OXYGEN DEMAND AND OXYGEN SUPPLY

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I. PURPOSE AND OUTLINE OF THE STUDY

BY OTTO RAHN

The oxygen supply of bacteria in our customary culture methods is known to be not quite adequate, and since that fact is generally admitted, further studies may seem unnecessary. Such a statement, however, is far too optimistic, as some simple calculations may show. To produce vinegar with the legal minimum of 4 per cent acetic acid, the medium should contain 3.07 per cent alcohol and 2.14 per cent of oxygen. The solubility of oxygen from air in water at atmospheric pressure is 0.0008 per cent. The disproportion is evident and explains the fact that the so-called German process of vinegar manufacture, with ample aeration, accomplishes in one day as much oxidation as the old Orleans process does in half a year. Tubercle bacteria require for the complete oxidation of 1 per cent glycerol 1.22 per cent oxygen. The medium (at 37°C.) contains only 0.00065 per cent. With 200 ml. of medium in a liter flask, the air above the medium contains 0.168 g. O_2. This air must be completely renewed 14 times to permit complete oxidation of the glycerol. The ratios for a nearly full flask or for a test tube culture are much more unfavorable. The disproportion between demand and supply is so striking that the large majority of cells in such bacterial cultures are starving for oxygen, and it seemed worth while to study the effect of this "undernourishment" upon the cells themselves, upon the shape of the so-called "normal growth curve," and upon the decompositions caused by bacteria under such circumstances.

Considerations of this kind have led to a study of various as-

pects of the oxygen supply problem. The first phases of this investigation are presented in the following pages, namely the oxygen demand of various species during active multiplication, the rate of oxygen diffusion in culture media, and the fate of aerobic species in the absence of oxygen. Studies on population growth with optimal oxygen supply, on the effect of convection currents, and on the oxidation systems of bacteria are under way. It is hoped that a reliable method of determining dissolved oxygen in presence of organic compounds can soon be included.

II. OXYGEN DEMAND OF MULTIPLYING BACTERIA

By Otto Rahn

Two methods can be used for the determination of oxygen consumption by multiplying bacteria. Clifton and Logan (1939) used the Warburg technique, by removing samples at various ages and preventing multiplication during the test by addition of sodium azide or dinitrophenol. This requires very large inoculation, and may not represent normal cell conditions. For the purpose of this investigation, the other method seemed preferable.

Method

The medium is saturated with air by standing with large exposed surface (150 ml. in a 2 l. Erlenmeyer) for two days at the temperature of the experiment. This temperature was 30° throughout the entire investigation. Into sterile test tubes is pipetted 1.0 ml. of the inoculum, 10 ml. of the air-saturated medium and 0.5 ml. of methylene blue solution, made by mixing 0.5 ml. of 0.1 per cent alcoholic solution with 9.5 ml. of sterile water. This culture is at once sealed with paraffine, or a vaseline-paraffine mixture. The bacteria of the inoculum are counted. The sealed culture is watched, and as soon as it shows a decoloration of 50 per cent (determined by color standards), the seal is broken and the bacteria are counted again.

The oxygen in the culture consists of the amount dissolved in the 10 ml. of medium which can be determined analytically, plus the oxygen contained in the sterile water used for preparing

the inoculum and the methylene blue solution. Both the medium and the water were kept for at least 24 hours at the temperature of the experiment and can be considered saturated with air. Thus we know the original oxygen content of the culture, the time required to consume all the oxygen, and the number of bacteria at that moment as well as at the start.

From these data, the oxygen consumption per cell per hour can be computed by the so-called Buchanan formula.

In order to make this formula apply correctly, the inoculum should be so young as to avoid lag. Some bacteria reduce methylene blue with difficulty, so that other indicators must be used. Decoloration is not instantaneous, but the time of decoloration can be measured quite accurately by comparison with color standards prepared from the same medium.

The actual experiments were carried out by making three successive decimal dilutions for the same organism. Commonly, the first tube received 1 ml. of the culture diluted 1:1,000, the second tube 1 ml. of the dilution 1:10,000 and the third 1 ml. of the dilution 1:100,000.

It can be shown that with small inocula, the number of cells at the moment of decoloration is independent of the inoculum because the amount of oxygen consumed by small numbers of bacteria is negligible. If we introduce the multiplication constant K into the Buchanan formula (Rahn, 1939, p. 6), the formula for the oxygen consumption becomes very simple.

$$x = \frac{K[O_2]}{b - a}$$

The oxygen concentration $[O_2]$ is the same in all three cultures, and the oxygen consumption per cell, x, and the multiplication constant K are also the same, and independent of the inoculum. Therefore

$$x = \frac{K[O_2]}{b_1 - a_1} = \frac{K[O_2]}{b_2 - a_2} = \frac{K[O_2]}{b_3 - a_3}$$

and hence $b_1 - a_1 = b_2 - a_2 = b_3 - a_3$.

As long as the inocula are sufficiently small to be neglected as compared with the final numbers, i.e., if a is less than about 1 per cent of b, we have $b_1 = b_2 = b_3$.

Results

The agreement between the parallel cultures made with different dilutions of the same species was usually fair, though striking deviations occurred occasionally which could not be accounted for. They are all included in the averages. Table 1 shows the Enterobacteriaceae (colon group) well confined within the limits of oxygen demand of 3 to 5 $\times$ 10^{-10} mgm. per cell per hour. Quite different is the demand of *Pseudomonas* which is of approximately the same cell size, but requires about 9 to 10 $\times$ 10^{-10} mgm. Much greater is the need of the sporeformers which are much larger in size. The great fluctuation of results in this latter group is largely due to the formation of chains which cannot be shaken apart in the ordinary technique of plating. Thus, what is counted as one cell because it was represented by one colony, may be a chain of a large number of cells. The very high values for *Bacillus megatherium* and *Bacillus peptogenes* are primarily the result of chain formation.

A slight retardation of the multiplication rate through methylene blue was noticeable in most bacteria, and averaged 6 per cent. Considering the great fluctuations of the oxygen consumption per cell, and the fairly uniform retardation, it seemed not important to correct for this retardation.

This simple method for the determination of the oxygen demand gave satisfactory agreement of results with simple media, but the results were in hopeless disagreement when milk was used. There was a very pronounced decrease in the final number of cells as the inoculum became smaller, and the computed oxygen consumption per cell increased correspondingly. The discrepancies obtained by our formula may be seen from the following data which give relative values for oxygen consumption by cultures inoculated with 0.01, 0.001, and 0.0001 ml. of stock culture.

INOCULUM	10^{-2}	10^{-3}	10^{-4}
For *Streptococcus lactis* 125	100	158	505
For *Streptococcus lactis* L21	100	168	396
For *Streptococcus zymogenes*	100	169	436
For *Streptococcus durans*	100	196	543
For *Streptococcus liquefaciens*	100	328	848
For *Streptococcus fecalis*	100	277	2310
Average	100	216	840

This could only mean that the milk as such had combined with oxygen. It seemed strange that this possibility had not been discovered earlier. Treating the earlier data in the same way, we find:

	WITHOUT GLUCOSE		WITH GLUCOSE	
A. aerogenes	100	105	100	220
A. levans	100	52	100	92
A. cloacae	100	103	100	210
E. coli	100	65	100	82
Proteus	100	91	100	237
Average	100	83.2	100	168.2

The tendency to combine with oxygen does not exist in the plain peptone solution, but is quite evident when glucose is added. In the presence of sugar, the oxygen consumption by the medium is so large as to affect the result in the short time of the experiment which, in the case of milk, was completely finished after 8 hours, in the case of the peptone solutions, after 12.3 hours.

It is evident that both the oxidation rate of the medium and the oxygen requirement of bacteria can be computed, since we have three different determinations and only two unknowns. Calling the oxygen uptake of the medium y mgm. per ml., and the oxygen consumption per cell x mgm., we have the total oxygen consumption during the experiment made up of two parts. The medium, in t hours, consumes $t \cdot y$ mgm. oxygen. The rest, ΔS, is used by the bacteria, and the consumption per cell is

$$x = \frac{\Delta S \cdot K}{b - a}$$

TABLE 1

Hourly oxygen demand per cell and per generation

	0.5 PER CENT PEPTONE			0.5 PER CENT PEPTONE + 0.5 PER CENT GLUCOSE		
	Generation time	Oxygen per cell per hour	Oxygen per generation	Generation time	Oxygen per cell per hour	Oxygen per generation
	minutes	*10^{-10} mgm.*	*10^{-10} mgm.*	*minutes*	*10^{-10} mgm.*	*10^{-10} mgm.*
Escherichia coli	38.6	4.7	3.0	40.7	3.8	2.6
	43.2	3.0	2.2	39.5	3.3	2.2
	41.8	5.2	3.6			
	47.6	3.4	2.7			
Aerobacter cloacae	33.0	3.1	1.7			
	34.0	3.1	1.8			
	33.0	3.3	1.8			
	39.8	3.2	2.1	42.3	1.8	1.3
	38.7	3.3	2.1	40.7	3.5	2.4
Aerobacter aerogenes	38.9	2.9	1.9	37.3	2.2	1.4
	37.9	3.2	2.0	35.9	4.9	2.9
Aerobacter levans	43.4	5.7	4.1	43.7	6.6	4.8
	46.0	2.9	2.2	42.7	7.1	5.0
Escherichia communior	60.7	3.6	3.6	53.5	3.1	2.3
				51.0	2.2	1.9
Proteus vulgaris	41.3	9.9	6.8			
	56.0	6.4	6.0			
	90.0	2.8	4.2			
	48.7	4.2	3.4	50.5	3.0	2.5
	46.0	3.9	3.0	44.0	7.3	5.4
Pseudomonas fluorescens	70.2	8.7	10.2			
	63.7	8.7	9.2			
	59.2	8.0	7.9			
Pseudomonas aeruginosa				64.5	7.7	8.3
				58.5	6.5	6.3
				59.3	11.0	10.9
Bacillus subtilis	60.7	19.8	20.0	42.0	9.6	6.7
	68.7	16.1	18.4	43.8	11.8	8.7
	45.5	23.8	18.0	42.7	12.8	9.1
	50.0	34.2	28.5	44.0	9.7	7.1
	58.9	47.8	46.5	52.8	20.6	18.1

TABLE 1—*Concluded*

	0.5 PER CENT PEPTONE			0.5 PER CENT PEPTONE + 0.5 PER CENT GLUCOSE		
	Generation time	Oxygen per cell per hour	Oxygen per generation	Generation time	Oxygen per cell per hour	Oxygen per generation
	minutes	10^{-10} *mgm.*	10^{-10} *mgm.*	*minutes*	10^{-10} *mgm.*	10^{-10} *mgm*
Bacillus cereus	47.9	39.8	31.8	42.9	38.2	27.3
	41.6	20.4	14.2			
	37.6	24.9	15.6			
	32.4	18.3	9.8			
	42.2	36.4	25.6			
	42.0	38.6	27.0			
	37.5	43.1	27.0			
Bacillus mesentericus	62.5	9.9	10.3	46.5	5.4	4.2
	90.0	8.0	12.0	50.9	22.0	18.6
	34.2	6.9	3.9	43.2	7.8	5.6
	42.0	11.0	7.7	44.2	7.9	5.8
	41.5	8.6	6.0	43.7	9.4	6.8
Bacillus megatherium	56.2	28.0	26.1	77.4	102.0	131.5
Bacillus peptogenes	57.0	77.3	73.4	82.6	87.5	120.0

where the multiplication constant K takes care of time and increase in numbers. The total oxygen consumed per ml. of culture until the moment of decoloration is

$$[O_2] = t \cdot y + \Delta S = t \cdot y + \frac{x(b - a)}{K}$$

The initial oxygen as well as K are the same in parallel experiments with different inocula. We obtain, therefore,

$$[O_2] = yt_1 + \frac{x(b_1 - a_1)}{K} = yt_2 + \frac{x(b_2 - a_2)}{K}$$

This permits us to express y in terms of x

$$y = \frac{x(b_2 - a_2 - b_1 + a_1)}{K(t_1 - t_2)}$$

This value for y in any of the above equations permits a simple solution for x. The final results are shown in table 2. The good agreement of the calculations of the oxygen uptake of the milk

indicates that the method is permissible. This is not the first calculation of this kind. Matuszewski and associates (1935)

TABLE 2

Streptococci in milk at 30°

Hourly oxygen demand per cell; hourly oxygen consumption per ml. of milk

	GENERATION TIMES				OXYGEN CONSUMPTION PER HOUR	
	Methylene blue			No methylene blue	Per cell	Per ml. of milk
	1	2	3			
	minutes	*minutes*	*minutes*		10^{-10} *mgm.*	10^{-4} *mgm.*
S. liquefaciens	85.0	73.7	64.5	56.7	7.22	7.83
S. fecalis	62.0	52.3	52.0	49.2	2.32	8.28
S. durans	63.0	56.5	52.3	55.3	1.07	7.02
S. lactis 125	57.9	46.5	42.3	43.2	0.46	8.65
S. lactis L21	59.1	50.5	42.4	38.7	2.10	7.40
S. zymogenes	68.2	54.8	49.8	55.8	2.33	8.28

TABLE 3

Oxygen and energy requirements for multiplication of bacteria

	CELL WEIGHT	O_2 PER CELL PER HOUR	O_2 PER 10^{-10} MGM. CELLS PER HOUR	O_2 REQUIRED TO PRODUCE 10^{-10} MGM. CELLS	LIVING CELLS PER GRAM OXYGEN	LIVING CELL PER CALORY OF RESPIRATION
	10^{-10} *mgm.*	10^{-10} *mgm.*	10^{-10} *mgm.*	10^{-10} *mgm.*	*grams*	*grams*
E. coli	7.5	3.9	0.52	0.36	2.78	0.87
A. cloacae	7.5	4.0	0.53	0.25	3.95	1.23
A. aerogenes	7.5	3.3	0.44	0.27	3.66	1.14
A. levans	7.5	5.6	0.75	0.53	1.85	0.58
E. communior	7.5	3.0	0.40	0.35	2.88	0.90
P. vulgaris	7.5	5.3	0.71	0.60	1.67	0.53
P. fluorescens	7.5	9.3	1.24	1.21	0.83	0.26
P. aeruginosa	7.5	8.4	1.11	1.13	0.88	0.27
B. subtilis	7.5	20.6	2.74	2.34	0.43	0.13
B. cereus	15.8	32.4	2.05	1.37	0.73	0.23
B. mesentericus	7.5	9.5	1.27	1.05	0.95	0.30
B. megatherium	32.6	65.0	2.00	2.16	0.46	0.14
B. peptogenes	4.5	82.4	18.30	21.4	0.05	0.01

have computed the number of bacteria present in milk from the reductase test.

The averages of all determinations for each species are shown in the second column of table 3. From these, we obtain the following averages for each genus or group:

Streptococci	$1.65\ 10^{-10}$ mg.
Enterobacteriaceae	$4.3\ 10^{-10}$ mg.
Pseudomonades	$8.9\ 10^{-10}$ mg.
Bacilli	$27.5\ 10^{-10}$ mg.

The difference of oxygen consumption between the Colon bacteria and the equally large *Pseudomonas* has already been mentioned. The streptococci require much less, but are also much smaller. To obtain a true comparison, the oxygen demand per cell should be divided by the weight of the individual cell, so that the oxygen demand per unit cell weight is obtained. In table 3, the volume of the cells rather than the weight is used because it can be more accurately determined. Even the value for the volume is at best an estimate, for we must realize that during the period of early growth and multiplication, the size of bacteria varies greatly. The values given in Bergey's Manual indicate no appreciable difference between the different species of the Enterobacteriaceae nor of *Pseudomonas*, so that for all of them, the average size of 0.75 μ^3 is taken as the cell volume (Rahn, 1932, p. 397).

In table 1 was also mentioned the generation time, and the amount of oxygen necessary for the doubling of one cell. These values are summarized in the fourth column of table 3. Since the doubling of a cell means the production of one new cell volume, we can find the amount of oxygen necessary for the unit volume of bacteria by dividing the oxygen per generation by the cell size. Thus, column 5 tells us how much oxygen is required to produce 1 μ^3 of cell substance. The reciprocal of this number shows the μ^3 of cell substance produced by a unit of oxygen; column 6 gives the values as ml. of cell substance per gram of oxygen. The last column expresses this in calories. Since the energy obtained from 1 gram of oxygen is 3.3 Calories regardless of the substance oxidized (with very few exceptions, see Pütter, 1910, or Rahn, 1932), the data of the preceding column must be divided by 3.3 to obtain the ml. of cell substance per Calory. In

bacteria, milliliters of cell substance are practically identical with grams, the density of bacteria being only slightly larger than that of water.

The streptococci are not included in this calculation because they cannot utilize the energy obtained from oxidation as will be shown in a later chapter. This group of bacteria behaves quite differently from the average types, and a special study will be devoted to the peculiarities of the oxygen uptake by streptococci.

III. OXYGEN DIFFUSION IN MEDIA

By Otto Rahn

Our standard laboratory media become saturated with air within a very few days after sterilization, partly through diffusion, partly through convection currents. The solubility of oxygen from the air in these media is not much different from that in water. However, most media combine slowly with oxygen as will be shown presently, and in this case, the oxygen content in deep layers, e.g., near the bottom of a completely filled flask, will be below the saturation point.

At best, solubility of oxygen is very small. It does not appear to be so little when given in cubic millimeters, but we should realize that the solubility of oxygen equals that of some compounds which we use in quantitative analysis because of their insolubility.

Solubility in mgm. per liter, or parts per million

$BaSO_4$	2.4	Ca oxalate	6.7
FeS	6.2	O_2 at 30°	7.5
MgO	6.2	O_2 at 20°	9.0
O_2 at 40°	6.4	Ag_2O	13.2
$Fe(OH)_2$	6.7	$Ca_3(PO_4)_2$	25.0
$FeCO_3$	6.7		

The preceding paper has shown that a medium saturated with air at 30° is depleted of all its oxygen when the bacteria have multiplied to about 2 to 10 million cells per ml. The maximal population reached in these cultures when grown in test tubes or flasks is about 500 to 2000 million. In other words the oxygen

becomes exhausted when only about one per cent of the maximal population is reached, and the question where the other 99 per cent of the final population get their necessary oxygen, demands an answer. It is customary to refer to the inexhaustable supply of oxygen in the atmosphere, but the point at issue is not only a question of ultimate quantity, but of rate. If 5 million bacteria consume all soluble oxygen in 8 hours, 10 million cells will consume it in 4 hours, 20 million in 2 hours, 40 million in 1 hour, 80 million in 30 minutes, 160 million in 15 minutes, and 320 million in 7.5 minutes. Can diffusion really supply oxygen so rapidly? And if not, what happens to the cells of aerobic bacteria?

The second question will be answered in chapter IV. In this chapter, the rate of diffusion of oxygen will be discussed. Besides diffusion, convection currents are also important factors in supplying oxygen to the lower strata. They cannot very well be standardized. But the rate of diffusion can be measured by eliminating convection currents through the addition of agar which does not interfere greatly with oxygen diffusion.

The method used was very simple. To the medium to be tested, besides about 0.5 per cent agar, sufficient methylene blue was added to color it distinctly (usually 1:200,000). This medium was filled in tall test tubes, plugged with cotton, and sterilized. Heating reduces the methylene blue. The tubes are then placed at once in cold water to solidify the colorless agar. As the oxygen diffuses in, it brings back the blue color of the dye, and the depth of the blue zone indicates how far the oxygen has advanced. The tubes are kept in a moisture-saturated atmosphere to prevent drying.

The results are shown in tables 4 to 6. The first table shows two experiments with water agar, made 3 years apart, with two different oxygen indicators, and at different temperatures. Water agar does not decolorize methylene blue during autoclaving. It was kept at 50 to 60° in oxygen-free atmosphere for about a week before it became colorless. This indicates that it contains hardly any compounds combining with oxygen, and the advance of the blue zone is representative of oxygen diffusion in water.

This rate is very slow, far too slow for oxygen supply of bacterial cultures, namely, less than 5 cm. in 24 hours.

With bacterial culture media, the advance of the blue zone is much slower because the oxygen combines with the medium.

TABLE 4

Depth of blue zone in water agar in millimeters

INDICATOR		METHYLENE BLUE		THIONINE, 1:100,000				
Time	$\sqrt{\text{Time}}$	0°	50°	0°	10°	20°	30°	40°
hours								
3.25	1.80			11	13.5	15	15.5	14
7.0	2.68	17	25					
15.25	3.90			23	29	33	31	30
18.75	4.33			26	32	34.5	34	34
22.75	4.77			29	35	37	41	(40)
24.00	4.90	33	47					
27.75	5.27			33	40	42		
40.25	6.34			41	(46)			
48	6.94	45		45				

TABLE 5

Oxygen diffusion at 30° in nutrient broth + methylene blue, 1:200,000 with different amounts of agar

Each number is the average of 3 experiments

HOURS	$\sqrt{\text{TIME}}$	DEPTH OF BLUE ZONE (MM.)						
		Agar concentration (per cent)						
		0.1	0.5	1	2	3	4	5
4	2.0	19.0	18.2	17.0	15.2	14.7	13.0	12.8
12	3.5	33.0	28.5	26.0	24.0	22.0	20.0	19.0
24	4.9	42.5	37.0	34.0	30.7	28.2	26.5	24.3
31	5.6	45.5	41.3	37.7	33.7	31.3	28.3	26.2
48	6.9	54.0	46.8	44.3	38.3	35.5	32.2	30.3
71	8.4	61.5	54.5	50.8	44.5	40.7	37.6	35.0
120	11.0	76.0	64.0	60.5	51.3	47.5	45.0	41.0

This has been shown in the preceding chapter where the oxygen uptake of milk had been calculated at about 8×10^{-4} mgm. O_2 per ml. of milk per hour. What we measure by the method employed is oxygen diffusion *minus* oxygen consumption. It is

this difference in which we are interested, because only the difference becomes available to bacteria.

Table 5 shows the effect of the agar concentration. It is noticeable, but not very important. Table 6 gives the effect of dilution upon the oxygen consumption of milk, since diffusion as such is probably not much affected by different concentrations of the milk constituents.

No way has been found as yet to calculate from these results the rate of oxygen uptake of the medium. In agreement with the theory of diffusion, the depth of the blue zone plotted against

TABLE 6

Oxygen diffusion at 0° in diluted milk + 0.5 per cent agar

Methylene blue, 1:200,000 (each value is the average of 6 experiments)

HOURS	$\sqrt{\text{TIME}}$	DEPTH OF BLUE ZONE (MM.)			
		Milk concentration (per cent)			
		40	20	10	5
7	2.6	15	17	17	17.7
10.5	3.2	18	19.5	20.5	
24	4.9	27.1	28.5	30.5	32.7
48	6.9	34.8	38.9	42.9	45.2
DAYS					
3	8.5	39.9	46.4	51.7	54.7
4	9.8	44.5	53.0	58.3	62.0
5	11.0	49.3	57.9	65.6	
6	12.0	52.5	62.6		
7	12.9	56.2	68.0		

the square root of time results in a straight line (fig. 1). This figure shows that carbohydrates increase the oxygen uptake considerably, verifying the comparison of broth and glucose broth in the preceding chapter.

Very interesting is the effect of temperature as shown in table 7. The rate of diffusion increases slightly with increasing temperature, and table 6 with water agar shows this quite distinctly. The glucose agar of table 7, however, reacts with oxygen, and this oxidation has a temperature coefficient of not less than 2, which for 50°C. means an increase in rate of oxidation of

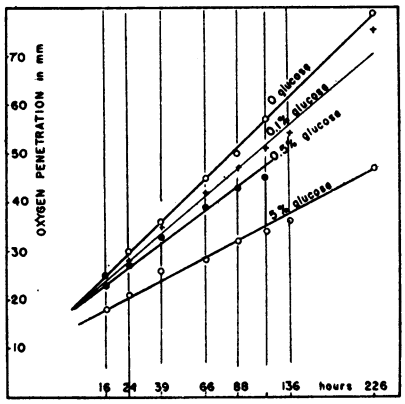

FIG. 1. THE GRADUAL RECOVERY OF REDUCED METHYLENE BLUE BY THE DIFFUSION OF OXYGEN IN NUTRIENT AGAR WITH AND WITHOUT GLUCOSE, PLOTTED AGAINST THE SQUARE ROOT OF TIME

TABLE 7

Oxygen diffusion at different temperatures in 0.5 per cent meat extract + 5 per cent glucose

Indicator: thionine 1:60,000

$\sqrt{\text{TIME}}$	HOURS	TEMPERATURE					
		0°	10°	20°	30°	40°	50°
		Depth of blue zone (mm.)					
1.8	3.25	8	9	10	9.5	9	7.5
3.9	15.25	17	17	16	16	15	11
4.2	18.75	18	18	19	17	16	12.5
4.8	22.75	20	20	20	19	16.5	12.5
5.3	28	21	22	22	20	17	13
6.3	40	23	24	23	22	19	14
7.0	48	26	27	25	23.5	19	14
7.9	63	29	30	27	25	20	14
9.4	88	34	34	31	27	21	14
10.6	112	37	38	33	28	21	15
11.6	136	41	40	34	29	22	14
13.5	184	47	47	39	32	23	15
18.2	330	54	51	42	33	23	15

$2^5 = 32$. This greatly increased oxygen consumption far outweighs the slight increase in diffusion, and oxygen penetrates less deeply at the higher temperature, contrary to table 4. At 50°,

a stable line became established at 15 mm. All oxygen that diffused into the agar was bound by the first 15 mm. of the medium, and this rate of oxidation was maintained for 14 days. We must expect, therefore, that the actual amount of dissolved oxygen in media containing sugar is lower, not because of a lesser solubility, but because the rate of oxygen uptake of the medium, especially at higher temperatures, is so great that the replacement by diffusion is insufficient.

It was found that phosphates are very active oxygen catalysts. Figure 2 shows the advance of the blue zone at 30°C. in methyl-

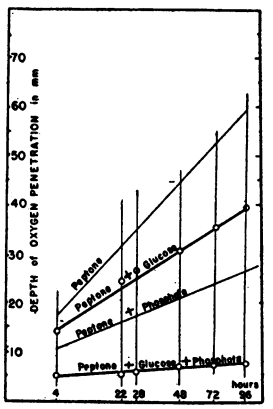

FIG. 2. THE RETARDING EFFECT OF GLUCOSE AND OF PHOSPHATES UPON THE OXYGEN SUPPLY OF PEPTONE AGAR (DEPTH OF BLUE ZONE PLOTTED AGAINST SQUARE ROOT OF TIME)

ene-blue agar containing 0.5 per cent peptone, with and without 2 per cent phosphate buffer, and in peptone plus glucose, with and without phosphate buffer. The addition of phosphate accelerated the oxygen uptake of the peptone much more than the addition of sugar. The combination of glucose and phosphate with peptone resulted in such rapid oxidation of the medium that no oxygen penetrated deeper than 1 cm. in a week. The pH of the media was 7.4 except in peptone glucose without phosphate which had decreased to 6.3 during sterilization.

It is not the phosphate alone that brings about this oxidation. Without peptone, glucose is not greatly attacked. Even during

autoclaving, the high temperature is not sufficient to bring about complete reduction of the dye, and the agar is pale blue after sterilization.

The effect of pH on the rate of oxidation of sterile media may be seen from table 8. Further details on the rôle of phosphates as catalysts of organic oxidations will be published elsewhere.

This rather simple method enables us to test how well a medium is fit for the easy cultivation of anaerobic bacteria. It is most desirable that the medium combines fairly rapidly with oxygen. Addition of reducing sugars, such as glucose or lactose, and buffering with phosphates are quite efficient means.

TABLE 8

Effect of pH on oxidation of 1 per cent peptone with 4 per cent phosphate buffer at 30°

$\sqrt{\text{TIME}}$	HOURS	DEPTH OF BLUE ZONE AT pH					
		5.2	5.6	6.3	7.5	8.1	8.3
2.34	5.5	20	21	19	14	10	9
4.18	17.5	37	35	30	20	14	
6.44	41.5	53	47	43	26	18	15.5
8.09	65.5	57	57	51	30	21	17.5
10.6	113.5	73	65	54	31	23	20

When 0.25 per cent Na_2SO_3 was added to standard nutrient agar, following the suggestion of Knaysi and Dutky (1934), the leuco methylene blue did not become blue in 10 days, except for about 1 mm. at the surface. This effect is not only due to the reduction potential as such, but also to the *rate* of oxygen consumption by the medium. Apparently, the SO_3 ion acts as catalyst.

Since the rate of oxidation increases rapidly with temperature while the solubility of oxygen decreases, a medium may support anaerobic growth at 40° while it is not nearly so good for this purpose at 20°.

Ultimately, media may become completely oxidized. It is general practise not to use old media for cultivation of bacteria preferring anaerobic conditions.

IV. THE FATE OF AEROBIC BACTERIA AFTER EXHAUSTION OF OXYGEN

By George Lloyd Richardson

In part II of these investigations, it had been shown that the dissolved oxygen of the usual culture media is completely exhausted by aerobic bacteria when the population has reached 2 to 100 million per ml. It seemed worth-while to study what happens to these bacteria from this moment on, if they remain under the airtight seal.

The investigation was started with the same technique as before. A number of parallel test-tube cultures were sealed with vaspar (a vaseline-paraffine mixture melting at 50°C.), and at increasing intervals after decoloration of the methylene blue, one of the tubes was opened and the bacteria counted. The results were not very satisfactory because of considerable fluctuation of the results. It is not really surprising that a number of cultures, though identical at the start, differ considerably after 10 to 24 hours of development. One large culture seemed far preferable to a succession of small parallel cultures. This was accomplished by a culture in a Florence flask with a very long neck filled as full as possible, and sealed with a layer of white mineral oil. It was known that the oil seal was not quite as safe as the vaspar seal because methylene blue milk sterilized under oil became blue again in 24 hours. But in parallel experiments with oil seal and vaspar seal, the decoloration times were very nearly the same, and it was hoped that the very small surface exposed in the neck of the Florence flask would make any oxygen diffusion negligible.

These experiments had been preceded a year ago by a somewhat different series of multiplication experiments by Miss E. G. Zimmerman. Three parallel cultures of each species were made in 75 ml. of milk. One of them was in a 125 ml. Erlenmeyer flask, one in a 2 liter Erlenmeyer, and one in a 125 ml. Erlenmeyer with a thick layer of mineral oil over it. The results are shown in figure 3. The graphs represent actual numbers, not logarithms. All cultures of any one species multiplied identically until the

dissolved oxygen was exhausted. This event did not effect the streptococcus which multiplied under oil, i.e., without oxygen, as rapidly and to the same numbers as with the good oxygen supply in the 2 liter flask.

The facultative *Escherichia coli*, having lactose for anaerobic growth, continued to multiply in all three flasks, but in the small flasks, the speed was considerably reduced because the anaerobic

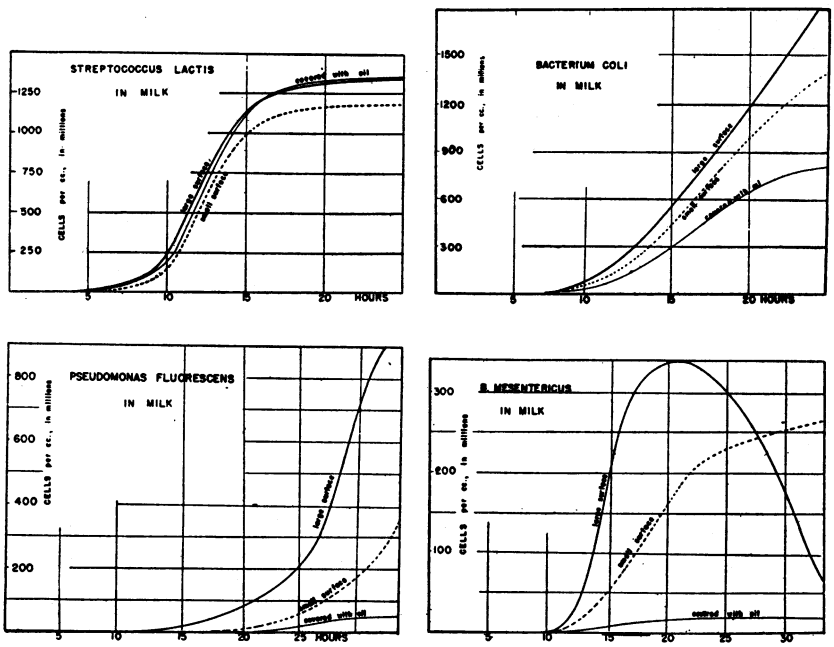

FIG. 3. MULTIPLICATION OF BACTERIA IN MILK WITH DIFFERENT OXYGEN SUPPLY
Heavy line = ample supply (75 ml. of milk in 2-liter flask). Dotted line = medium supply (75 ml. of milk in 125 cc. flask). Thin line = no supply (75 ml. of milk under oil). The ordinates are cells per cubic centimeter in millions, not logarithms.

fermentation does not furnish as much energy as the aerobic oxidation. The two strict aerobes, *Pseudomonas fluorescens* and *Bacillus mesentericus* did not multiply at all under oil, and they increased more slowly in the small flask than in the large one, because of the poorer oxygen supply.

It would appear from these observations that a layer of mineral oil at least 1 centimeter thick should provide adequate protection

against oxygen diffusion into the media beneath. Several experiments extending over fairly long times were made with three bacilli. *Bacillus subtilis* and *B. mesentericus* behaved very much alike, dying rapidly under the oil seal as soon as the methylene blue became decolorized. After 8 hours, 90 to 99 per cent of all cells were dead. *Bacillus cereus* acted differently. After decoloration, it continued to multiply for one, and sometimes 2 and even 3 generations. After that, it decreased in numbers, but more slowly than the others.

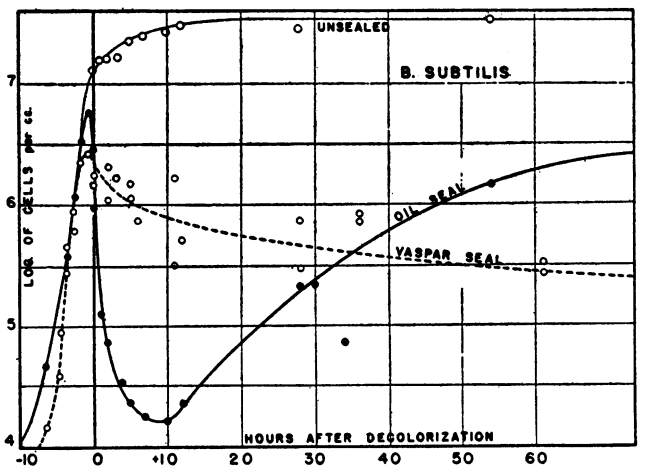

FIG. 4. BACILLUS SUBTILIS IN 1 PER CENT PEPTONE SOLUTION, IN SEALED AND OPEN CULTURES

The zero hour is the time when the dissolved oxygen was exhausted

When the experiments with oil seal were continued beyond 10 hours after decoloration, *Bacillus cereus* continued to die slowly and regularly, but the other two bacteria began to increase again in number. The rate of multiplication was one-half of what it had been with oxygen, and 3 to 4 days after decoloration, the number of cells per ml. was nearly as high, or even higher than it had been at decoloration. The heavy line of figure 4 shows one of several experiments with *B. subtilis* made several months apart, all of which gave similar results. The results obtained with *B. mesentericus* were quite similar, the only difference being a slight difference in rates of death and recovery.

While these results with oil seal were consistent in repeated experiments, they differed from occasional parallel tests made with vaspar seal. It was therefore decided to solve the problem by a long-continued parallel experiment with oil-sealed flasks and vaspar-sealed test tubes. Added to this was an oil-sealed flask which was kept in vacuum except for the removal of samples for plating which required less than ½ hour. A further addition was the "double vaspar seal." A test tube was about half filled with the inoculated medium, sealed with vaspar, about 2 ml. of the inoculated medium were poured on top of the hardened vaspar, and this was sealed again with vaspar. If any oxygen leaked through the top seal, it would be consumed immediately by the culture underneath and could not possibly pass through the lower seal. The culture at the bottom of the test tube could be considered absolutely anaerobic as soon as it became decolorized.

A large number of tubes were thus prepared, and frequent plate counts were made of the different types of cultures. Sometimes two tubes with vaspar seals were opened simultaneously to determine the deviation in duplicate cultures. The results are given in table 9.

The comparison shows very plainly that there is no suggestion of an increase in the cultures of *B. subtilis* after the oxygen is completely removed. The cultures with double vaspar seals which are absolutely anaerobic do not differ essentially from the single vaspar seal cultures, which is a good proof that the single vaspar seal can be considered oxygen-tight for the purpose of bacterial cultivation. On the other hand, it is very evident that a mineral oil seal should not be considered oxygen-tight, because it permits multiplication of bacteria which cannot multiply in the complete absence of oxygen.

However, this is not all that is to be said about the oil. It is quite remarkable that in all experiments with all aerobic bacteria, the rate of death after the exhaustion of oxygen is much greater under oil than under vaspar, although oil permits more oxygen to come through. It suggests that the oil affects the physiology of the cell directly, and not only by preventing oxygen diffusion.

To determine the nature of this influence, mineral oil was

TABLE 9

Number of cells of B. subtilis in 1 per cent peptone solution under various anaerobic conditions

HOURS	OIL SEAL	OIL SEAL + VACUUM	VASPAR SEAL	DOUBLE VASPAR SEAL
0	2,967	2,967	1,900	1,900
9			1,707,000	1,123,000
10	171,000		1,130,000	
11	192,700		2,170,000	963,300
			1,130,000	743,300
12	168,300		1,643,000	1,365,000
13	152,700			
14			1,500,000	1,773,000
			1,150,000	1,040,000
15	87,000		752,000	
16	56,300			
19				1,340,000
				1,067,000
20			1,680,000	1,780,000
			320,000	
21	49,670	90,670	523,000	
37			754,300	455,300
			207,300	417,800
45	85,500	592,200	1,173,000	243,300
			853,000	180,000
70	1,277,000	2,867,000	336,600	284,700
			276,000	250,700
95	3,163,000	1,252,000	209,000	54,670
			165,700	46,330
111			113,700	121,300
			67,000	46,670
141	1,870,000	2,657,000	221,000	45,000
			110,700	29,900
156			121,000	206,700
			120,300	95,300
214			114,700	74,670
			78,300	23,000
230	5,130,000	2,753,000	188,300	58,300
279	613,000	690,000	296,000	58,300
			246,700	17,130
300			192,300	16,270
330	910,000	663,000	243,700	77,000
			62,700	10,130

shaken with water and with peptone solution; the emulsion was centrifuged, the water used for preparing peptone solution, and

these media were used for the cultivation of the bacilli under vaspar. No significant difference was observed between the media which had been in intimate contact with oil, and parallel media made with pure water. It seems therefore that the peculiar effect of the mineral oil cannot be ascribed to a water-soluble component.

As a different type of aerobic species, *Pseudomonas fluorescens* was tested under the same conditions. This bacterium proved to be capable of multiplying very, very slowly under the vaspar

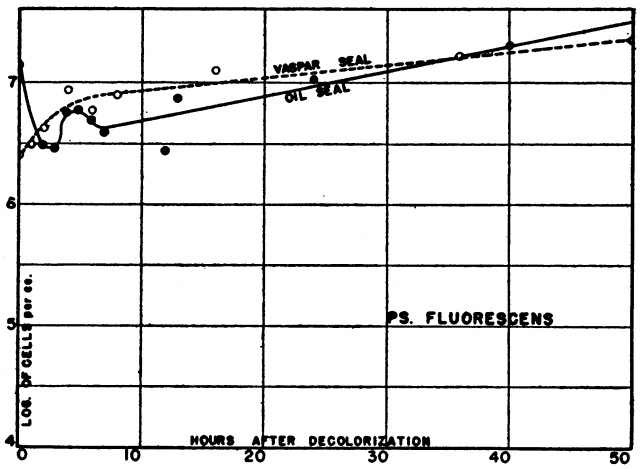

FIG. 5. PSEUDOMONAS FLUORESCENS IN 1 PER CENT PEPTONE SOLUTION, IN SEALED CULTURES
The zero hour is the time when the dissolved oxygen is exhausted

seal after decoloration. There is no indication of death in 1 per cent peptone solution, even 4 days after the oxygen had been exhausted. Figure 5 shows the organism under oil and under vaspar.

Again, the two seals show a different effect. Again there is a rapid death of bacteria under oil as soon as the oxygen is exhausted while there is no indication of death, merely a great decrease in the multiplication rate, under vaspar. The bacteria under oil recover soon (the sudden rise and fall around 5 hours shown in figure 5 may be due only to an accidental scatter-

ing of the experimental data although the same scattering was observed in two separate experiments) and their multiplication rate is greater than that under vaspar, suggesting leaking of oxygen through the oil.

These experiments force us to the conclusion that the tolerance of anaerobic conditions varies with different groups of aerobic organisms. The three species of *Bacillus* which were tested died in the absence of oxygen. The initial rate of death, expressed as per cents of survivors dying per hour, was

	Under oil *per cent*	Under vaspar *per cent*
For *B. mesentericus*	85	51
For *B. subtilis*	60	13
For *B. cereus*	40	23

The latter differed from the first two species by its ability to multiply for one or two generations after the exhaustion of oxygen. The same phenomenon had been observed in 1912 by Müller, who grew *E. coli* and *Pseudomonas fluorescens* in very dilute, synthetic media. In every one of the many experiments published in detail, multiplication continued for about one hour after the complete exhaustion of oxygen. *B. mesentericus* and *B. subtilis* never showed this phenomenon in our experiments. On the contrary, *B. subtilis* frequently began to decrease before the culture was decolorized (see fig. 4).

Pseudomonas fluorescens differs from the sporeformers by its ability to multiply slowly in the complete absence of oxygen when cultivated in a solution of 1 per cent Bacto-peptone.

SUMMARY

I. Our knowledge of the oxygen consumption of multiplying bacteria is incomplete, and little is known about the utilization of the energy derived from this oxidation. A definite knowledge of the actual oxygen supply in so-called aerobic cultures is also missing. The following studies are intended to fill some of the gaps in our knowledge.

II. A simple method has been used to measure the oxygen con-

sumption of multiplying bacteria per cell per hour. The results of more than 100 determinations can be summarized as follows:

Streptococci	0.5	to	2×10^{-10} mgm. O_2 per cell per hour
Enterobacteriaceae	3	to	5×10^{-10} mgm. O_2 per cell per hour
Pseudomonades	8	to	9×10^{-10} mgm. O_2 per cell per hour
Bacilli	10	to	80×10^{-10} mgm. O_2 per cell per hour

The oxygen consumption does not depend on the size only; *Pseudomonas* requires more oxygen than the Enterobacteriaceae group, for the same amount of cell substance.

The efficiency of utilization of energy in bacteria is not much greater than that of animals. It appears to be of the same magnitude as that of cold-blooded animals.

Media containing sugars take up oxygen in measurable amounts in a short time. It is possible to compute the oxygen uptake of media separately from the uptake by growing bacteria.

III. The rate of oxygen diffusion is very slow. It can be measured by a very simple method, by adding agar and methylene blue to the medium, autoclaving until colorless, and measuring the advance of the blue zone with time. In water agar, the oxygen diffused as far as 5 cm. in 24 hours which is entirely insufficient to replace the oxygen consumed by 10^7 or 10^8 cells per ml. In culture media, the blue zone advances more slowly because the medium itself combines with oxygen. Media containing carbohydrates take up more oxygen than media without them. Phosphates are active catalysts for oxidation, especially at pH higher than 6.

IV. In a medium saturated with oxygen from the air, all dissolved oxygen is exhausted when aerobic bacteria have multiplied to 2 to 10 million cells per ml. The rate of oxygen diffusion is not nearly sufficient to provide the entire culture with oxygen.

The fate of aerobic bacteria at this point was studied by comparison of ordinary test tube or flask cultures with similar cultures sealed with oil or paraffine using a 1 per cent solution of peptone as medium. The rate of multiplication in both types of cultures was identical until the oxygen was almost completely exhausted. After exhaustion, not all types of bacteria behaved alike. The aerobic sporeformers died at a fairly rapid rate (13 to 50 per cent

of survivors per hour) while *Pseudomonas fluorescens* continued to multiply very slowly in complete absence of oxygen.

It could be shown that a vaspar (vaseline plus paraffin) seal was sufficient to keep the oxygen of the air away from the culture while mineral oil was not sufficient. Besides, mineral oil appeared to become suddenly toxic to bacteria as soon as all oxygen was exhausted, although the bacteria recovered later. The cause of this has not been ascertained.

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