LXII. THE EFFECT OF HYDROGEN ION CONCENTRATION ON SOME OXIDATIONS BY *B. COLI COMMUNIS*.

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THE effect of treatment with toluene on the aerobic and anaerobic oxidations of various substrates has been noted in a previous communication [Cook, 1930]. The present paper is the outcome of experiments which were undertaken to investigate the most favourable conditions of hydrogen ion concentration for the aerobic and anaerobic oxidations of formate, succinate and lactate. Apart from the immediate practical value of these experiments, the results are of interest in relation to the kinetics of the action of oxidative enzymes and the interaction of the dehydrogenases and oxygenases in the bacterial cell.

The p_H -activity curves of the oxidases have not been studied in such great detail as those of the hydrolytic enzymes. For a summary of work on the latter Haldane's monograph [1930] should be consulted. Ohlsson [1921] made a very careful study of extracted muscle succinoxidase, the results of which in relation to the present work will be discussed later. Quastel and Whetham [1924] investigated the effect of p_H on the dehydrogenation of succinate by normal *B. coli*. Dixon and Thurlow [1924] and Bernheim [1928] studied the p_H -activities of xanthine oxidase and the potato aldehyde oxidase respectively. In all the above instances, with the exception of one experiment by Bernheim who used nitrate, methylene blue was used as the hydrogen acceptor. There appears to be little work on the oxidases in which oxygen either activated or not is involved. Hare [1928] made an interesting study of the p_H -activity curve of tyramine oxidase in which unactivated oxygen is involved. Cook and Stephenson [1928] studied the effect of p_H on the oxidation of lactate by activated oxygen by normal *B. coli*. References to peroxidase, catalase and some other oxidases are given in Haldane's monograph.

It had been noted earlier [Cook, 1930] that above a temperature of about 30° the rate of oxygen reduction appeared to be the limiting factor in the aerobic oxidation of formate and lactate. The aerobic oxidation of these substrates was therefore studied at 16° and 40°. The anaerobic experiments in which the reduction of methylene blue in the Thunberg tube was observed and which are a measure solely of the dehydrogenases were carried out at 40°.

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From the earlier results it was shown that the reaction velocity increased in a linear manner with increase of temperature until destruction of the enzyme occurred at a temperature of about 50°. Some experiments carried out at a lower temperature showed that the shape of the p_H -activity curves remained substantially the same as that of those at 40°.

EXPERIMENTAL.

The bacterial suspension used for these experiments was derived from a strain of *B. coli communis* Escherich obtained from the National Collection of Type Cultures. Some experiments were carried out on a strain obtained from the Pathology Department, Cambridge, but apart from slight differences in the velocities of respiration no significant differences were noted in the shapes of the curves. The method by which the stock preparation is prepared has been described previously [Cook, 1930]. This preparation retains its activity for several weeks if kept in the refrigerator, but if left in the open autolysis sets in and it rapidly deteriorates. The effect is most marked on the reduction of oxygen. The reduction of methylene blue especially by formate and lactate shows only a slight falling off in activity. From the stock preparation of bacteria the toluene-treated organism is prepared by making a suitable dilution so that a convenient velocity may be obtained. The dilution is best made with frog Ringer's solution containing 0.65 % NaCl, 0.014 % KCl and 0.012 % CaCl_2 . No NaHCO_3 is added as it would cause an increase in p_H due to loss of CO_2 . It has been found that, while preparations in which the dilution has been made with water are apt to show some falling off in activity after several hours, those made up with Ringer's solution keep well even at room temperature for 6–8 hours, which is well within the time necessary for one series of experiments to be made. The preparation is kept on ice during the course of the experiments. With regard to the action of the Ringer's solution it is necessary to note that the effect of the added salts on the shape of the curves is very slight indeed. The actual amount of salts present in the aerobic experiments is a third of those present in normal Ringer's solution and in the anaerobic experiments at most a tenth. If a comparison is made of the p_H -activity curve of the methylene blue reduction in the presence of well-washed bacteria in distilled water and bacteria in the presence of a concentration of salts corresponding to Ringer's solution, the velocity is reduced by about 10 % in the latter case, but the curve follows that obtained for the washed preparation. The aerobic oxidations show a very slight difference between the initial rates of respiration, but a difference is noted in the activity after several hours. The activity of the washed preparation has fallen by about 15–20 % after 6 hours. This difference is not nearly so marked with the reduction of methylene blue, so that some alteration in the oxygenase is possibly indicated. The study of the oxidation of succinate is complicated by the fact that after two or three days the organism loses its capacity for the aerobic oxidation at 16°. Fresh preparations of bacteria oxidise succinate at

this temperature and the oxidation at 40° and the methylene blue reduction are unaffected. The technique followed for the aerobic and anaerobic experiments is essentially the same as that described previously.

The buffers used were the Clark-Lubs series of potassium hydrogen phthalate-NaOH, KH_2PO_4 -KOH and boric acid-KCl, NaOH. A borax- KH_2PO_4 buffer (Kolthoff) was also employed. This buffer is to be highly recommended as it may be used to check any effects of the phosphate or borate ions *per se* over a considerable portion of their range. The p_H of the reaction mixtures was checked electrometrically at the beginning and end of numerous experiments. The p_H of the undiluted bacterial suspension was in general about 7.4. The bacteria if present in large amounts have a considerable buffering capacity but, as they are used in relatively small amounts in the actual experiments and the solutions of buffer added were always present in a concentration of at least $M/120$, the p_H of the mixture remained practically that of the buffer solutions. The substrate concentration and the total nitrogen of the bacteria present are given in the separate experiments. The substrate concentration taken was always very much greater than that necessary for saturation of the enzyme. The total nitrogen of the bacteria may be used as a measure of the dry weight of bacteria present, as noted previously.

As a test of the aerobic method the following experiment was undertaken. A preparation of bacteria was made by dilution of the stock with Ringer's solution and treated with toluene for exactly half an hour. This time was made the standard time for treatment. The procedure followed was to shake the suspension well with toluene at the beginning of the treatment and to shake at intervals of 5 minutes. The treatment was always made at room temperature. The lower layer of bacteria was then pipetted free from the toluene layer. A small amount of toluene is always present in the material used for experiment and although this does not seem to affect the anaerobic experiments there is a possibility that the presence of the toluene would affect the manometric readings. The same amount of bacterial suspension was therefore placed in both cups of the Barcroft compensating manometer. The suspension thus prepared was diluted to various extents, and the oxidation of lactate at p_H 7.6 and 40° was studied. After 4 hours the stock suspension of the treated organism was again diluted and the experiments repeated. The results are shown on Fig. 1. It will be seen that the velocity of the oxidation is almost proportional to the amount of bacteria. It will also be noted that there is no significant difference between the results obtained half an hour and 4 hours after treatment with toluene.

Anaerobic experiments.

The reduction of methylene blue at 40° in the Thunberg tube in the presence of a standard concentration of substrate and a known amount of bacteria was taken to a standard of 90 % reduction of the methylene blue present. The concentration of this dye used throughout was 1/30,000 for each experiment.

The toluene-treated organism does not reduce methylene blue in the absence of substrate even after several days. A standard consisting of the same amount of bacteria used in the experiments and an amount of dye corresponding to 90 % reduction may be preserved for some time. Fig. 2 represents the oxidation of formate by methylene blue at 40°. The buffer solutions used for obtaining various parts of the curve are indicated on the figure. It will be seen that the curve from p_H 4 to 7 rises steadily. There is an optimum at about

Fig. 1. The aerobic oxidation of *M/60* lactate at p_H 7.6 and 40°.

Ordinate: oxygen uptake in initial 30 minutes.

Abscissa: dry weight of bacteria in mg.; obtained by taking the total nitrogen as representing 8.3 % of the dry weight of the bacteria.

Values shown by dots represent the velocities half an hour after treatment and those shown by crosses 4 hours after treatment.

⊙-⊙-⊙ Phthalate. ×-×-× Phosphate. ⊗-⊗-⊗ Borax-Phosphate. △-△-△ Borate.

Fig. 2. The anaerobic oxidation of *M/120* formate by toluene-treated *B. coli* in the presence of 1/30,000 methylene blue at 40°.

Ordinate: velocity, this being the reciprocal of the reduction time for 90 % reduction of the methylene blue $\times 100$.

Abscissa: p_H .

Bacterial nitrogen present for each experiment 0.192 mg.

p_H 7 after which the curve falls slightly, rising again though not to the same level from p_H 7.8 to 9.0. It will also be noted that the velocity in borate buffer at p_H 7.8 is considerably less than in phosphate at this point. In the more alkaline region the curve falls rapidly owing to irreversible inactivation of the enzyme as will be shown later. Fig. 3 shows the anaerobic oxidation of lactate, and it will be seen that the curve is similar in shape to the formate curve, but no peak is to be noted. Fig. 4 represents the anaerobic oxidation of succinate. The curve is of the same shape as that obtained for lactate but

the maximum is not reached until a p_H of 8.6, and the plateau is only of short duration.

The effect of the borate buffer at the same p_H as the phosphate buffer is to be noted, though in a different degree, with all three dehydrogenases. It was at first thought that the difference in velocity at the point where the p_H was the same might be explained by (a) an inhibiting effect of the borate ion or (b) an accelerating effect of the phosphate ion. But this latter effect is

Fig. 3. The oxidation of *M*/120 lactate at 40°. Ordinate and abscissa as for Fig. 2. Bacterial nitrogen 0.48 mg.

Fig. 4. The oxidation of *M*/120 succinate at 40°. Bacterial nitrogen 0.96 mg.

rendered unlikely in that no appreciable difference is noted at the change from phosphate to phthalate buffers. Experiments carried out with a borax-phosphate buffer gave a velocity equal to that obtained for the phosphate buffer. The effect of the borate buffer is not due to the presence of the KCl. Experiments carried out with diluted borate buffer and phosphate buffer to which KCl was added did not show any alteration in velocity. It will be noted that the curve obtained with borate rises to give approximately the same

velocity as that for borax-phosphate buffer. The effect of the borate buffer is always most marked at p_H 7.8–8.0. It would perhaps seem that the decrease in velocity was due to an effect of the undissociated boric acid. The p_K of boric acid is 9.24 so that at p_H 7.8 the acid is only slightly dissociated.

Aerobic oxidations.

The toluene-treated organism has no endogenous oxidation, so that the results obtained represent oxidation of the substrates only. The method employed has been described previously. The Barcroft manometers when set up were swung open to the air for exactly 5 minutes. The taps were then shut and the initial readings taken. Readings were then taken at 15-minute intervals. The velocity was obtained from the difference in the readings after 15 and 30 minutes. A reading at 45 minutes served to show whether the velocity was still constant. At the more acid and alkaline p_H values the velocity fell off indicating enzyme inactivation. At values ranging from p_H 5 to 9 there was no appreciable diminution. The p_H was checked electrometrically at the beginning and end of the experiments. There is no appreciable alteration in the oxidation of lactate and succinate, but with formate there is a change of about 0.2 of a unit with the concentration of buffer used. This change is due to the fact that the formate is oxidised to bicarbonate. This dissociates at p_H 7.4, the CO_2 being taken up by the KOH in the manometer. Thus the effect on p_H of the oxidation of formate is the same as that of adding an equivalent of NaOH. The actual change in p_H during the course of the experiment is probably not more than 0.1 of a unit. The extra difference was probably due to the fact that further oxidation had occurred during the interval between removing the solution from the manometer pot and measuring the p_H . At the time when the velocity was measured, oxidation of 50 % at most of the formate had taken place. This would raise the p_H of $M/20$ buffer solution by about 0.1 of a unit. In general this figure has been added to the initial p_H reading of the aerobic formate experiments.

Figs. 5, 6 and 7 represent the aerobic oxidations of formate, lactate and succinate respectively. It will be seen that the curves obtained for formate and lactate at 16° and 40° show little difference in shape with increase of temperature. It will also be noted that there is practically no difference between the velocities of oxidation where the change is made from phosphate to borate buffer. A similar result was found by Cook and Stephenson [1928] for the aerobic oxidation of lactate by the untreated organism.

Reversibility of the effect of acid and alkaline p_H on the aerobic and anaerobic systems.

It is necessary before any attempt can be made to interpret the nature of the curves to observe the action of the more acid and alkaline buffers on the reactions. The curves show that the velocity is considerably diminished at p_H 3 and 10. The procedure adopted was as follows: 50 cc. of the toluene-

○-○-○ Phthalate. ×-×-× Phosphate. △-△-△ Borate.

Fig. 5. The aerobic oxidation of *M/120* formate in atmospheric oxygen at 16° and 40°.

Ordinate: velocity, being difference between oxygen uptakes in the readings at 15 and 30 minutes.

Abscissa: p_H .

Bacterial nitrogen for each experiment at 40° 0.6 mg.; at 16° 1.2 mg.

Fig. 6. The oxidation of *M/120* lactate at 16° and 40°.

Ordinate and abscissa as for Fig. 5. Bacterial nitrogen at both temperatures 1.2 mg.

Fig. 7. The oxidation of *M/120* succinate at 40°.

Bacterial nitrogen 1.2 mg.

treated organism was prepared and divided into 10 cc. samples. To *A* were added 20 cc. of Ringer's solution; to *B* 20 cc. of phthalate buffer at p_H 3.2, the resultant p_H being 3.38; to *C* were added 20 cc. of phosphate buffer at p_H 7.2; to *D* 20 cc. of borate buffer at p_H 10, the resultant p_H being 9.7; *E* the residual 10 cc. served as a second control and was kept on ice. The suspensions were well mixed and allowed to stand at 18° for 1½ hours. The mixtures were then centrifuged and each sample made up to the original volume of 10 cc. The aerobic oxidation of *M*/120 formate at p_H 6.3, and of *M*/120 lactate and succinate at p_H 7.6 and 40° was then investigated. The methylene blue reductions were carried out with the same concentrations of substrates, but at p_H 7.6 in each case. The results are shown in Table I. In these experi-

Table I. *Percentage of original velocity remaining after exposure of the bacteria to the p_H shown for 1½ hours at 18°.*

Methylene blue reductions: p_H 7.6.

Oxygen reduction: succinate and lactate, p_H 7.6; formate, p_H 6.3.

Temperature 40°.

	Bacteria exposed to							
	<i>A</i>		<i>B</i>		<i>C</i>		<i>D</i>	
	Ringer		p_H 3.38		p_H 7.2		p_H 9.7	
	O ₂	M.b.	O ₂	M.b.	O ₂	M.b.	O ₂	M.b.
Formate	100	100	12.2*	56.5	72.7	100	36	80.5
Succinate	"	"	7.0	<3.0	86.3	95.7	58.6	48.3
Lactate	"	"	23.6	"	109	88.1	61.6	74

* Initial velocity about 60 % of original velocity.

ments a reduction of less than 15 % is not to be considered significant, as it is somewhat difficult to ensure the presence of equal amounts of bacteria in the several experiments. The results show that exposure to an acid p_H diminishes considerably the activity of lactic and succinic dehydrogenases, formic still retaining rather more than half its activity. At p_H 7 none of the dehydrogenases is appreciably affected, but at p_H 9.7 the dehydrogenases are affected in the order of activity succinic, lactic and formic. These results agree with those obtained by Quastel and Wooldridge [1927] for the untreated organism. In the aerobic experiments the effect of the acid p_H is most marked with succinate, this being followed by formate and lactate. At p_H 7.2 the oxidation of lactate and succinate is not appreciably affected, but that of formate is decreased by 23 %, while at a more alkaline p_H the effect is in the order formate, succinate, and lactate.

DISCUSSION.

The results obtained in the aerobic oxidations are more complicated in that they involve a second factor in the activation of the oxygen. An interpretation must first be given to the anaerobic oxidations in which it is clear that dehydrogenation of the substrate is the main factor concerned. A glance

at Figs. 2, 3 and 4 will show that the p_H -activity curves of the dehydrogenases are of quite a different type from those obtained with hydrolytic enzymes.

Michaelis and Davidsohn [1911] suggested from the results obtained on the p_H -activity curve of invertase that the enzyme was an amphoteric electrolyte. This conception has been extended by Woolf [1931] who has based his conclusions on the results obtained with fumarase [Mann and Woolf, 1930]. Woolf points out that the symmetrical p_H -activity curve obtained for hydrating enzymes is not given by dehydrogenases, and suggests that the curves represent the action of the enzyme as an ionised acid or an un-ionised base. The curves shown in Figs. 2, 3 and 4 therefore represent the dissociation curve of an acid or the dissociation residue curve of a base. But the curves obtained, especially those in Fig. 2, show deviations from this ideal form. A further study of the formate oxidation is in progress. The curves obtained for lactate and succinate are of the same type as that obtained for formate, although the point at which the curve reaches the plateau is different with the different substrates (Table II).

Table II.

	Formate	Lactate	Succinate
M.b. plateau starts (p_H)	7.0	7.4	8.5
O ₂ optimum (p_H)	6.0	6.3	7.6
p_K of acid	3.68	3.86	5.28

The figures obtained for the maximum velocity in oxygen will be referred to later. It will be seen from Table II that there appears to be some relation between the degree of dissociation of the acid concerned and the activity of the dehydrogenase. The p_K of succinic acid has been taken as that of the second ionisation. It may be postulated tentatively that the dehydrogenases are acting as un-ionised bases, the activity being dependent on combination with the fully dissociated acid. This active enzyme-substrate complex then breaks down, the hydrogen of the substrate being transferred to the methylene blue.

The curve obtained for succinate dehydrogenation is similar to that obtained by Ohlsson for extracted muscle succinoxidase. This writer also observed the inhibitory effect of the borate buffer, but continued his experiments in glycine-phosphate buffer. This buffer is not satisfactory for work with bacteria, as deamination may occur. The curve obtained by Quastel and Whetham for resting untreated *B. coli* rises to an optimum at about p_H 7.2 and continues as a plateau to p_H 10. No inhibitory effect is noted with borate buffer. These results may be explained by the fact that the p_H -activity curve of the toluene-treated organism resembles more closely that of an extracted enzyme. It is perhaps significant that the velocity of dehydrogenation is increased by about 30 % after toluene-treatment. In the aerobic experiments with formate and lactate there is no significant difference in the shape of the curves at 16° and 40°. It would therefore appear that when activation of oxygen is the limiting factor, hydrogen ion concentration does not alter the relation between

dehydrogenase and oxygenase. Figs. 5, 6 and 7 show that the curves obtained in some respects resemble those for the dehydrogenases, but that the oxygen optima have been shifted in relation to the edge of the methylene blue plateau to the acid side in all three cases. In Table II the oxygen optima are given and it will be seen that the shift is in each case equal to approximately one p_H unit. It will also be noted from the figures that at the point where a change is made from borate to phosphate buffer no appreciable change in velocity occurs, although there is a difference of about 40 % in velocity at this point in the anaerobic process. A similar effect has been noted by Cook, Haldane and Mapson [1931] with the action of 8-hydroxyquinolinesulphonic acid. From these experiments it is evident that for the aerobic oxidations the activity of the dehydrogenase is not always the limiting factor in the reaction. The shift of the p_H optima in the aerobic oxidations would perhaps indicate that it is the activity of the oxygenase that is limiting the reaction. It will also be seen from Table I that after exposure to p_H 3.38 although there is a considerable diminution in the activity of the lactic and succinic dehydrogenases there is still an appreciable oxygen uptake. With formate the initial velocity corresponds to about 60 % of the original velocity, but the value given represents the velocity when the system is in a steady state.

It is difficult to give any complete interpretation of the curves obtained in the aerobic experiments—as in all cases the process is complicated by the action of the dehydrogenase. A study of the oxidation of indophenol or some related compound in which activation of the oxygen is the sole process concerned would possibly throw some light on this process.

It is evident from these results that the process of oxidation is one of considerable complexity and that the study of the effect of hydrogen ion concentration on the oxidative mechanisms gives us one method of attack on the mechanism of correlation of the various enzymes concerned.

SUMMARY.

1. A study has been made of the effect of hydrogen ion concentration on the aerobic and anaerobic oxidations of formate, lactate and succinate by toluene-treated *B. coli*.

2. The anaerobic oxidations in the presence of methylene blue give p_H -activity curves which rise steadily from the acid region to a plateau which starts at about p_H 7.0 with formate, p_H 7.4 with lactate and p_H 8.5 with succinate. There is a steady drop from the plateau at about p_H 9.5 in all cases.

3. The p_H -activity curves obtained in the aerobic oxidation of the three substrates resemble in general shape the curves for the dehydrogenases, but the optima have been shifted to the acid side.

4. Exposure to solutions of low p_H inactivates the dehydrogenases in the order succinate, lactate and formate. The aerobic mechanism is affected about equally in each instance.

5. The effect is not so marked in the alkaline region. The oxidation of succinate both anaerobically and aerobically and the aerobic oxidation of formate are the mechanisms most affected.

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