BACTERIAL UTILIZATION OF LOW CONCENTRATIONS OF ORGANIC MATTER[1]

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Although several investigators have studied the effect of concentration of organic nutrients upon the growth of microörganisms, most of the experiments have been concerned with concentrations which are much higher than those which occur in soil solutions, sea water and other natural environments. According to Rahn (1932) the concentration of food does not influence the rate of growth of bacteria except when the concentration is very low, 0.01 to 0.1 per cent. Penfold and Norris (1912), Hucker and Carpenter (1927), Friedlein (1928), Bigger (1937) and others report that the minimum concentration of organic nutrients required for the multiplication of heterotrophs ranges from 0.001 to 0.01 per cent or 10 to 100 mgm./l.

That many bacteria multiply and are otherwise physiologically active in very dilute nutrient solutions is manifest from the abundance of bacteria in lake water (Henrici, 1939) and sea water (Benecke, 1933). The organic content of sea water is generally less than 5 mgm./l., yet during the storage of sea water in the laboratory the bacterial population usually increases from a few hundred to several million bacteria per ml. (Waksman and Carey, 1935a). Bacterial multiplication is accompanied by the evolution of carbon dioxide, oxygen consumption, ammonia production, nitrate reduction and other biochemical changes (ZoBell and Anderson, 1936). Similar changes have been noted in lake water (ZoBell, 1940) which contained less than 10 mgm. of organic matter per liter. Butterfield (1929) found that after an extended lag period *Aerobacter aerogenes* multiplied slowly in a solution containing only 0.5 mgm./l. each of glucose and peptone. Heukelekian and Heller (1940) found that when glass beads were present to augment the area of solid surface, *Escherichia coli* grew in either glucose or peptone solutions as dilute as 0.5 mgm./l.

Recently ZoBell and Grant (1942) reported that marine bacteria multiply in mineral media when the concentration of organic nutrients is less than 0.1 mgm./l. There were indications that lower concentrations might provide for bacterial activity. The following experiments were designed to determine the effect of the concentration of various kinds of organic matter upon its rate of utilization by bacteria.

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EXPERIMENTAL METHODS

Since it is a physiologically balanced salt solution which provides for the mineral requirements of all microörganisms which have been tested, aged sea water was used in the preparation of the nutrient solutions. It must be diluted 1:10 to 1:20 when used for fresh-water or soil bacteria but it is satisfactory without dilution for marine bacteria (ZoBell, 1941). The organic content of sea water was reduced to a fraction of a milligram per liter by "ageing" it in the dark for several months at room temperature. The water was collected from unpolluted areas in the sea in glass receptables and passed through a fine fritted glass filter to remove particulate material. After five or six months storage the utilizable organic matter content was found to be around 0.2 mgm./l., and by successively transferring the water to acid-cleaned bottles, autoclaving, reinoculating with a few bacteria and incubating the water, the organic content could be reduced to 0.02 mgm./l.

Bacto-peptone to give concentrations ranging from 0.1 to 100 mgm./l. (0.000,01 to 0.01 per cent or 0.1 to 100 p.p.m.) was added to the water. The media were dispensed in 10 ml. quantities in test tubes, sterilized in the autoclave and inoculated with a 2 mm. loopful containing 10 to 100 bacteria suspended in organic-matter-free water. The inoculated media were incubated at 22°C. and examined daily for evidence of multiplication. Plating procedures revealed that all thirty of the different species of common marine bacteria under investigation multiplied in all concentrations of peptone used. Little or no multiplication occurred in the control medium to which no peptone was added. There was not the faintest clouding of the media containing less than 10 mgm./l. of peptone although most of the cultures clouded the medium which contained 100 mgm./l. of peptone. Eight of the cultures produced a perceptible turbidity in the medium containing 10 mgm./l. of peptone.

Similar results were obtained with glucose. When glucose and other non-nitrogenous substrates were used, 1 mgm./l. of ammonium phosphate was added to the water to provide a source of nitrogen.

It becomes obvious from a few simple calculations that one could not expect media containing less than 10 mgm./l. of organic matter to be clouded by the growth of bacteria. If quantitatively converted into bacterial protoplasm, 1.0 mgm. of organic carbon would yield around 10^{10} bacteria. A medium containing 1.0 mgm./l. of organic carbon could yield no more than 10^7 bacteria per ml. Actually the number would be less because, as will be discussed below, only part of the organic matter is converted into bacterial protoplasm. Since it requires 10^7 to 10^8 bacteria to render a clear medium perceptibly turbid, it follows that ordinary turbidimetric methods are not applicable to the study of bacterial multiplication in dilute nutrient solutions. This may explain why certain workers have reported that bacteria do not multiply in dilute nutrient solutions.

OXYGEN CONSUMPTION

The effect of concentration of organic matter upon the rate of its utilization by bacteria was investigated by noting oxygen consumption. Concentrations of glucose ranging from 0.25 to 100 mgm./l. were added to organic-matter-free water inoculated with a few hundred bacteria per ml. After thorough shaking to provide for uniformity in composition and to insure its saturation with oxygen, the water was dispensed in 60 ml. glass-stoppered bottles. The oxygen content of the water in duplicate pairs of bottles was determined at once, using a modified Winkler technique which was accurate to ±0.01 mgm./l. The oxygen content of the remaining bottles of inoculated water was determined after different periods of incubation in the dark, in a water bath at 22°C. From these data the amount of oxygen consumed by bacteria after different periods of time in the presence of different concentrations of glucose was calculated after making corrections for the controls. The results of a representative experiment are summarized in table 1.

TABLE 1

Amount of oxygen consumed by marine bacteria in water containing different concentrations of glucose after different periods of incubation at 22°C.

PERIOD OF INCUBATION	INITIAL CONCENTRATION OF GLUCOSE IN MGM./L.							
	0.25	0.50	1.0	2.5	5.0	10.0	100	1000
hours	*mgm./l.*	*mgm./l.*	*mgm./l.*	*mgm./l.*	*mgm./l.*	*mgm./l*	*mgm./l.*	*mgm./l.*
21	0.01	0.02	0.02	0.02		0.13	0.03	0.24
45	0.17	0.27	0.20	0.59	0.99	1.78	2.35	7.13
57					1.97	3.51	7.79	8.85*
69	0.19	0.31	0.39	1.05	2.21	4.20	8.85*	
95	0.19	0.33	0.52	1.50	2.42	4.72		
168	0.18	0.34	0.63	1.49	2.80	5.08		

* All of the dissolved oxygen was utilized.

Similar results were obtained with peptone as shown graphically by figure 1. In either peptone or glucose solutions the rate of bacterial activity, as indicated by oxygen consumption, is more or less directly proportional to the initial concentration of substrate. Plate counts indicate that the rate of bacterial multiplication as well as the rate of oxygen consumption is a function of the concentration of the substrate. However, we do not have sufficient data to appraise quantitatively the effect of concentration of substrate upon multiplication and respiration. It has not been feasible to prepare suspensions of "resting cells" which are sufficiently free of oxidizable organic matter to test the effect of concentrations of organic substrates, which range from 0.25 to 10 mgm./l., upon bacterial respiration. Geiger-Huber (1934) found that the rate of respiration of yeasts was proportional to the concentration of glucose until the latter exceeded M/500, above which the rate was independent of the glucose concentration. However, the lowest concentration of glucose tried by Geiger-Huber was M/10,000 or 18 mgm./l.

It is significant that while bacteria multiply more rapidly in the higher concentrations, the substrate disappears first in media containing the lowest initial concentration of the substrate. This is illustrated by the data in table 2 in which the per cent of the glucose oxidized is given, calculated upon a basis of 1.07 mgm. of oxygen being required to oxidize 1.0 mgm. of glucose to carbon dioxide and water. It will be observed that more than half of the glucose was oxidized in five days or less when the initial concentration was less than 1 mgm./l., whereas progressively longer periods of time were required to oxidize half of higher concentrations.

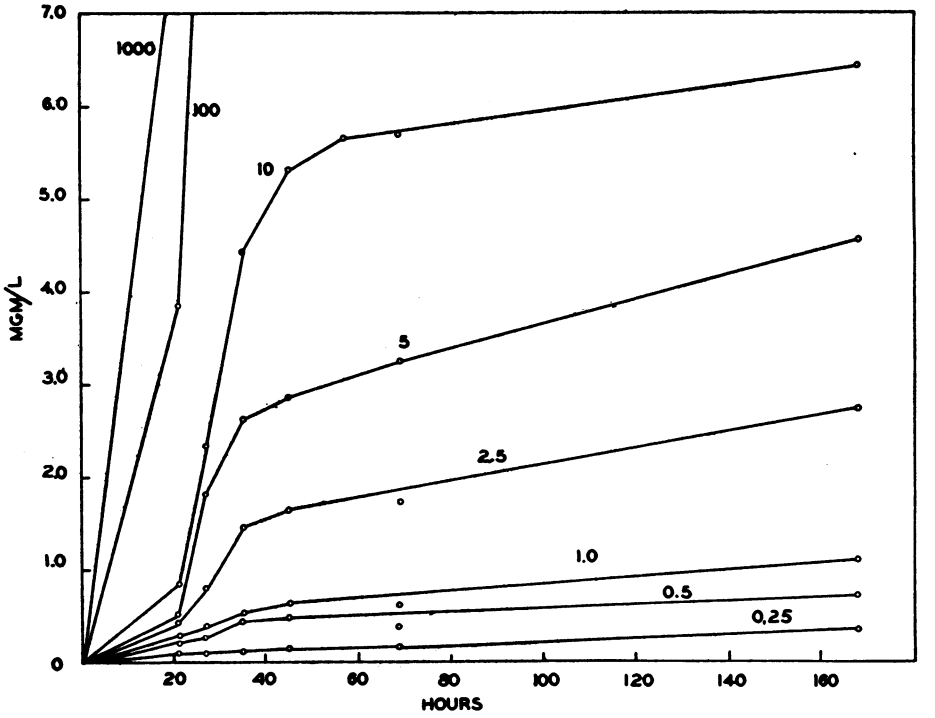

FIG. 1. AMOUNT OF OXYGEN CONSUMED BY BACTERIA GROWING IN A MINERAL SOLUTION ENRICHED WITH 0.25 TO 1000 MGM./L. OF PEPTONE AFTER DIFFERENT PERIODS OF TIME AT 22°C.

After 50 to 65 per cent of the glucose is oxidized, the rate of oxygen consumption decreases sharply regardless of the initial concentration of glucose, although there is still plenty of dissolved oxygen left. The work of ZoBell (1940) established that the rate of oxidation of organic matter by bacteria is independent of the oxygen tension until the latter is reduced to less than 0.3 mgm./l.

After 30 days incubation no glucose could be detected in the media, which initially contained 0.1 to 10 mgm./l., although oxygen consumption indicated that only 60 to 70 per cent of the glucose had been oxidized. The explanation was found in the bacterial populations of the respective media as determined by the direct microscopic procedure. The calculated weight of bacteria in the

medium and on the walls of the culture vessel approximated the weight of the rest of the glucose. In other words, in dilute solutions approximately 60 to 70 per cent of the glucose is oxidized in 3 to 30 days at 22°C. and 30 to 40 per cent is converted into bacterial protoplasm. According to Rahn (1932) when the initial concentration of fermentable substance is low, considerably less CO_2 or alcohol is produced than should be expected, probably due to the utilization of a comparatively large amount of fermentable material for cell construction in a poor medium. Waksman and Carey (1935b) reported that during the storage of sea water, bacteria oxidize around 60 per cent of the organic matter and convert 40 per cent of it to bacterial protoplasm.

With prolonged incubation (60 to 120 days) oxygen consumption continues at a very slow rate. This is attributed to the oxidation of cell substance although experimental proof is lacking due to the small concentrations of reactants

TABLE 2

Per cent of glucose oxidized by bacteria in water treated with different concentrations of glucose based upon oxygen consumption after different periods of incubation at 22°C.

PERIOD OF INCUBATION	INITIAL CONCENTRATION OF GLUCOSE IN MGM./L.						
	0.10	0.25	0.50	1.0	2.0	5.0	10
days	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
2	56.8	44.9	33.6	27.2	12.7	5.6	5.5
3	60.1	64.1	39.2	33.6	34.4	19.4	12.8
4	67.3	65.8	52.4	42.0	39.2	27.8	14.7
5		71.2	64.9	50.1	42.6	40.3	29.2
7	68.0	69.9	57.8	60.7	51.2	44.7	34.6
10	74.8	64.1	58.5	57.0	53.8	52.7	39.3
16		72.3	56.1		58.4	61.2	46.7
20	69.5	71.2	61.7	61.6	61.5	63.2	57.9
30	66.8	71.2	67.2	60.7	65.5	60.4	63.8

With low concentrations of substrate the experimental error is relatively high.

and the difficulties involved in estimating the bacterial populations. The decrease in the number of viable bacteria as indicated by plate counts suggests that some of the cells are dying and undergoing decomposition. This process can be accelerated by heating the old cultures to 50 or 60°C. for ten minutes.

Besides glucose and peptone, it has been demonstrated that low concentrations of glycerol, ethanol, lactate, succinate, asparagine and starch are quantitatively oxidized by bacteria occurring in sea water. The per cent of different concentrations ranging from 0.25 to 10 mgm./l. of each compound which was oxidized after 16 days incubation at 22°C. is summarized in table 3. The per cent of each compound oxidized was calculated from the amount of oxygen consumed by the bacteria in the media and the amount of oxygen which would be required to oxidize the compound completely to carbon dioxide and water. As with glucose, it was found that from 50 to 70 per cent of each compound tried was oxidized within 16 days. No significant differences were noted in the

degree of oxidation of the different compounds probably because mixed cultures were used, but differences were found in the rate at which the different compounds were oxidized. Determinations made after 2, 5 and 10 days showed that ethanol, lactate, succinate, glycerol and glucose were utilized most rapidly in the order listed, while starch and asparagine were utilized more slowly.

As little as 0.1 mgm./l. of the compounds listed in table 3 was sufficient to initiate the multiplication of marine bacteria, and apparently this concentration was quantitatively utilized but with such low concentrations the experimental error is high.

TABLE 3

Per cent of different organic compounds oxidized by bacteria in water as determined by oxygen consumption after 16 days incubation at 22°C.

ORGANIC SUBSTRATE	INITIAL CONCENTRATION OF ORGANIC SUBSTRATE IN MGM./L.					
	0.25	0.50	1.0	2.5	5.0	10.0
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Glucose	68.1	67.1	60.0	62.7	57.9	36.2
Succinate	89.3	83.7	81.0	85.7	71.6	40.6
Glycerol	67.6	58.9	71.3	73.0	49.9	
Starch	58.7	62.4	55.1	56.3	51.2	31.6
Lactate	78.6	84.7	67.1	63.6	64.4	
Ethanol	79.1		63.5	62.5	56.7	
Asparagine	64.3	58.2	47.2	42.8	45.1	

EXPERIMENTS WITH PURE CULTURES

Suspensions of pure cultures of marine bacteria in organic-matter-free water were used to inoculate sterile glass-stoppered bottles which were then filled with sterile oxygenated organic-matter-free water. Eight bottles inoculated with each culture were filled with water containing 5 mgm./l. of glucose and eight were filled with water containing no glucose. Duplicates of each were analyzed for dissolved oxygen at the beginning of the experiment and the remaining bottles were placed in a sterile water bath. Oxygen consumption was determined on duplicates after 5, 10 and 15 days incubation at 22°C. Table 4 shows the amount of oxygen which was consumed in 15 days in the water with and without glucose.

It will be observed that every culture consumed significantly more oxygen in water enriched with 5 mgm./l. of glucose than in water to which no glucose was added. The data in table 4 are representative of the results obtained with 70 different cultures of marine bacteria. Not only do all of the cultures oxidize glucose; they attack concentrations as low as 5 mgm./l., and some of them quantitatively utilize this amount of glucose in 15 days. Incidentally, most of these cultures do not produce acid in glucose media and according to standard methods of the Committee on Bacteriological Technic of the S. A. B. (1940), they would be described as non-glucose-fermenters. They may not produce any gas and no acid except carbonic (which is too poorly dissociated to alter perceptibly the pH of the medium) but oxygen consumption provides incontrovertible evidence that they oxidize glucose.

An extension of the experiments with lower concentrations of glucose revealed that as little as 0.1 mgm./l. promoted the multiplication and respiration of all ten pure cultures of marine bacteria which were used as inocula. Although there were marked differences in the rate of utilization, some of the cultures quantitatively oxidized 0.1 to 5 mgm./l. of glucose in 30 days at 22°C.

It has been suggested that marine bacteria coming from an environment which is notoriously low in organic matter may be specially adapted to utilize low concentrations of organic nutrients. Therefore a few well-known cultures which are ordinarily found associated with organic matter were used to inoculate dilute glucose solutions in glass-stoppered bottles. *Escherichia coli*, *Staphylococcus citreus*, *Bacillus megatherium*, *Proteus vulgaris* and *Lactobacillus lactis* were all found to consume oxygen in glucose solutions as dilute as 0.1 mgm./l. Plate

TABLE 4

Oxygen consumed by pure cultures of marine bacteria in sea water with and without the addition of glucose, after 15 days at 22°C.

CULTURE	GLUCOSE CONTENT		CULTURE	GLUCOSE CONTENT	
	None	5 mgm./l.		None	5 mgm./l.
	mgm./l.	*mgm./l.*		*mgm./l.*	*mgm./l.*
None	0.03	0.01	524	0.78	3.18
502	0.26	0.65	530	0.55	2.71
505	0.18	3.24	533	0.54	1.57
510	0.19	0.78	541	0.34	2.10
511	0.34	2.29	542	0.73	2.06
513	0.00	1.52	545	0.40	1.76
514	0.09	0.54	546	0.32	1.45
516	0.30	1.70	547	0.77	2.67
517	0.17	1.30	549	0.62	1.93
518	0.12	3.82	552	0.65	2.88
520	0.51	2.91	553	0.39	3.08
521	0.32	3.10	555	0.27	2.30
522	0.18	3.03	556	0.26	1.32

counts showed that the bacteria had multiplied, although none of the cultures caused turbidity in solutions containing less than 10 mgm./l. of glucose.

Using turbidity as a criterion of growth Bigger (1937) noted that coliform bacteria failed to "grow" in concentrations of peptone lower than 1:5000 (200 mgm./l.), but in later work in which plate counts were employed to detect growth (Bigger and Nelson, 1941), coliform bacteria were observed to multiply in glass re-distilled water which contained no more than one part of nutrient in 2,500,000 parts of water (0.4 mgm./l.). Incidentally Bigger and Nelson believed that the coliform bacteria obtained their energy autotrophically from the oxidation of minute traces of ammonium absorbed from the atmosphere, but regardless of whether the coliform bacteria utilized ammonium or peptone, the fact remains that their energy requirements were satisfied by very low concentrations. According to Heukelekian and Heller (1940) *E. coli* failed to grow when the concen-

tration of organic nutrients was less than 0.5 to 2.5 mgm./l. unless glass beads were added to increase the solid surface.

DISCUSSION

There is no reason to believe that 0.1 mgm./l. is the lowest concentration of food which will provide for the multiplication and respiration of bacteria although lower initial concentrations have not been tried experimentally due to the difficulties of preparing mineral solutions containing lower concentrations and testing for their utilization. However, since 0.1 to 0.25 mgm./l. of food is quantitatively utilized, concentrations considerably lower must be utilized. Any single molecule of food which comes into contact with the bacterial cell may be utilized. If the molecules reach the cell faster than they are needed to maintain basal metabolism, some of the food may be converted into bacterial protoplasm. When very low (less than 10 mgm./l.) the concentration of food seems to be of importance only in so far as it influences the rate of the reaction. The rate of utilization is almost directly proportional to the concentration of food although when the concentration is very low, proportionately more of it seems to be converted into bacterial protoplasm and less is oxidized.

Anything which tends to bring the food into contact with bacterial cells will accelerate its utilization. Increasing the concentration of food or increasing the size of the inoculum accelerates the utilization of the food. When the concentration of food is very low, solid surfaces such as sand, glass beads or the walls of the culture receptacle which adsorb food and bacteria promote the multiplication of bacteria and the oxidation of organic matter. This explains why bacteria often multiply faster in water samples stored in small receptacles which present much more adsorbing surface per unit of water than in large receptacles (Whipple, 1901, ZoBell and Anderson, 1936, Lloyd, 1937). This view is substantiated by the fact that neither water volumes nor solid surfaces influence the rate of bacterial activity in water rich in organic nutrients or in water containing only dissolved nutrients such as glucose, glycerol or lactate, for example, which are not readily concentrated on solid surfaces by adsorption. The observations of the senior author that the colloidal organic matter occurring in lake water or sea water is concentrated by adsorption on glass have been confirmed by the work of Stark *et al.* (1938), Heulelekian and Heller (1940) and Harvey (1941). In the experiments of Bigger and Nelson (1941) referred to above, they found that ignited talc, asbestos, kaolin, silica, unglazed porcelain, kieselguhr, permutit and other inert solids rendered dilute nutrient solutions growth-promoting.

Much information on the factors which influence the metabolism of microorganisms could be gained by observing them in dilute nutrient solutions approximating the concentrations found in their native habitats. In dilute nutrient solutions the organisms grow more slowly, and it may be more difficult to test for end products, but the results would probably be more representative of the behavior of the microorganisms in their native habitats. Moreover, in dilute nutrient solutions there is little likelihood of the accumulation of end products influencing the activity of enzymes or possibly preventing the regeneration of enzymes (Rahn, 1932).

According to Hastings (1923) *Streptococcus lactis* and allied microorganisms behaved quite differently in dilute nutrient solutions than in the artificial nutrient solutions ordinarily used in the laboratory. In a recent personal communication Professor Hastings comments, "It has always seemed to me that we tend to grow microorganisms in the laboratory under conditions which are vastly different from those existing in nature, and that therefore the viability and vitality of the organisms as we study them may be quite different from those in nature."

SUMMARY

1. Plate counts reveal that marine heterotrophic bacteria multiply in mineral solutions containing only 0.1 mgm./l. of peptone or glucose but the media are not clouded unless the concentration of initial organic nutrient is 10 to 100 mgm./l.

2. The rate of multiplication and oxygen consumption in solutions containing less than 10 mgm./l. is more or less directly proportional to the concentration of the substrate.

3. Concentrations of glucose, glycerol, ethanol, lactate, succinate, starch and asparagine ranging from 0.25 to 5 mgm./l. were quantitatively utilized by bacteria in 16 to 30 days at 22°C. From 60 to 70 per cent of the organic substrate is oxidized and 30 to 40 per cent is converted into bacterial protoplasm.

4. Using oxygen consumption as a criterion, it was found that 70 different pure cultures of marine bacteria utilize glucose although very few of them produce acid in standard methods media. The cultures attack concentrations of glucose as low as 0.1 mgm./l.

5. *Escherichia coli*, *Staphylococcus citreus*, *Bacillus megatherium*, *Proteus vulgaris* and *Lactobacillus lactis* multiply and consume oxygen in glucose solutions as dilute as 0.1 mgm./l. Lower concentrations of nutrients were not tried due to experimental difficulties.

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