THE CATALYTIC EFFECT OF DYES ON THE OXYGEN CONSUMPTION OF LIVING CELLS

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It has been shown (1) that methylene blue added to living cells increases their oxygen consumption, the increase being partly due to the oxidation of some of the decomposition products of the carbohydrate metabolism (2). It was then suggested (3) that the action of methylene blue was due to its reversibility with respect to oxidation, which enabled it to play the rôle of catalyst in the presence of atmospheric oxygen. It was of interest to study the relation between the reduction potential of the dye and its catalytic power and to correlate these properties with the reduction intensity in the cell.

The most suitable material for this purpose is the mature unfertilized eggs of starfish (*Asterias forbesii*).

Their pH value and reduction intensity have been studied and their active respiration allows accurate measurements of oxygen consumption; furthermore, the more or less transparent protoplasm of the eggs makes it easy to observe the penetration of the dye through the cell wall. The eggs of the same starfish were used in each series of experiments. They were collected in flat-bottomed dishes, washed once with sea water, and after 40 to 50 minutes (time necessary for maturation) the experiments were started. The oxygen consumption was measured at 25°C. ±0.01 in Warburg's vessels with Barcroft micromanometers. Freshly prepared dyes were used at a concentration of 1.253 millimols per liter. The oxygen consumption of the cells was measured for an hour, after which time, the dye kept in the side arm of the respiration vessel was thrown into the main vessel containing the cells, and the oxygen consumption again measured for 1 hour.

Relation Between the Reduction Potential of Dyes and the Increase in the Oxygen Consumption of Living Cells

We possess now a fair number of reversible dyes which can be employed as indicators of reduction intensity. We selected them,

having in consideration these two factors: (1) permeability of the cell membrane to dye and (2) the toxicity of the dye to the cell. We, therefore, had to discard those reversible dyes which either did not penetrate the cell membrane or were clearly toxic. Cohen, Chambers and Reznikoff (4), in their studies on the reduction potentials of *Amoeba dubia*, studied the toxicity of some of these dyes after microinjection. The following dyes which cover a wide range of reduction intensity were employed: (1) Phenolindophenol; (2) toluylene blue chloride;* (3) cresyl blue† (4) methylene blue† (5) cresyl violet; (6) safranine; (7) Janus green; and (8) neutral red.

TABLE I

The Effect of Reversible Dyes on the Oxygen Consumption of Starfish Eggs (Mature, Unfertilized). Dyes Which Penetrate through the Cell Membrane

Name of dye	Oxygen consumption per hour in c.mm.		Number of experiments	Per cent increase
	Before dye addition	After dye addition		
Phenolindophenol	19.2	39.9	12	104
Toluylene blue chloride	32.0	121.6	9	280
Cresyl blue	22.3	82.5	11	270
Methylene blue	23.5	86.7	18	269
Cresyl violet	25.2	66.6	15	165
Safranine	24.3	30.7	14	26
Janus green	23.8	26.2	13	10
Neutral red	25.0	24.5	14	None

A number of experiments were performed with each dye and the results tabulated in Table I represent the averages of the figures obtained. There is always a difference in the catalytic power of the same dye upon different samples of eggs, one cause being the number of cells employed, which alter the concentration of the dye in relation to the individual cells. This difference, however, becomes small when the same quantity of eggs is used. The relation between the E'_0

* We are indebted to Dr. Barnet Cohen for this sample of toluylene blue chloride.

† No claim is made for the purity of the methylene blue from azure and it may be open to further investigation whether the effect of methylene blue is in part to be ascribed to the content of azure.

(the electrode potential difference between the normal hydrogen electrode and an equimolecular mixture of oxidant and reductant at pH 7.00) of the dye at the cellular pH (pH of the protoplasm of mature starfish eggs, 6.8±0.1) and its power to act as a catalyst for cellular oxidations, can be seen in Table II where the E'_0 of the dyes has been tabulated with the corresponding increment produced in the cell oxygen consumption. The statement made previously (1) that

TABLE II

The Effect of Reversible Dyes on the Oxygen Consumption of Mature, Unfertilized Starfish Eggs. Relation between Their Catalytic Power and E'_0

Name of dye	E'_0 at pH 7.00 in volts	Measured by	Increase in the O_2 consumption per cent per hour
Phenolindophenol	+0.229	Cohen, Gibbs and Clark (5)	104
Toluylene blue chloride	+0.115	Phillips, Clark and Cohen (11)	280
Cresyl blue	+0.035	Rapkine, Struyk and Wurmser (12)	270
Methylene blue	+0.011	Clark, Cohen and Gibbs (5)	269
Gallocyanine	+0.013	Measured in Professor Michaelis' Laboratory (not yet published)	46
Indigodisulphonate	−0.125	Sullivan, Cohen and Clark (5)	None
Cresyl violet	−0.165	Rapkine, Struyk and Wurmser (12)	165
Safranine*	−0.255	Vellinger (13)	26
Janus green*	−0.257	Rapkine, Struyk and Wurmser (12)	10
Neutral red*	−0.315	Vellinger (13)	None

* The values for the E'_0 of safranin, Janus green and neutral red, seem subject to caution. We have titrated commercial preparations of Janus green and neutral red and found that both of them were partially irreversibly oxidized; therefore no definite value for the E'_0 could be found. A single experiment with safranin gave identical results. Professor Clark informs us he has had the same results with these dyes.

reversible dyes with an E'_0 close to that of methylene blue are the best catalysts, holds true.

As a result of the fundamental researches of Clark and his coworkers (5), who have laid the foundations upon which to build our knowledge of oxidation-reduction systems of biological interest, many attempts have been made to measure the reduction intensity of living cells.

The reduction potential of the eggs of starfish has been measured by the method of microinjection of reversible dyes by Needham and Needham (6), Rapkine and Wurmser (7) and recently by Chambers, Pollack and Cohen (8). While the first two investigators place the aerobic rH of starfish eggs at 19 to 20, Chambers and coworkers have found it to be approximately 12. We accept the last figure as the reduction potential of the eggs of starfish for they are of the same species we have been employing. As an rH of 12 corresponds to a potential of -0.060 volt when referred to the standard hydrogen electrode, it can be seen that the dyes with optimum catalytic power are those whose E'_0 lies on the positive side of the reduction potential of the cell we are studying. When the E'_0 of the dye is much more positive than the reduction intensity of the cell, the effect of the dye will depend on its concentration. This easily can be observed, using phenolindophenol as catalyst. Accidentally the concentration we employed was just enough to overcome the reduction capacity of the cell and phenolindophenol then acted as a catalyst. But upon diminishing the concentration from 1.253 mM to 0.79 mM, the dye was completely reduced by the starfish eggs, and kept reduced. No catalytic effect was then observed and the oxygen consumption remained at the same rate as before the addition of the dye. The same eggs with the usual concentration of phenolindophenol (1.253 mM) gave an increase of 106 per cent. When the E'_0 of the dye lies on the negative side of the reduction potential of the cell, the catalytic power becomes lower, until with dyes whose E'_0 is -0.200 volt more negative than the cell reduction potential the catalytic effect ceases.

The Effect of the Permeability of the Cell Membrane

The experiments of Wieland and Bertho (9) on the fermentation of alcohol to acetic acid by *Bacterium orleanenses* in the absence of free oxygen due to the dehydrogenating action of dyes, have shown that while benzoquinones, which readily penetrate the cell walls, produce a rapid dehydrogenation, methylene blue, which does not penetrate, acts slowly, as in this case the dehydrogenation takes place only on the outer surface. The same phenomenon can be observed in the catalytic action of dyes on the oxidative processes of starfish eggs in an atmos-

phere of oxygen. Taking into consideration the relation of the E'_0 of the dye to its catalytic power, we arranged the experiments in the following manner: (1) A dye which does not penetrate the cell membrane and with an E'_0 more positive than the reduction potential of the starfish eggs, was compared with a dye with similar E'_0 but which does penetrate. The pairs of experiments were always performed with the same sample of eggs. The effect of gallocyanine was accordingly compared with that of methylene blue (Table III). While the latter increased the oxygen consumption by 260 per cent in these experi-

TABLE III

The Effect of Reversible Dyes on the Oxygen Consumption of Starfish Eggs (Mature, Unfertilized). The Effect of the Membrane Permeability

Name of dye	E'_0 at pH 7.00 in volts	Oxygen consumption per hour in c.mm.		Permeability of membrane	Per cent increase
		Before dye addition	After dye addition		
Gallocyanine*	+0.013	23.1	33.7	Does not penetrate	46
Methylene blue	+0.011	23.3	85.5	Penetrates	267
Indigodisulphonate	−0.125	25.4	24.6	Does not penetrate	None
Cresyl violet	−0.165	25.3	65.8	Penetrates	160

* A detailed investigation on the properties of this reversible dye will be published by Dr. L. Michaelis.

ments, the increase produced by gallocyanine was only 46 per cent. As gallocyanine does not penetrate through the cell surface of the starfish egg, this observed increase must be ascribed to the action of the dye on the oxidative processes taking place at the outer surface of the cell. (2) In a similar manner, experiments were performed with dyes possessing an E'_0 more negative than the reduction potential of starfish eggs, namely, indigodisulphonate and the slightly more negative cresyl violet (Table III). In this latter case, even those oxidative processes taking place at the outer surface of the cell were not activated by the dyes, their effect being negative.

DISCUSSION

1. Relation Between the Catalytic Power of the Dyes and Cellular Metabolism

The ability of reversible dyes to catalyze certain oxidative processes taking place in the living cell seem to us of importance for the understanding of the mechanism of some phases of cellular metabolism.

There are within the cell two kinds of oxidative processes: (1) The respiratory process which we can define with Warburg (10) as the combustion of organic substances through molecular oxygen in the living cells, a process catalyzed by an iron-containing enzyme, which, so far as Warburg's researches show, is the same for a wide variety of cells and probably for all cells. (2) The oxidative processes which require the activation of the organic substances and the presence of a hydrogen acceptor. The former, *i.e.*, the respiratory process, is inhibited by cyanide and CO and is activated by a ferment present in the same form in every cell; the latter is not inhibited by cyanide and is effected by multiple hydrogen acceptors. The long still existing controversy between Warburg's conception of respiration and Wieland's theory of oxidative dehydrogenation has been kept alive because of the different and distinct definitions given to the term respiration. The oxidative processes activated by reversible dyes belong to the second category of oxidations. They have in common with the respiratory process, the consumption of oxygen, the production of carbon dioxide, and the inhibiting effect of strongly adsorbable substances (narcotics), but they differ from respiration in that they are intimately related to the fermentative process. The effect of the dye appears to be proportional to the fermentative power, *i.e.*, the anaerobic glycolysis of the cell, as we will show in a later communication.

The relation of the E'_0 of the dye and its catalytic power to the reduction intensity of the cell is suggestive. Those dyes possessing an E'_0 on the positive side of the reduction potential of the cell are the more active, their activity being diminished or even abolished only when the dye is far beyond the reduction intensity or when the amount of dye used has been lower than the reduction capacity of the cell. The dyes whose E'_0 is on the negative side of the reduction potential of the cell have less effect, and, beyond certain limits, none. If we

plot on the abscissa the E'_0 of the dyes at pH 7.00 (pH of the protoplasm) and on the ordinate the per cent increase of the oxygen consumption of the cell due to the addition of the dye (Fig. 1), we have a plateau-like curve extending from the reduction potential of the starfish eggs, equivalent to -0.06 volt (referred to the standard hydrogen electrode), to $+0.115$ volt. On either side of this plateau the curve

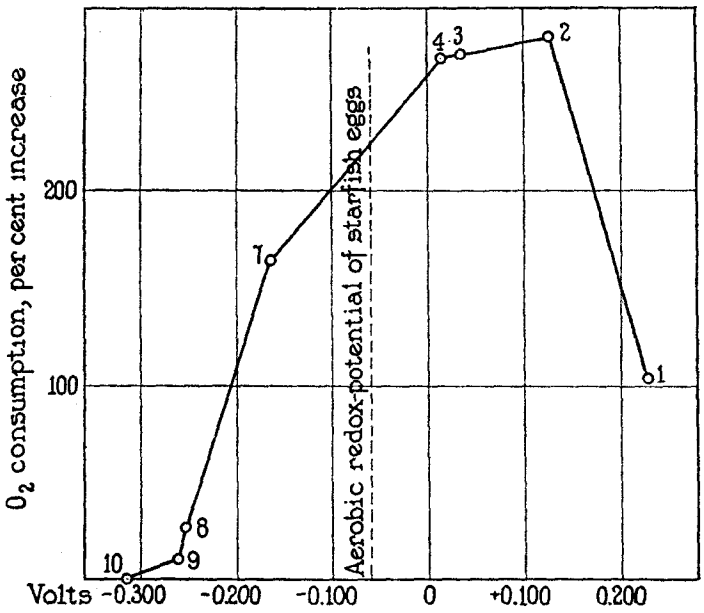

FIG. 1. Relation between the catalytic power of dyes and its E'_0 to the reduction potential (aerobic) of starfish eggs. (Dyes which penetrate through the cell wall.) Abscissa = E'_0 of dyes at pH 7.00. Ordinate = per cent increase of O_2 consumption after dye addition. 1, Phenolindophenol. 2, Toluylene blue chloride. 3, Methylene blue. 4, Cresyl blue. 7, Cresyl violet. 8, Safranine. 9, Janus green. 10, Neutral red.

falls abruptly. It seems possible that the substances responsible for the reduction potential of the cell are those activated by the dyes as catalysts. If the aim of those investigators studying the reduction potentials of cells is to correlate them with certain phases of the cellular metabolism it is indeed natural that we should look for a correlation between the appearance of certain metabolic products and the attain-

ment of certain levels of reduction intensity. It has been shown by one of us and Harrop (2) that one of the substances oxidized by the catalytic effect of reversible dyes is a carbohydrate, as the amount of lactic acid produced by the blood cells when incubated in the presence of methylene blue is less than the normal control. If the carbohydrates were the only substances oxidized by the action of dyes, it would be possible, from the entropies of sugar and lactic acid, to determine the free energy given by this catalysis and compare with the reduction potential of the cell; but the fact that the respiratory quotient of the red blood cells incubated with methylene blue is always less than one ((±) 0.75) shows that other substances besides carbohydrates are also oxidized, which complicates the problem.

II. General Remarks on the Relationship Between the Catalytic Power of a Dye and Its Potential Range

It is justly assumed in thermodynamics that there is in general no necessary relationship between the speed and free energy change of a reaction. Thus oxygen gas, the potential of which is extremely positive as calculated for the oxygen electrode, is in many cases a very sluggish oxidant or no oxidant at all without a catalyst; whereas oxidants of much lower range of potential very often can be used as efficient oxidants. In general, the rate of oxidation of an oxidizable substance does not depend on the potential of the oxidant, but on specific chemical properties and can greatly be varied by catalysts. The action of catalysis, indeed, is a much more important factor for the rate of oxidation than the potential range of the oxidant. From this standpoint it seems difficult to understand how the speed of oxygen consumption in living cells by the action of dyes could be referred to the potential of the dye. This contradiction may find an explanation in the following way. Though, in general, speed and potential have no relationship, yet there are quite definite cases in which such relationship can be recognized.

1. We have first the case described by Conant (14) when he reduces a substance capable of an irreversible reduction (a number of azo-dyes, nitrocompounds and unsaturated 1,4,diketones) by an equimolecular mixture of the reduced and oxidized forms of a reversible reductant. No equilibrium between these two substances can be established.

What happens is that the substance in question will be reduced first with a certain velocity, then this velocity will gradually slow down and finally become so slow that we may speak practically of a quasi equilibrium. This is not a thermodynamic state of equilibrium, but it is a state in which the reaction velocity is almost zero. Conant has found that the reduction in an homogenous solution of such compounds is a function of the potential of the reducing agent. He designates that potential which just produces a measurable speed of reaction, as "the apparent reduction potential" of the substance being investigated.

2. A second example is found in Voegtlin, Johnson and Dyer's experiments (16) on the reducing power of normal and cancer tissue. These authors observed that under standard conditions the reduction time of the various oxidation-reduction indicators decreases with an increase in the electrode potential of the indicators; and the indicators, if tested by the biological method, arrange themselves in the same order as that obtained by means of the purely physical electrode measurements. From their experiments they conclude that "the reduction time is approximately a logarithmic function of the electrode potential."

3. A third example is the following: The speed of oxidation of a leuco-dye by molecular oxygen seems to depend on the potential range of the dye. There are scarcely accurate data available for this assertion, and the velocity of oxidation depends in this case also on other factors. For instance, Clark found (15) that the speed of oxidation of methylene white by oxygen is approximately proportional to the fifth root of the hydroxyl ion concentration, but, though other accurate data are not known, there is one striking fact which can be used in this connection. The leuco-dyes in a solution of approximately the same pH are oxidized by molecular oxygen at a very different rate, which quite obviously depends on the potential range of the dye. Reduced indophenol is rather slowly oxidized in the air, and in a gas mixture of very low oxygen pressure is even re-oxidized very sluggishly. Methylene white is oxidized at reduced oxygen pressure quicker than indophenol indeed, but only gradually too. Reduced safranine is reduced even at low oxygen pressures with great speed. It is very sensitive indeed for traces of oxygen, so it seems likely that the speed

of oxidation by molecular oxygen is related to the potential range of the dye.

In these considerations there is involved an explanation for the different catalytic effects of the various dyes on cellular oxidations. When a dye is added to a suspension of cells, the dye will be reduced by the cell with a definite velocity, and the reduced dye will be oxidized by the air with a different velocity, also depending on the conditions. The catalytic power of the dye depends on these two velocities. If the velocity of the oxygen consumption brought about by the dye as a catalyst is greater than the one produced by the natural catalyst of the cell, then the dye will increase the oxygen consumption. In the case of indophenol the speed at which the dye is reduced by the cell is very great; but the speed at which the reduced indophenol is reoxidized by air is very slow, so slow indeed that indophenol does not increase the oxygen consumption unless in high concentrations. In the case of methylene blue the dye is easily reduced by the cell, and the reduced dye is easily oxidized by the air, a fact which makes methylene blue a good catalyst for cellular oxidations. On the other hand the reduction of safranin by the cell is extremely slow though its reoxidation by air is very rapid. Therefore, the dye will not act as catalyst. This seems a satisfactory explanation for the fact that the potential range of a reversible dye has a recognizable relationship to its ability to work as an oxidation enzyme.

SUMMARY

From the experiments described in this paper and in those previously published it can be concluded that dyes which can be reversibly oxidized and reduced, act as catalysts for some oxidative processes taking place in the living cells, as is manifested by an increase in their oxygen consumption.

It has been found that the catalytic power of the dyes on the oxygen consumption of starfish eggs (mature, unfertilized) is conditioned by two factors: the reduction potential of the dye and the permeability of the cell surface. Dyes whose E'_0 is towards the positive side of the aerobic reduction potential of the starfish eggs have a maximum catalytic effect. This catalytic power decreases as the E'_0 becomes more negative than the reduction potential of the cell and becomes

nil beyond certain limits. When a dye cannot penetrate into the cell, its effect is greatly diminished as in this case only those oxidative processes taking place at the outer surface of the cell can be activated.

Whether a dye can act as a catalyst or not is dependent on whether the normal consumption of oxygen by the cell is slower or quicker than the oxidation activated by the dye. The speed of this activation is correlated to (1) the speed at which the dye is reduced by the cell, and (2) the speed at which the leuco-dye is oxidized by the atmospheric oxygen. If one of these two processes is slower than the normal respiration, the dye cannot increase the rate of oxygen consumption (phenol indophenol at low concentrations which is kept reduced by the cell is very slowly reoxidized by atmospheric oxygen, on the other hand safranin and neutral red which are not reduced by the cell or at least too slowly reduced, though rapidly reoxidized by air). It will depend on these two reactions velocities whether a dye will act as catalyst (methylene blue and dyes with similar E'_0 which are quickly reduced by the cell and the leuco-dyes of which are relatively quickly reoxidized). Though this relationship between the reduction potential of the dyes and its catalytic power would seem in contradiction with the well known thermodynamic assumption that there is in general no distinct relationship between the potential and velocity of the reaction, we have pointed out from the literature some of the various experiments where one does recognize this connection.

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