2	244	19

TABLE II

Effect of Ostruthin on Oxygen Consumption by Succinate–Methylene Blue Reductase

Manometry was done at 37°C. The reaction mixture (3 ml) contained 0·15M phosphate buffer of pH 7·3, 25 mM sodium succinate, 9 mM KCN, 3·2% ethanol, 0·9 mM methylene blue, enzyme and ostruthin as indicated.

Enzyme mg protein	Ostruthin 10^{-4}M	Oxygen consumption μl/h	Activity %
2	0	278	100
2	3	180	65
10	0	432	100
10	3	524	121

oxidation of ascorbate by MB is inhibited by ostruthin and only very little by menaquinone, the auto-oxidation of ascorbate by atmospheric oxygen is markedly stimulated by menaquinone and almost unaffected by ostruthin.

DISCUSSION

The unusual dependence of oxygen consumption by the succinate–MB reductase is not a specific property of the enzyme system since a similar course may be observed even during nonenzymic reduction of MB by ascorbate. It can be thus concluded that the decrease of this rate at higher amounts of reducing agent is due to MB. Simultaneously with this decrease a partial bleaching of MB was observed which suggests that the reoxidation of MBH_2 is inhibited. Arrigoni and Singer[4] showed for PMS that the solubility of oxygen in the reaction medium can become a limiting factor when measuring the rate of oxygen consumption by enzymic oxidoreduction systems. However, the shape of the curves in Fig. 1 and 2, *i.e.* their descending part, cannot be explained in this fashion. It was possible to assume the formation of a nonreactive form of MBH_2. This assumption is supported by the results of Table I. MBH_2 formed by reduction of MB before the start of measurement is much less active in transferring hydrogen to oxygen than is MB. The increased concentration of MBH_2 thus yields a nonreactive form. Similarly, formation of a nonreactive form is assumed[5] with $PMSH_2$. A necessary condition for its formation is the coexistence of protonated forms of PMS and $PMSH_2$. The values of dissociation constants[6] for MB and MBH_2 permitted to calculate that at pH 7·3 some 3% of MBH_2 is protonated while at pH 9 only 0·07%. If the mechanism of formation of the nonreactive form of MBH_2 were analogous with $PMSH_2$, at higher pH values the drop of oxygen consumption rate would have to be less apparent. However, a change of buffer pH had no substantial effect on the dependence shown in Fig. 2, only a general increase

TABLE III

Effect of Ostruthin on Ascorbate Oxidation Catalyzed by Methylene Blue

The reaction conditions as in Fig. 2 but the reaction mixture also contained 5% ethanol or $5 \cdot 10^{-4}$ M ostruthin as shown.

Ascorbate μmol	Oxygen consumption, control	μl/h ostruthin	%
10	689	684	99
40	1 104	1 236	112
100	626	950	151
400	575	591	103

in oxygen consumption being observed at higher pH. Hence it follows that the formation of a similar nonreactive form as the case is with $PMSH_2$ is not likely with MBH_2.

Since MBH_2 is much less soluble than MB it could be assumed that an increase of MBH_2 concentration due to heavy reduction of MB results in precipitation of MBH_2 from the solution

TABLE IV

Effect of Some Coumarins, Quinones and Detergents on Ascorbate Oxidation Catalyzed by Methylene Blue

The reaction conditions are as in Fig. 2, with 100 μmol ascorbate. The oxygen consumption by the control = 100%.

Compound	mM	Activity %	Compound	mM	Activity %
Ostruthin	0·25	122	phylloquinone	1·0	135
Ostruthin	0·5	152	menaquinone	0·5	208
Coumarin	0·5	100	menadione	1·0	110
Umbelliferone	1·0	93	ubiquinone 30	0·25	127
Daphnetin	1·0	93	ubiquinone 50	0·1	108
Aesculin	1·0	116	α-tocopherol	0·5	97
Geraniol	0·5	89	Tween 40	[a]	105
Geraniol	5·0	118	Tween 80	[a]	96

[a] 1% solutions were used.

TABLE V

Effect of Ostruthin and Menaquinone on Ascorbate Oxidation

Anaerobic oxidation of ascorbate with MB as the terminal electron acceptor was estimated by Thunberg's method at 40°C. The reaction mixture (2 ml) contained 0·15M phosphate buffer of pH 7·4, 100 μmol ascorbate, 1 mg MB, 5% ethanol and $5 \cdot 10^{-4}$M ostruthin or menaquinone. Oxidation of ascorbate with atmospheric oxygen was followed manometrically under the same conditions as with anaerobic estimation but MB was not present.

Added	Anaerobic oxidation		Aerobic oxidation	
	T[a]	%	μl O_2/h	%
—	5·0	100	147	100
Ostruthin	7·7	65	157	107
Menaquinone	5·5	91	395	269

[a] T is the time (min) required for bleaching of methylene blue by 95%.

If the concentration of MB was decreased so that after its reduction all the MBH_2 remained dissolved, no drop in the rate of oxygen consumption due to higher amount of reducing agent should be observed. Fig. 3 shows that when the MB concentration is decreased to 50 μg/ml the drop is still apparent. Only when the concentration of MB is decreased to 25 μg/ml does the drop disappear and a gradual attainment of the limiting oxygen consumption rate is observed. Both concentrations of MB are much lower than the concentration of saturated solution of MBH_2 in water but are higher than the concentration of saturated solution of MBH_2 in 0·15M phosphate. Since all of the MB is not reduced during the aerobic measurement, at 25 μg MB/ml the concentration of MBH_2 apparently does not exceed its solubility in 0·15M phosphate, *i.e.* 18 μg/ml. MBH_2 does not precipitate from the solution and the rate of oxygen consumption is not decreased. At higher concentrations of MB (*e.g.* 50 μg/ml) heavy reduction increases the concentration of MBH_2 above the limit of its solubility, MBH_2 begins to precipitate and the rate of oxygen consumption decreases. The nonreactive form of MBH_2 which is formed due to heavy reduction of MB is thus MBH_2 that has precipitated from a solution. The rate of oxygen consumption is then limited by the solubility of MBH_2.

The activity of succinate-MB reductase is inhibited by ostruthin in the presence of 2 mg enzyme protein but at 10 mg protein ostruthin stimulates the rate of oxygen consumption. To exclude the effect of ostruthin on the enzyme system, ascorbate was used as a hydrogen donor. Even with this nonenzymic system, the rate of oxygen consumption was stimulated. At the same time, in the enzyme system without MB, ostruthin did not bring about any oxygen consumption and also its effect on autooxidation of ascorbate was only minute (Table V). Hence it follows that the basis of this phenomenon is to be sought in the interaction of ostruthin with MB. If the results shown in Fig. 1 and 2 and in Tables II and III are compared it will be seen that ostruthin is inhibitory (negligibly with the nonenzymic system) in the region of direct relationship between oxygen consumption and the amount of reducing agent whereas it is stimulatory in the region of indirect relationship. This phenomenon can be explained if ostruthin inhibits not only the enzymic but also the nonenzymic reduction of MB. This second effect of ostruthin was demonstrated here (Table V). Thus, under conditions when only little MBH_2 is formed (ascending parts of curves in Fig. 1 and 2) the inhibition of MB reduction is reflected in a decrease of the oxygen consumption rate. This is apparent particularly with the enzyme system (Table II). In the case of the nonenzymic system the decrease is minute, apparently due to autooxidation of ascorbate. If an excess of MBH_2 is formed which then precipitates from the solution (descending parts of the curves) the inhibiton of MB reduction decreases the formation of MBH_2 and its precipitation. This is reflected in an increased rate of oxygen consumption, similarly to a decrease in the amount of reducing agent. This interpretation also accounts for the inefficiency of ostruthin at high concentrations of ascorbate (horizontal part of the curve). Here the partial inhibition of MB reduction is not reflected in a change of oxygen consumption rate.

It is of interest that ostruthin inhibits the nonenzymic reduction of MB. If ostruthin interacts with MB in such a manner that it decreases its reducibility this can distort the results. The values of inhibition found are then higher than would correspond to the inhibition of the enzyme reac-

tion alone. In this way, one can explain the increased sensitivity of succinate-MB reductase to ostruthin. With this system of electron transfer it was not possible to demonstrate a release of the inhibition by ostruthin when adding albumin[1] although albumin is an effective reactivator[7] of ostruthin-inhibited succinate oxidase. It is likely that ostruthin bound to albumin cannot interact with the enzyme preparation but it retains its ability to interact with MB. Likewise, some other compounds used for studying natural redox systems affect the nonenzymic system of ascorbate–MB. Of the compounds studied, menaquinone caused the highest stimulation of the rate of oxygen consumption. The mechanism of its effect is different from that of ostruthin and consists in an increased auto-oxidation of ascorbate, having no effect on the reduction of MB. Mutual interactions of compounds which are not a natural component of the enzyme system can cause errors of measurement when artificial electron acceptors are used.

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