CLXXII. BACTERIAL OXIDATIONS BY MOLECULAR OXYGEN.

I. THE AEROBIC OXIDATION OF GLUCOSE AND ITS FERMENTATION PRODUCTS IN ITS RELATION TO THE VIABILITY OF THE ORGANISM.

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IN recent studies much attention has been directed towards bacterial oxidations in which compounds have been oxidised at the expense of reducible dyes, nitrates, fumarates, etc. It is obvious however that for any organisms growing aerobically the most important hydrogen acceptor is molecular oxygen and that consequently a study of aerobic oxidations is essential if a true picture of the normal life of the cell is to be obtained.

The power to effect the oxidation of their own cell material by molecular oxygen was shown by Callow [1924] to be common to aerobes and facultative anaerobes; this was demonstrated by measuring the oxygen uptake of washed bacteria suspended in buffer solution in a Barcroft manometer; organisms in these conditions take up 5 to 25 cc. of oxygen per g. of dry cell per hour; two organisms only amongst those examined were practically lacking in this faculty, viz. *B. sporogenes*, the only strict anaerobe reported on, and *S. acidi lactici*, a facultative anaerobe resembling the strict anaerobes in possessing no catalase.

It is obvious that many aerobes and facultative anaerobes possess the power of oxidising not only the simple carbohydrates but also their fermentation products since aerobic growth is known to occur on lactate, acetate, succinate, glycerol, etc. where these form the only source of carbon [Braun and Cahn-Bronner, 1922; Stephenson and Whetham, 1924]. In the course of the present inquiry we have confined ourselves chiefly to oxidations by *B. coli*, though experiments have also been carried out with *B. alkaligenes* and with *B. sporogenes*; we have sought to discover (1) the degree of oxidation sustained by glucose and some of its fermentation products, viz. lactate, acetate, formate and ethyl alcohol, in conditions highly favourable to aerobic oxidation; (2) the state of the organisms responsible for the process, i.e. whether they be living or dead, and if the former whether multiplying, at rest or dying off.

METHOD.

The organisms were grown on tryptic broth agar containing 0.5 % sodium lactate; after 24 hours the growth was washed off with phosphate buffer and centrifuged; the organisms were washed twice with Ringer's solution and finally suspended in about 100 cc. of Ringer's solution, aerated for 15 minutes to make an even suspension, and kept at 0°. It is important to use Ringer's solution for this purpose in preference to saline or phosphate buffer as by this means a more actively oxidising suspension is obtained. This stock suspension obtained from 12 agar plates of 5½ inches diameter contains from 6×10^{10} to 8×10^{10} organisms per cc. The suspension is diluted with an equal volume of Ringer's solution before use and 1 cc. employed for each oxidation. The oxidisable materials were usually made up in solutions of $M/100$ strength; glucose was used in $M/200$ strength in order to compare directly with $M/100$ lactate. The purest chemicals obtainable were used; the strength of the acids was checked by titration; in the case of lactic acid a known volume (excess) of $N/10$ NaOH was added to 10 cc. of approximately $N/10$ lactic acid and the mixture autoclaved in order to hydrolyse the lactone present and the excess of sodium hydroxide titrated back with HCl and made up so as to contain exactly $N/100$ sodium lactate; glucose was estimated in $M/10$ solutions by the method of Wood-Ost.

The oxidations were carried out in the Barcroft differential manometer using the flat-bottomed cups employed in tissue oxidations. The cups were filled as follows.

Left-hand cup 1: 1 cc. buffer (usually p_H 7.4); 1 cc. bacterial suspension; 1 cc. water.

Right-hand cup 2: 1 cc. buffer; 1 cc. bacterial suspension; 1 cc. oxidisable material $M/100$. The experiments were carried out in a bath at 40°; 3 minutes were usually allowed for the cups to acquire the temperature of the bath before the taps were closed off; this renders the results somewhat too low, the extent of the error may be gauged by extrapolation. A large number of observations showed that the method was subject to an error of ± 10 %.

The organisms themselves in the absence of any oxidisable material take up oxygen amounting to about 70 mm.³ per hour; it is assumed that in our experiments this endogenous uptake is balanced in each pot; *i.e.* that the presence of the oxidisable material in cup 2 causes no change; no way of checking this assumption has suggested itself but the possibility that our experimental error may be due to an unbalanced endogenous oxidation is not excluded.

The oxidation of glucose, lactate and pyruvate.

The amount of oxygen taken up by glucose and lactate is almost identical amounting to 433 mm.³ of oxygen per cc. $M/200$ glucose (average of 28 observations) and 438 mm.³ per cc. $M/100$ lactate (average of 15 observations) (see Table I).

Table I.

mm. ³ O ₂ taken up by 1 cc. <i>M</i> /200 glucose.													
436	426	396	460	456	452	454	380	388	405	510	497	495	476
395	452	412	513	415	431	385	432	396	377	413	412	412.	Average 433
mm. ³ O ₂ taken up by 1 cc. <i>M</i> /100 lactate.													
475	442	400	448	460	490	444	470	463	392	398	460	417	421
Average 438													

This approximates fairly closely to 4 atoms of oxygen per molecule of lactate or per half molecule of glucose (448 mm.³), 6 atoms being required for complete oxidation in each case.

Fig. 1. Oxidation of 1 cc. *M*/200 glucose. At the point indicated by the arrow the curve A shows the effect of the addition of 1 cc. *M*/200 glucose. Curve B shows the effect of adding 1 cc. of fresh bacterial suspension. I and II correspond to the theoretical values for 8 and 12 atoms of oxygen respectively per mol. of glucose.

The initial rate in the case of glucose is somewhat slower than in the case of lactate; this is apparent from the slope of the curve of two oxidations taken simultaneously; it was also demonstrated by oxidising *M*/200 glucose in one Barcroft cup and *M*/100 lactate in the other; the readings of the manometer showed a negative pressure on the lactate side in the early stages of the experiment though later an equilibrium was achieved.

M/100 pyruvate (prepared from pyruvic acid freshly distilled at 1 mm. pressure) takes up oxygen approximating to 3 atoms of oxygen per molecule (Fig. 2) agreeing with the fact that lactate passes through the stage of pyruvate during aerobic oxidation by loss of 2 atoms of hydrogen [Stephenson, 1928].

Fig. 2. Oxidation of *M/100* lactate and *M/100* pyruvate. I and II correspond to the theoretical values for 3 and 4 atoms respectively of oxygen per mol. of pyruvate and lactate.

The oxidation of acetate, ethyl alcohol and acetaldehyde.

Acetate is rapidly oxidised, the uptake amounting to 3 atoms of oxygen per molecule of acetate, 4 being required for complete oxidation (Table II, Fig. 3).

Table II.

mm. ³ O ₂ taken up by 1 cc. <i>M/100</i> acetate.											
355	386	371	350	308	347	325	350	337	346	314.	Average 335

Ethyl alcohol (*M/100*) remains completely unoxidised; this might be attributed to two causes, either failure of the organism to activate the molecule or to toxic properties; in order to decide between these alternatives *M/100* ethyl alcohol was added to *M/100* lactate in order to show whether the presence of the former at this concentration inhibited the oxidation of the latter; no inhibition was observable (see Fig. 4) and we must therefore conclude that its failure to become oxidised is due to the lack of the appropriate enzymes in the organism concerned.

Acetaldehyde also gives no oxygen uptake; this is not due to its toxic nature since in *M/100* and *M/200* concentrations it exerts no inhibition on

the oxidation of *M*/200 glucose (Fig. 5). Its failure to become oxidised is unexpected in view of the generally accepted theory that ethyl alcohol and acetic acid arise in fermentation through the dismutation of acetaldehyde; this would lead us to expect that in the conditions of our experiments 2 molecules of acetaldehyde would give rise to one molecule of ethyl alcohol which would remain unoxidised and one molecule of acetic acid which would take up 3 atoms of oxygen as already shown.

Fig. 3. Effect of exposure of the bacterial suspension to ultra-violet light on the subsequent oxidation of 1 cc. *M*/100 acetate.

Time of exposure (in mins.)	...	0	20
Total number of organisms present		3.04×10^{10}	3.04×10^{10}
Number of viable organisms present		1.04×10^{10}	2.80×10^7

I corresponds to the theoretical value of 3 atoms of oxygen per mol. of acetate. Four atoms represent complete combustion.

The fate of the acetaldehyde under the influence of the organism was then investigated. In order to avoid the possibility of loss due to evaporation these experiments were carried out in 25 cc. Erlenmeyer flasks, the same quantities of reagents being taken as in the Barcroft experiments; these were cooled to 0° before being mixed, and immediately corked and sealed with paraffin wax; they were then incubated at 37° for 6 hours. The acetaldehyde was estimated by the method of Friedemann, Cotonio and Shaffer [1927] in control experiments without incubation both in the presence and absence of the organism; as the presence of the bacterial suspension made no appreciable difference to the results it was not considered necessary to distil off the acetaldehyde before making the estimation.

Fig. 4. Effect of alcohol on oxidation of $M/100$ lactate.

Fig. 5. Effect of acetaldehyde on the oxidation of $M/200$ glucose.

Table III.

	A cc. <i>N</i> /1000 iodine	$A \times 0.05 =$ cc. <i>M</i> /100 acetaldehyde
1 cc. <i>M</i> /100 acetaldehyde + 1 cc. Ringer + 1 cc. water	18.1 } 18.0 }	18.05 0.902
1 cc. <i>M</i> /100 acetaldehyde + 1 cc. Ringer + 1 cc. bacterial suspension	18.5 } 18.5 }	18.5 0.925
1 cc. <i>M</i> /100 acetaldehyde + 1 cc. Ringer + 1 cc. boiled bacterial suspension	18.5	0.925
Error due to bacterial suspension	0.45	0.022
1 cc. <i>M</i> /100 acetaldehyde + 1 cc. Ringer + 1 cc. boiled bacterial suspension incubated in stoppered flask for 6 hours	18.8 } 18.7 }	18.75 0.937
1 cc. <i>M</i> /100 acetaldehyde + 1 cc. Ringer + 1 cc. bacterial suspension incubated in stoppered flask for 6 hours	10.2 } 10.1 }	10.15 0.507
Disappearance of acetaldehyde due to incubation with bacterial suspension = 47 %	—	0.937 - 0.507 = 0.43

From the figures in Table III it is clear that at the temperature and concentration of our experiments acetaldehyde undergoes no diminution in the absence of the bacteria nor in the presence of boiled bacteria; in the presence of active organisms about 47 % disappears in the course of 6 hours; in the absence of an oxygen uptake this cannot be due to an oxidation unless this be balanced by a simultaneous reduction which must give an oxidisable material (acetic acid), whether it be due to polymerisation or to some other cause is undecided.

The oxidation of formate.

Formate alone among the substances studied is oxidised to completion (Fig. 6). This is noteworthy in view of the fact that Pakes and Jollyman [1901] found that *B. coli*, in the semi-aerobic conditions obtaining in a test-tube and in the presence of peptone, decomposes formates with liberation of hydrogen; had this occurred in our experiments we should have obtained an apparently lower oxygen uptake than that required for complete oxidation due to the positive pressure exerted by the liberated hydrogen. In order to

Fig. 6. Oxidation of 1 cc. *M*/100 formate. 1 corresponds to the theoretical value for 1 atom of oxygen per mol. formate, i.e. complete oxidation.

be sure that this did not occur we repeated the oxidations at an acid reaction (p_H 5.75) still obtaining an oxygen uptake corresponding to complete combustion. We also carried out three experiments in an atmosphere of nitrogen; in these anaerobic conditions liberation of hydrogen would be unbalanced by absorption of oxygen and therefore be clearly apparent; for this purpose we used a Barcroft apparatus having a side-tube and tap inserted above the junction of the cup and the manometer; by means of this tube the cups were evacuated after the solutions had been placed in them and nitrogen was passed in through a 3-way tap; this was repeated three times, the taps were then closed and the apparatus was placed in the water-bath; after 5 minutes the taps were opened for a moment for equilibration and the experiment was carried on as usual; no evolution of hydrogen was observed in any of the three experiments and we therefore concluded that in the conditions of our oxidations no evolution of free hydrogen occurred. It is noteworthy that the oxidation of formate was not more rapid than that of other substrates studied; this is interesting in view of the fact that in the case of methylene blue the rate of reduction by formate is 700 times that of acetate though only 1.2 times that of lactate [Quastel and Whetham, 1925].

The oxidation of glycollate and oxalate.

Glycollate is very slowly oxidised, the oxidation ceasing when 2 to 3 atoms of oxygen have been taken up per molecule of glycollate, 3 being required for complete oxidation. Oxalate is unoxidised; this again is not due to toxicity since $M/100$ oxalate causes no inhibition in the oxidation of $M/200$ glucose. Malonic acid is also unoxidised.

All experiments with boiled bacterial suspension were negative.

Experiments with B. alkaligenes and B. sporogenes.

B. alkaligenes was selected as being an example of a strict aerobe and therefore more likely than *B. coli* to carry oxidations to completion. This organism differs from *B. coli* in being unable to oxidise glucose; this accords with the fact that it is unable to activate glucose to act as a hydrogen donator in the presence of methylene blue [Quastel and Wooldridge, 1925]. Its behaviour with lactate however corresponds with that of *B. coli*, i.e. 1 molecule of lactate takes up approximately 4 atoms of oxygen, 6 being required for complete oxidation. With acetate and formate also its behaviour corresponds with that of *B. coli*, i.e. three-fourths oxidation of acetate and complete oxidation of formate.

B. sporogenes was chosen as an example of a strict anaerobe. The organism was grown in tryptic broth to which 0.1 % of cysteine hydrochloride previously neutralised was added. The organism was grown for 48 hours and centrifuged and washed in the usual way; the suspension consisted mainly of vegetative cells with but few spores.

No oxygen uptake on any of the substrates tested was obtained. The following substances were tried:

broth, $M/200$ glucose, $M/100$ lactate, pyruvate and formate.

In view of the probability that aerobic oxidations involve the activation both of the hydrogen donator and of molecular oxygen we considered that the failure to obtain oxidations by *B. sporogenes* might be due to its inability to effect the activation of oxygen; if this were the case the oxidation might be brought about by the addition of an autoxidisable substance capable of re-oxidation by molecular oxygen as is the case with the lactic dehydrogenase and methylene blue [Stephenson, 1928]. Accordingly we tried the addition of $M/3000$ methylene blue to lactate, pyruvate and also $M/3000$ glutathione (reduced form) to glucose and broth. No significant oxygen uptake was observed in any of these cases.

It has been suggested [M'Leod and Gordon, 1923] that strict anaerobes produce hydrogen peroxide through the aerobic oxidation of their substrates and that this accumulates owing to the absence of catalase from these organisms. If this were so it must surely be evidenced by an oxygen uptake whether the peroxide accumulates as in the case of the *Pneumococcus* or whether it causes secondary oxidations as in the case of the xanthine oxidase system [Thurlow, 1925]. An oxygen uptake equivalent to a production of hydrogen peroxide amounting to 1 part in 50,000 would be clearly demonstrated in our experiments; if peroxide production occurs therefore, it must be within these limits.

We therefore conclude that in the case of this strict anaerobe the oxidative mechanisms are either absent or require totally different conditions for their manifestation than in the case of aerobes and facultative anaerobes.

The influence of hydrogen ion concentration.

The influence of hydrogen ion concentration on the rate of oxidation of lactate by *B. coli* was studied, the rate being measured by the oxygen uptake during the first half hour of the experiment. The exchange of phosphate for phthalate and borate buffers of the same p_H made no difference to the rate as is shown by the following example.

Experiment.

Time (hrs.)	p_H	Buffer	O ₂ uptake 1 cc. $M/100$ lactate (mm. ³)
3	5.75	Phosphate	391
3	5.88	Phthalate	399
3	7.95	Phosphate	355
3	7.90	Borate	359

The p_H of the buffers was determined electrometrically. It is seen (Fig. 7) that an optimum occurs about p_H 5.6 but that little fall occurs on passing to the alkaline side till p_H 9 is reached; below p_H 4.2 and above p_H 11.0 no oxidation occurs; a similar curve for glucose follows approximately the same course. This corresponds with the findings of Sherman and Holm [1922], who

showed that in optimum salt concentration the growth rate varied but little between p_H 5.3 and 8.3.

The physiological condition of the organisms (B. coli) responsible for the oxidation.

As is apparent from the curves, the course of the oxidations exhibits in the main the features of an enzyme system, there being no evidence that the reaction is dependent on bacterial multiplication; it remains however to determine whether the cells responsible for the oxidation are alive or dead in the bacteriological sense, *i.e.* capable or incapable of multiplication when subcultivated; and if the former, whether during the oxidation period they are static, multiplying or dying off.

Fig. 7. Effect of hydrogen ion concentration on the rate of oxidation of lactate.

To determine these points both total and viable counts were made on the suspension and viable counts were repeated after the organism had been shaken in the Barcroft apparatus (1) with and (2) without oxidisable substrate, *i.e.* in each Barcroft cup at the end of the experiment. The total counts were carried out by a method described by Wilson [1922] in which the suspension, after suitable dilution in 1 % phenolised saline, is counted directly in a specially calibrated slide using dark ground illumination. The viable count was done by the roll tube method, serial dilutions of the suspension being made in sterile Ringer's solution and drops being withdrawn by means of standard dropping pipettes into test-tubes containing about 2 cc. of nutrient agar at 45° which was immediately rolled.

Example of results.

Total count.

1 cc.	original suspension + 9 cc. phenolised saline = 10 cc.	Tube (1)
1 cc. (1)	„ + 9 cc. „ = 10 cc.	Tube (2)
1 cc. (2)	„ + 9 cc. „ = 10 cc.	Tube (3)

The organisms in tube (3) were counted direct on the slide, 100 small squares being counted in each case and the average number of organisms in one small

square thus obtained. The results of three counts were as follows: 4.30, 3.86, 4.12; average 4.09.

The volume of one small square being $1/20,000$ mm.³ the number of organisms per 0.5 cc. of the original suspension (which is the quantity used in each Barcroft cup) = 4.09×10^{10} . (Error ± 5 %.)

Viable count.

1 cc.	suspension from Barcroft cup	+ 9 cc. Ringer	= 10 cc. Tube (1)
1 cc. (1)	"	+ 9 cc. "	= 10 cc. Tube (2)
1 cc. (2)	"	+ 9 cc. "	= 10 cc. Tube (3)
1 cc. (3)	"	+ 9 cc. "	= 10 cc. Tube (4)
1 cc. (4)	"	+ 9 cc. "	= 10 cc. Tube (5)
1 cc. (5)	"	+ 9 cc. "	= 10 cc. Tube (6)

Serial no. of tube	Drops taken	Colonies found	Colonies per drop	Colonies per drop $\times 76.1^* =$ colonies per cc.
6	20	270	13.5	1027
6	20	268	13.4	
6	10	137	13.7	

* 76.1 = drops of Ringer's solution per cc. delivered by dropping pipette.

In considering the results of our viable count we have allowed for a possible error of ± 10 %; differences of less than 20 % have therefore no significance.

It is noteworthy that contaminating colonies were only rarely observed in any of the roll tubes.

We first determined whether keeping the organisms aerated in a Barcroft apparatus at 40° resulted in any change in the number of living cells. 1 cc. bacterial suspension in Ringer's solution, 1 cc. phosphate buffer (p_H 7.4) and 1 cc. water were placed in each cup of the apparatus and shaken for 4 hours; 1 cc. was then withdrawn from each cup, diluted, and a viable count taken and compared with the count before treatment; the results given throughout this paper represent the number of organisms in the Barcroft cup, see Table IV.

Table IV.

Number of organisms in each cup of Barcroft apparatus				
Total	Viable			Increase
	Before experiment	After shaking in buffer for 4 hours with no oxidisable material		
4.30×10^{10}	3.09×10^9	3.16×10^9	4.92×10^9	49 %
4.12×10^{10}	3.24×10^9		4.72×10^9	
3.86×10^{10}				
4.09×10^{10}				

From Table IV it is clear that mere aeration in Ringer and buffer at 40° causes some viable organisms to divide, the results agreeing with the supposition that about half the number of viable organisms divide once in 4 hours—the course of an average experiment. We next ascertained whether the presence of oxidisable material causes a further increase in this multiplication.

For this purpose counts were made at the end of the oxidation experiments in each cup, the increase on the left representing that which took place in Ringer's solution and buffer, that on the right in Ringer's solution, buffer and oxidisable material. The results are given in Table V.

Table V. *Number of organisms in each cup of Barcroft apparatus.*

Oxidisable material	Total Each cup	Beginning of experiment		End of experiment		% increase during experiment	
		Viable		Viable		Viable	
		Left cup without oxidisable material	Right cup with oxidisable material	Left cup without oxidisable material	Right cup with oxidisable material	Left cup without oxidisable material	Right cup with oxidisable material
Glucose <i>M</i> /200 3½ hrs	4.09×10^{10}	3.16×10^9	3.16×10^9	5.58×10^9	7.35×10^9	76	132
Glucose <i>M</i> /200 7 hrs.	"	"	"	4.74×10^9	7.35×10^9	50	132
Glucose <i>M</i> /200 5 hrs.	3.04×10^{10}	1.04×10^{10}	1.04×10^{10}	1.70×10^{10}	2.06×10^{10}	63	98
Lactate <i>M</i> /100 2½ hrs.	4.09×10^{10}	3.16×10^9	3.16×10^9	4.29×10^9	5.46×10^9	41	73
Lactate <i>M</i> /100 2½ hrs.	"	"	"	5.25×10^9	6.06×10^9	66	92
Formate <i>M</i> /100 6½ hrs.	"	"	"	4.95×10^9	4.92×10^9	57	59

From Table V it is clear that aeration at 40° without oxidisable material causes an increase of 40–76 %; the presence of oxidisable material raising this to 59–132 %. One may regard these increases as being due to a proportion of the viable organisms being in a state almost ready to divide and requiring only the stimulus of the temperature and salts to cause them to complete one division; a further number require the extra stimulus of the carbon food material. It must be pointed out that no nitrogenous food material is present except that obtained from the dead organisms.

The next question to be answered is whether the oxygen uptake measured is due to the living or to the dead cells; in order to ascertain this we reduced the number of living organisms by exposure to ultra-violet light from a mercury lamp. The lamp used was a K.B.B. atmospheric quartz mercury vapour lamp; the culture in Ringer's solution was placed in a quartz test-tube enclosed in a second quartz tube to avoid overheating and clamped 6 inches from the lamp; exposures of 20 min. and 30 min. were made, the suspension being turned and shaken at intervals; after exposure the culture was diluted and a viable count made; oxidations were carried out with the suspension immediately after exposure and the rates compared with those of the normal organism. Tables VI and VII show the effect of the exposure on the number of live organisms; 20 minutes' exposure leaves approximately 2.74 organisms alive out of 1000; 30 minutes' exposure leaves 2 in 10,000. Fig. 8 shows the rate of oxygen uptake on glucose, by untreated organisms and by those

exposed for 20 and 30 minutes respectively. The greatest effect is observed in the initial stages, the uptake at the end of 30 minutes being reduced in the case of the suspension previously exposed for 20 minutes to slightly over one-half and in that exposed for 30 minutes to slightly under one-half; by the end of one hour the rates are approximately equal. Since the number of living organisms has been divided by 370 and 5000 respectively by the 20 minutes' and 30 minutes' exposure it is quite clear that the living organisms exercise no

Fig. 8. Effect of exposure of the bacterial suspension to ultra-violet light on the subsequent oxidation of 1 cc. *M*/200 glucose.

Time of exposure (mins.)	0	20	20	30
Total number of organisms present	3.04×10^{10}	3.04×10^{10}	3.04×10^{10}	3.04×10^{10}
Number of viable organisms present	1.04×10^{10}	2.85×10^7	2.80×10^7	2.16×10^6

measurable influence on the oxidation process, the very slight falling off in the rate by the exposed cultures being attributable to a slight injury to both dead and living organisms similar to that experienced by any enzyme system on exposure to ultra-violet light. This experiment also shows that the increase in viable organisms in the Barcroft apparatus during the process of oxidation has no measurable connection with the oxygen uptake, since in the case of organisms previously exposed to ultra-violet light the oxidation is attended by a substantial death-rate in the few surviving organisms (Tables VI and VII).

The oxidation of lactate by exposed suspensions suffers much the same slight diminution as in the case of glucose (Fig. 9); that of formate is reduced to about one-half (Fig. 10) but even in this case shows no quantitative relationship to the number of living cells. It is interesting to compare the effect of reducing the number of living organisms by radiation with that of reducing the total number by dilution (Fig. 11). In the latter case the rate of oxidation is roughly proportional to the number of organisms present.

Table VI.

Time of exposure (min.)	Number of viable organisms in Barcroft cup before and after exposure to ultra-violet light		
	Before shaking	After shaking	
		Without oxidisable material	With oxidisable material
0	1.04×10^{10}	1.70×10^{10}	2.06×10^{10}
20	2.85×10^7	6.61×10^6	3.57×10^6
30	2.16×10^6	5.35×10^6	1.84×10^6

Table VII.

Time of exposure (mins.)	Relative number of viable organisms before and after exposure to ultra-violet light			
	Before exposure	Before shaking in Barcroft apparatus	After exposure	
			Without oxidisable material	With oxidisable material
20	1000	2.74	0.63	0.51
30	1000	0.207	0.034	0.017

We may conclude from these experiments that the oxidation phenomena studied are the work of dead equally with living organisms.

Problem of the incomplete oxidation of glucose, lactate and acetate.

These experiments show that *B. coli* is capable of oxidising glucose and the fermentation products which it produces, with the exception of ethyl alcohol and acetaldehyde. The problem opened up by our results is contained in the observation that neither glucose, lactate nor acetate is oxidised to completion, the two former taking up about two-thirds and the latter about three-fourths the amount of oxygen required for complete oxidation. In the case of glucose and lactate this ratio has been found to persist throughout very varying conditions; it holds throughout the range of hydrogen ion concentration at which oxidation occurs and also at varying dilutions; it is unaffected by growing the organisms anaerobically instead of on agar plates or by killing off the living organisms by ultra-violet light.

Fig. 9. Effect of the exposure of the bacterial suspension to ultra-violet light on the subsequent oxidation of 1 cc. $M/100$ lactate.

Time of exposure (min.)	...	0	20
Total number of organisms present	...	3.04×10^{10}	3.04×10^{10}
Number of viable organisms present	...	1.04×10^{10}	2.80×10^7

I corresponds to the theoretical value for 4 atoms of oxygen per mol. of lactate; 6 atoms represents complete combustion.

Fig. 10. Effect of exposure of the bacterial suspension to ultra-violet light on the subsequent oxidation of 1 cc. $M/100$ formate.

Time of exposure (min.)	...	0	20
Total number of organisms present	...	3.04×10^{10}	3.04×10^{10}
Number of viable organisms present	...	1.04×10^{10}	2.80×10^7

I represents the theoretical oxygen uptake for complete oxidation.

Several explanations for this behaviour suggested themselves. The first was the possibility that the oxidation fell short of completion owing to the destruction of the enzymes responsible. This was tested by removing the Barcroft cups after the oxidation had ceased and adding to each 1 cc. of fresh bacterial suspension; no further oxidation was however obtained (Fig. 1, p. 1370). The alternative device of adding 1 cc. of fresh oxidisable material to cup 2 and 1 cc. of water to cup 1 resulted in an immediate renewed uptake of oxygen at approximately the original rate (Fig. 1). The idea that the oxidation had ceased owing to the destruction of the enzyme was therefore untenable.

Fig. 11. Effect of the dilution of the bacterial suspension on the oxidation of 1 cc. of $M/200$ glucose.

	Total number of organisms $\times 10^{10}$
A = Normal dilution	3.04
B = $A/2$	1.52
C = $A/5$	0.608
D = $A/10$	0.304

A second explanation which suggested itself was that by the time two-thirds of the glucose or lactate was oxidised the concentration had fallen below the threshold at which oxidation could occur. If this were the case, oxidation of $M/600$ (or less) glucose should not occur; oxidation however occurs with $M/800$ glucose, two-thirds the theoretical complete uptake of oxygen being again achieved (Fig. 12). It seems fairly certain therefore that oxidation does not cease whilst a proportion of the original substrate remains unoxidised and we must look in another direction for an explanation of the constant incomplete uptake.

In the case of acetic acid the uptake corresponds very closely to that required to oxidise acetic acid to oxalic acid:

Moreover, since oxalate itself is unoxidised by the organism it seemed likely that it might be accumulating as an end-product; should this be the case it would be present in the Barcroft cup at the end of the oxidation in *M*/300 strength. The possibility of detecting oxalate in this concentration was explored; for this purpose 1 cc. buffer + 1 cc. water + 1 cc. *M*/100 oxalate were acidified with one drop of concentrated sulphuric acid and titrated with *N*/100 KMnO_4 using a micro-burette; the presence of 1 cc. of bacterial suspension strongly reduces permanganate and it was therefore necessary to remove the organisms before titration; to do this the contents of the Barcroft cup were transferred by means of a curved pipette to a small centrifuge cup, the Barcroft cup was washed out twice with 1 cc. of water, and 1 cc. of 20 % phosphotungstic acid added to the combined contents and washings and the whole centrifuged until the supernatant liquid was clear; 5 cc. of the liquid was then withdrawn, heated to boiling and titrated with *N*/100 KMnO_4 .

Fig. 12. Oxidation of 1 cc. of *M*/100, *M*/400 and *M*/800 glucose.

Results.

		cc. <i>N</i> /100 KMnO_4
1 cc. <i>M</i> /100 oxalate } 1 cc. buffer } 1 cc. water }		1.75
		1.70
		1.65
		1.75
		1.65
		1.70
1 cc. <i>M</i> /100 oxalate } 1 cc. buffer } 1 cc. bacterial suspension } 2 cc. water }	The whole centrifuged and 5 cc. of the super- natant liquid taken for titration	1.47
		1.58
		1.54
		1.50
		1.42
		$1.50 \times \frac{5}{3} = 1.80$

The experiment was then repeated with the contents of Barcroft cups in which 1 cc. *M*/100 and 1 cc. *M*/50 acetate had been oxidised; no measurable reduction of the permanganate occurred and we have therefore provisionally abandoned the hypothesis that acetate is oxidised to oxalate.

As previous work has shown that in the case of the Timothy Grass bacillus the addition of acetate to the media greatly increased the fat content of the cell [Stephenson and Whetham, 1922] it seemed worth while to test whether in our experiments the unoxidised acetate might be giving rise to fat. For this purpose the oxidation was carried out in a 1000 cc. Erlenmeyer flask through which a rapid stream of air was led; two experiments were made, one with and one without acetate; the contents of the flasks were as follows:

Flask 1	Flask 2, control, half-quantity
100 cc. bacterial suspension	50 cc. bacterial suspension
100 cc. Ringer's solution	50 cc. Ringer's solution
200 cc. phosphate buffer p_H 7.4	100 cc. buffer
200 cc. acetate (=1.2 g. acetic acid)	100 cc. water

As the experiment was run for 20 hours special precautions were taken to ensure that no contaminating organisms were present; the bacteria were grown on agar in Roux bottles, washed off with sterile Ringer's solution and centrifuged and washed with sterile precautions; the suspension was plated at the end of the experiment and found to be in pure culture. The experimental flasks and the rubber stoppers fitted with entrance and exit tubes were separately sterilised and the sterile solutions together with the bacterial suspension introduced into the flasks by means of sterile pipettes; finally the stoppers were placed in position and sealed with paraffin wax. The entrance and exit tubes were protected from contamination by cotton wool plugs and the exit tube was fitted with a Kjeldahl trap to act as a condenser and prevent the plug from becoming wet. The liquid in the flasks was aerated by a steady stream of air passed through by means of an aspirator. The experiment was run for 20 hours; a drop was then withdrawn, diluted and plated and the culture was found to be pure. The whole was then centrifuged and the bacterial deposit dried *in vacuo* at 96° treated with 98 % alcohol, evaporated to dryness and then extracted with ether in a Soxhlet apparatus. The supernatant liquid from the centrifuge cups was passed through a bacterial filter, evaporated on a water-bath to about 50 cc. and extracted from neutral solution with ether in a liquid extractor.

Results.

	Experiment (with acetate) (mg.)	Control without acetate (mg. $\times$ 2)
Fat from bacteria	32.5	50
Fat from solution	38.0	64

From these results there is clearly no indication that the unoxidised acetate gives rise to fat. It is now clear that the fate of the unoxidised portions of glucose, lactic and acetic acids can only be investigated by means of work on a large scale and we have therefore decided to allow it to form the subject of a separate investigation.

SUMMARY.

1. *B. coli* is capable of oxidising, by means of molecular oxygen, glucose and certain of its products of fermentation, viz. lactate, pyruvate, acetate, and formate; ethyl alcohol and acetaldehyde are not oxidised, though in the concentrations used they do not act as inhibitors to the oxidation of other substances.

2. The oxidation of formate is carried to completion; that of glucose and lactate to two-thirds and of acetate to three-fourths of completion.

3. *B. alkaligenes* differs from *B. coli* in being unable to oxidise glucose; its behaviour to lactate, acetate and formate is similar to that of *B. coli*. *B. sporogenes* is unable to oxidise any of the substrates tried.

4. Oxidations by *B. coli* are not dependent on living cells; these may be originally present in proportions varying from one-third to one-tenth of the total number of organisms; on reducing their number by exposure to ultra-violet radiation to 0.27 % and 0.021 % of their original number the rate of oxidation is only slightly affected.

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