THE RATE OF REDUCTION OF METHYLENE BLUE BY BACILLUS COLI.

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It has recently been shown (1) that the velocity (v) of the hydrolysis of starch by the amylase of germinated barley varies with the concentration (c) of the starch according to the equation

$$v = a + b \log c$$

where a and b are constants. A similar relationship has been described (2) for the reduction of methylene blue by "resting" *B. coli* using succinic acid as substrate. As these are the only instances described of this relationship it seemed of interest to compare them more closely, and for this purpose it was necessary to elaborate the data for the latter case.

Methods.

The organism used was "resting" *B. coli* prepared as described by Quastel and Wooldridge (3); *i.e.*, it was grown on tryptic broth, centrifuged and washed with 0.95 per cent sodium chloride several times, and a current of air was passed through the suspension for several hours. It was kept at 0° until diluted and used.

The buffers were phosphate (Clark and Lubs) and the pH value was checked electrometrically. Succinic acid was brought to the required pH by the addition of sodium hydroxide before being used.

The various reagents were mixed in several tubes of the kind described by Thunberg (4), and finally the suspension of bacilli was added. After putting in the stoppers all the tubes were connected to the same pump and evacuation was begun as quickly as possible. After thorough evacuation the tubes were filled with nitrogen and the evacuation repeated. They were then placed in the water bath at 45° as nearly at the same time as possible. Before this plan of simultaneous evacuation was adopted, great difficulty was experienced in obtaining results of any regularity, since the degree of evacuation varied, *e.g.* with the water pressure in the pump, and even minute traces of oxygen affect the reduction times very markedly. It may be noted that the amount of methylene blue used in a

tube was only 2×10^{-5} gm. An experiment was done to show the effect of incomplete evacuation and will be given later. In order to minimize the danger of leakage etc., the suspension of the organism was adjusted so that the reduction was complete within 2 hours.

As shown by Quastel and Whetham (5), the rate of decolorisation of the methylene blue is linear until approximately 95 per cent of the methylene blue is reduced, and accordingly a standard was used containing 0.05 or 0.10 cc. of the methylene blue solution with the same amount of bacterial emulsion and saline made up to volume. This of course was not evacuated. Consequently the time for 90 or 95 per cent reduction was used as a measure of the rate.

Even with these precautions there remain numerous sources of error, and control experiments show that differences in similar tubes are often 5 per cent, but seldom exceed 10 per cent.

TABLE I.

Succinate concentration	Reduction time	
	Found	Calculated
M/120	9′ 30″	8′ 33″
M/240	10′	11′ 5″
M/360	12′ 55″	12′ 49″
M/480	14′ 10″	14′ 20″
M/600	16′ 20″	15′ 58″
M/720	17′ 20″	17′ 32″
M/840	20′ 20″	19′

Succinic Acid.

As Quastel and Whetham discovered this relationship with succinic acid, I first repeated their experiments. A typical result is given in Table I.

The calculations in Table I are made from the equation

$$v = 25.74 - 7.01 \log c.$$

Since the agreement is quite satisfactory, and since in none of the curves obtained is there any definite trend of the divergences between found and calculated values, it may be concluded that equations of this type accurately describe the relations found over this range of concentrations.

An interesting part of the amylase curve is with low concentrations of starch where zero velocity is obtained. Unfortunately I was unable to compare the two curves in this region, because in the case of *B. coli* the danger of slow leakages, etc., render prolonged experiments unsatisfactory; and although I have obtained with low concentrations of succinate results that appeared to indicate that the time for reduction was becoming infinitely long and the velocity therefore zero, I have no confidence that I have completely excluded the possibility of serious errors.

A note may be inserted here on the effect of incomplete evacuation. Four identical sets, each of three tubes, were prepared, in which the final succinate concentrations were M/100, M/300 and M/500. Each succeeding set was evacuated more thoroughly than the preceding. The results are given in Fig. 1 where the velocity ($= 100/t$ where t is the time in minutes for 95 per cent reduction) is

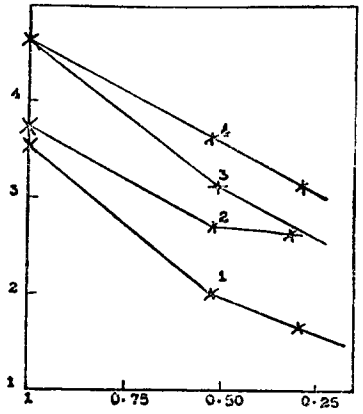

FIG. 1. The effect of incomplete evacuation. Curve 1 belongs to the least evacuated and so in order to No. 4 the most thoroughly evacuated.

plotted against the logarithm of the succinate concentration. It is obvious that with more thorough evacuation there is a decrease in reduction time and an approach to regularity. The slope of the curve also changes a good deal, and considerable caution must be used in drawing deductions from alterations in the slope of the curve.

Glucose.

To test the limits of validity of this relation another substrate of different chemical character was now substituted for succinic acid. Results with glucose are given in Fig. 2. The smooth curve is drawn from the equation

$$v = 22.8 - 4.83 \log c.$$

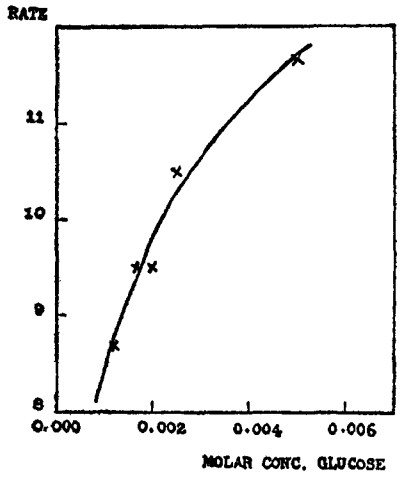

FIG. 2. The reduction velocity of glucose at moderate concentrations. x = experimental points. The smooth curve is drawn from the equation.

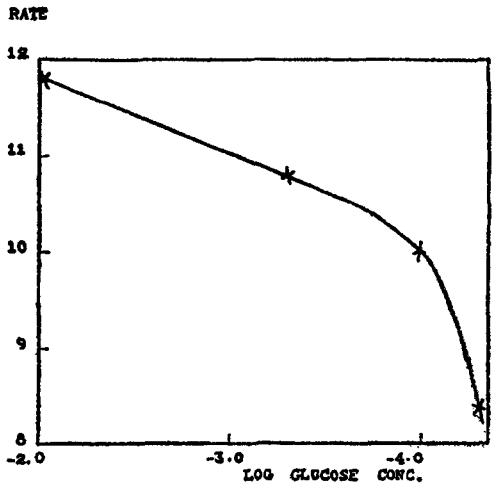

FIG. 3. The reduction velocity of glucose at low concentrations, showing how the velocity falls off more rapidly than is predicted by the equation. Values fitting the equation form a straight line as on the left of the diagram.

and it is obvious that this correctly describes the relations over this range of concentrations. However with concentrations less than this there is a rapid decrease in velocity, until one reaches concentrations at which the amount of glucose is less than sufficient for the reduction of the methylene blue present. This is illustrated in Fig. 3. The logarithm of glucose concentration is used so that deviations from the logarithmic relation will show as deviations from a straight line. The cause of this decrease in velocity with low concentrations may possibly be a difference in the rate of transference of the different hydrogens of glucose. The number transferred, however, is (as might be expected) independent of the concentration of methylene blue.

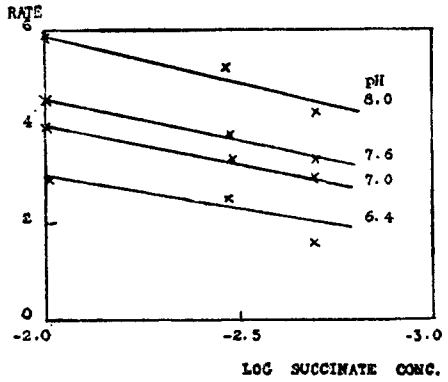

FIG. 4. The effect of pH on the relation between substrate concentration and reduction velocity.

It may be noted incidentally that here we have complete reduction of 1 cc. of 1:5,000 methylene blue in the presence of 5 cc. of M/40,000 glucose; this means that each molecule of glucose gives up almost exactly 5 atoms of hydrogen to the methylene blue. Since it is highly improbable that the number of hydrogens donated is odd, we assume that one molecule of glucose donates six hydrogens, one being transferred to the oxygen still remaining. This would require 0.0007 cc. of oxygen. Quastel and Whetham have demonstrated that four or more atoms were donated and considered that the number is probably six.

Effect of Hydrogen Ion Concentration.

Differences between the amylase system and the *B. coli* system are clearly shown when one varies the pH. Results from one experiment are shown in Fig. 4. Four series of three tubes were prepared, the pH of the different series being 6.4, 7.0, 7.6 and 8.0, and the succinate concentrations of the different tubes of each series being M/100, M/300 and M/500. In plotting, the logarithms of these concentrations were used, and the result is four parallel lines. In other words, the effect, within these limits, of making the solution more alkaline is to increase the rate of reduction; and this increase, being proportionately the

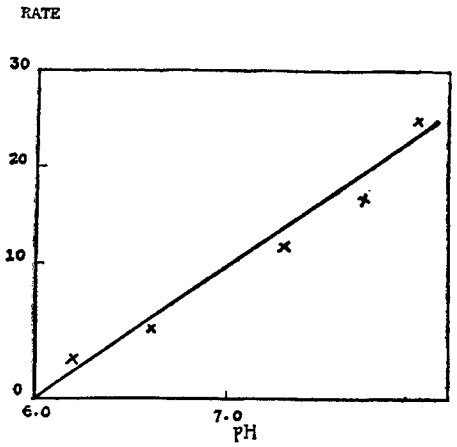

FIG. 5. The effect of pH on reduction velocity.

same, is independent of the concentration of the substrate. With barley amylase the effect on the rate is relatively more marked at lower concentrations, and the results when plotted in this way show lines which are not parallel.

Quastel and Whetham (5) found that when the reduction time was plotted against pH, a curve resembling a rectangular hyperbola was obtained. I have used a much narrower range of pH than they did. With glucose as donator there was little or no difference in rate between pH 6.3 and 7.7; on the acid side of this the rate falls off. The results with succinic acid are shown in Fig. 5. Here velocity is directly proportionate to the pH value.

In attempting to analyse this relationship, we may consider the succinate, the methylene blue and the organism. Alteration of pH over this range has no effect on the succinate except to increase slightly its degree of ionisation, a change too slight to produce these results.

Clark, Cohen and Gibbs (6) have shown that the E_h for 50 per cent reduction of methylene blue alters in a linear way with the pH over this range, the former going from positive to negative with increasing alkalinity. Now, if (Cannan, Cohen and Clark, (7)) methylene blue and similar dyes adjust practically instantaneously to induced levels of oxidation-reduction potential, and the lag in reduction in experiments such as these is due to the fact that the cell system gradually develops greater negative potential, then we should expect, others things being equal, that the reduction will occur first in those solutions in which the E_h for 50 per cent reduction of the methylene blue is least negative. In other words the least alkaline solutions will become reduced first. The exact opposite, however, is the case, and we must conclude that the effect of the alteration in pH is on the organism and its enzymes, and that the effect on the methylene blue is completely masked. The only alternative to this is to suppose that under these conditions the behaviour of cell suspensions differs from that predicted by W. M. Clark and his coworkers. In order to test this point I used their series of indicators. (I am indebted to Dr. M. Dixon for these dyes. I understand that they were sent to Dr. J. Needham by Dr. W. M. Clark.) The concentrations of dye solutions were equivalent to the concentration of the methylene blue used in previous experiments. The substrate used was succinate. Table II gives the results of three experiments. The concentration of the *B. coli* suspension and succinate was different in each of them.

These experiments show (*a*) that Nos. 1, 2 and 3 are not reduced; (*b*) that No. 7 is the fastest, being markedly faster than No. 8 (this dye contains no halogen nor sulfonic acid group); (*c*) that methylene blue (No. 3*a*) in Experiment *a* occupies a place close to No. 8, in Experiment *b* close to No. 7, while in Experiment *c* it is slower than No. 6. Thus not only does it not occupy its proper place in the series but its position is variable. Apparently when the rate of

reduction of the whole series is faster, the rate for methylene blue is relatively faster still.

The anomalous behaviour of Nos. 3*a* and 7 thus seems to indicate that the reduction of the dye is not entirely independent of the reducing system or the substance acting as hydrogen donator, as Clark appears to think. On this point see Dixon (8). The variable position of methylene blue may perhaps be accounted for by attributing to it a slight toxic action. It should be noted that this would be more pronounced in experiments of longer duration and it is in these that the reduction of methylene blue is relatively slowest. This, however, will not explain its very rapid reduction in some cases.

TABLE II.

Indicator	Rate of reduction (= 100/*t*)		
	Experiment *a*	Experiment *b*	Experiment *c*
1. Indigotin disulphonate	0	0	—
2. Indigotin trisulphonate	0	0	—
3. Indigotin tetrasulphonate	0	0	—
3a. Methylene blue	5.3	30.7	Less than 0.5
4. 1-naphthol-2-sulphonate-2-6-dichloroindophenol	0.5	1.0	—
5. 1-naphthol-2-sulphonate indophenol	1.4	3.0	—
6. o-cresol, 2-6-dichloroindophenol	4.0	9.2	1.0
7. o-cresol indophenol	14.3	50.0	4.4
8. 2-6-dibromophenol indophenol	5.0	11.1	1.2

Effect of Temperature.

This was studied with succinate as substrate. A typical result is as follows.

Succinate concentration	Q_{10} (37° – 47°)
M/20	2.3
M/120	1.8

The slight falling off of the Q_{10} with lower concentration (where the duration of the experiment was necessarily longer) suggests a slight injurious effect of the higher temperature on the organism. There is here again a striking difference from the amylase-starch system.

DISCUSSION.

Summing up the comparison of this system with that of barley amylase and starch, we find that, within limits, the same relation between substrate concentration and velocity holds for both of them. Beyond this differences appear; and the effects of alteration in pH and temperature on this relation are quite different in the two cases.

There still remains the question of the significance of the equation relating velocity to substrate concentration. If we assume that the mechanism of the reaction is essentially a combination of the substrate with its enzyme, and that this combination decomposes to give the products of the reaction, we may regard the velocity as proportional to the amount of this combination existing in the mixture. This combination may be chemical (*cf.*, *e.g.*, invertase (9), liver amylase (10)), or physical, *i.e.* of the nature of adsorption. If chemical, it will be governed by the mass law, and we should expect the relation between velocity and substrate concentration to be of the type described by Michaelis and Menten (9). Since it very obviously does not conform to this, we may consider the possibility of adsorption. Several equations have been proposed to describe the relation of the amount of a substance adsorbed to its concentration in solution, such as those of Freundlich, Arrhenius, Langmuir; but as Garner (11) points out all these equations have only a limited range of validity. Some of them, *e.g.* that of Freundlich, as pointed out by Arrhenius (12) have no theoretical basis. Arrhenius in the same paper deduced an equation from a characteristic property of adsorption, *viz.*, that the amount of substance adsorbed reaches a maximum value. If we suppose that this limit is reached asymptotically we may deduce an equation giving the same general sort of curve. Let us suppose, then, that on increasing the amount of substance in solution the amount adsorbed (x) shows gradually smaller increases as the concentration in solution (c) rises. This may be written as a differential equation:

$$\frac{dx}{dc} = \frac{p}{c}$$

p being a constant. On integrating this we obtain

$$x = p \cdot \log c + q$$

where q is the constant of integration. This, of course, is identical with the equation I found empirically. The significance of the equation therefore appears to be that there is a gradual approach to a saturated condition. The constant q indicates that there is no adsorption ($x = 0$) until the concentration reaches a definite value ($c = e^{-q/p}$). In other words there is a sudden break in the curve at this point. In this connection it is of interest to note that Garner (11) finds breaks in the adsorption isotherms of certain alcohols.

The equation therefore probably means that adsorption plays an essential part in the reaction.

SUMMARY.

This paper deals with the relation between substrate concentration and velocity in the case of the reduction of methylene blue and of the other oxidation-reduction indicators of Clark by *B. coli* in the presence of succinic acid and glucose. This system is compared with starch and barley amylase. Reasons are given for considering the mechanism as an adsorption phenomenon.

It is a pleasure to acknowledge my indebtedness to Prof. Sir F. G. Hopkins and to Dr. J. H. Quastel.

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