FACTORS WHICH CONTROL MAXIMAL GROWTH OF BACTERIA

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ABSTRACT

SINCLAIR, N. A. (Washington State University, Pullman) AND J. L. STOKES. Factors which control maximal growth of bacteria. J. Bacteriol. **83**:1147–1154. 1962.—In a chemically defined medium containing 1% glucose and 0.1% $(NH_4)_2SO_4$, both of these compounds are virtually exhausted by the growth of *Pseudomonas fluorescens*. If these carbon, energy, and nitrogen sources are added back to the culture filtrate, maximal growth to the level of the original culture is obtained. This process can be repeated several times with the same results. Eventually, however, the supply of minerals in the culture limits growth. When the nutrient levels are raised to 3% glucose and 0.3% $(NH_4)_2SO_4$, lack of oxygen and low pH limit growth before the supply of nutrients is exhausted. There is no evidence that specific autoinhibitory substances are produced either in chemically defined or complex nitrogenous media or that physical crowding of the cells limits growth. The results with *Escherichia coli* are similar to those with *P. fluorescens*. However, after a few growth cycles aerobically and after only one growth cycle anaerobically, inhibitory substances, probably organic acids, accumulate and limit growth.

Several explanations have been offered for the cessation of growth in bacterial cultures. These include accumulation of toxic metabolic products, exhaustion of essential nutrients and oxygen, development of an unfavorable pH, and physical crowding or Bail's M-concentration hypothesis.

The early literature in this field has been reviewed carefully by Henrici (1928). The most generally accepted idea at that time was that growth in bacterial cultures is limited by the accumulation of toxic metabolic products. In most cases, these toxic materials were not identified and appeared to be either thermolabile, nonfilterable substances (Eijkman, 1904; Rahn, 1906) or heat-stable filterable substances (Hajos, 1922). Hydrogen peroxide, however, was identified as the toxic compound in *Diplococcus pneumoniae* cultures (McLeod and Gordon, 1922), and undissociated lactic acid in *Streptococcus lactis* cultures (Rogers and Whittier, 1928). In addition, Rogers and Whittier found that exhaustion of essential nutrients and O_2 may also limit growth.

Bail's contention that bacterial cells stop multiplying when they become too crowded has received scant experimental support. The fact that cultures on solid media are much denser than those in liquid media, and that the cell crop increases with increase in the nutrient content of the medium constitute strong evidence against Bail's hypothesis. Van Niel (1949), in his critical review on the kinetics of microbial growth, suggested that cessation of growth in Bail's cultures was probably owing to exhaustion of O_2, a limiting factor which, according to van Niel, has not received adequate attention in growth studies. The more recent investigations of Dagley and Hinshelwood (1938), Monod (1942), and of Ecker and Lockhart (1961*a*) with the coli-aerogenes group show clearly that the total yield of cells is proportional, over a wide range, to the amount of food available.

The present investigations were undertaken to obtain additional information on the factors which limit the growth of bacteria in cultures. Chemically defined media were used and, in contrast to previous investigations, quantitative analyses were made to determine the extent of disappearance of the carbon, energy, and nitrogen sources from the medium during growth. Also, attempts were made to induce the accumulation of toxic metabolites in the cultures by a serially repeated process of growing the organism, filtering off the cells, and reinoculating the spent culture medium.

MATERIALS AND METHODS

Medium and cultural conditions. A psychrophilic strain of *Pseudomonas fluorescens* and a fecal

strain of *Escherichia coli* were used. Stock cultures were maintained on slants of the chemically defined medium described below, solidified with 1.5% agar.

The chemically defined medium consisted of 1% glucose, 0.1% $(NH_4)_2SO_4$, 0.02% $MgSO_4 \cdot 7H_2O$, 1.25% K_2HPO_4, and 0.15% KH_2PO_4 in distilled water. For the experiments with *P. fluorescens*, 0.05% Na citrate $\cdot 2H_2O$ was added to the above medium, since it decreased the lag period. All media were adjusted to pH 7.0. In some experiments, the glucose and $(NH_4)_2SO_4$ concentrations were varied and also Trypticase soy broth was used.

Inocula were prepared by suspending the growth from an 18-hr slant culture in 8 ml of distilled water; 0.2 ml of this suspension was used to inoculate 50 ml of medium. Aerobic experiments were carried out on a New Brunswick rotatory shaker at 30 C for *P. fluorescens* and 35 C for *E. coli*. Shallow layers of medium were used to facilitate aeration. The ratio of medium to flask volume was approximately 1:10. Anaerobic studies with *E. coli* were made in glass-stoppered bottles, 30-ml to 230-ml capacity, completely filled with medium and incubated at 35 C. In a few experiments, anaerobiosis was established in flask cultures by use of an atmosphere of 95% N_2 and 5% CO_2.

Growth was determined turbidimetrically with a Klett photometer (red filter) and also by surface and pour-plate counts for *P. fluorescens* and *E. coli*, respectively, with Trypticase soy agar. The anthrone method (Umbreit, Burris, and Stauffer, 1957) was used to analyze for carbohydrate. Ammoniacal nitrogen was determined by nesslerization after collection of NH_3 by steam distillation or by the microdiffusion technique of Conway (1950). All chemical assays were made immediately after the cells entered the stationary phase. In this way, the complicating effect of further utilization of nutrients by the nongrowing cultures was eliminated.

Growth-cycle technique. After a culture had reached maximal growth, the cells were removed by centrifugation. The supernatant fluid was adjusted to pH 7.0 with 1 N NaOH, and the volume, after correction for sampling, was adjusted to the original level with distilled water. The supernatant fluid was then divided into four equal portions and sterilized by passage through bacterial sintered-glass filters. To the four portions were added, respectively, no nutrients, sterile glucose, sterile $(NH_4)_2SO_4$, and both glucose and $(NH_4)_2SO_4$. The nutrients were added back to the filtrates in amounts sufficient to raise them to the original levels of 1% glucose and 0.1% $(NH_4)_2SO_4$.

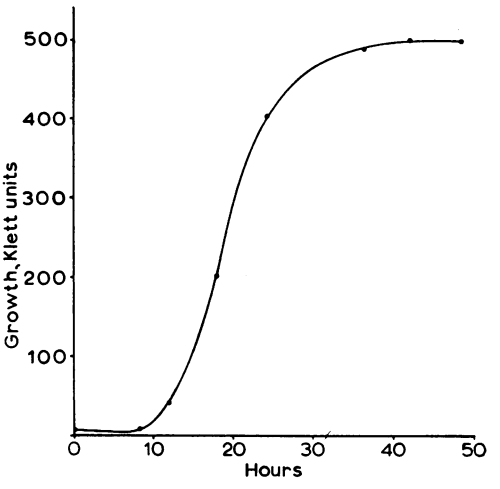

FIG. 1. *Growth curve of Pseudomonas fluorescens in a chemically defined medium.*

TABLE 1. *Aerobic growth and utilization of glucose and ammonium-nitrogen by Pseudomonas fluorescens*

Determination	Initial	Final
Turbidity, Klett units	6	450
Viable cells, millions per ml	3.4	3,700*
pH	7.0	6.2
Glucose, mg per ml	9.5	0.2
$NH_4^+ - N$, mg per ml	0.27	0.01

* Maximal crop.

RESULTS

Aerobic growth of P. fluorescens. When *P. fluorescens* is grown in shallow layers of chemically defined medium on the shaker, maximal growth occurs in about 2 days. The growth curve of a typical culture is shown in Fig. 1. The results of a representative experiment in which chemical analyses as well as plate counts were made are given in Table 1. A maximal count of 3.7 billion cells per ml was obtained, the pH decreased from 7.0 to 6.2, and 98% of the glucose and 96% of the $(NH_4)_2SO_4$ in the medium

TABLE 2. *Aerobic growth of Pseudomonas fluorescens in its own culture filtrates, with and without nutrient supplementation*

Determination	Growth cycle 2				Growth cycle 3			
	Addenda				Addenda			
	Nil	Glucose	$NH_4^+ - N$*	Glucose + $NH_4^+ - N$*	Nil	Glucose	$NH_4^+ - N$	Glucose + $NH_4^+ - N$
Turbidity, Klett units	36	54	236	307	20	10	9	425
Viable cells, millions per ml	140	240	1,300	2,300	170	30	32	4,500
pH, final	7.4	7.3	7.9	6.4	7.0	7.0	7.1	6.5
Glucose, mg per ml	0.2	9.6	0.09	0.54	0.2	9.2	0.08	0.84
$NH_4^+ - N$, mg per ml	0.006	0.01	0.01	0.01	0.003	0.01	0.25	0.01

* Only 0.5 mg per ml rather than the usual 1.0 mg per ml was present initially.

were consumed. These data indicate that maximal growth of *P. fluorescens* is controlled by the supply of carbon, energy, and nitrogen and that growth stops when these are virtually exhausted. The initial levels of glucose and $(NH_4)_2SO_4$ in the medium were 1% and 0.1%, respectively. Since these are the amounts which are used customarily in culture media, the above data suggest that exhaustion of these nutrients may be the principal growth-limiting factor in the usual bacterial cultures.

The cells of *P. fluorescens* were removed from the culture by centrifugation and filtration. Samples of the sterile filtrate received no supplementation, or glucose, or $(NH_4)_2SO_4$, or both, in amounts sufficient to bring these nutrients to the original medium levels. Inadvertently, and only in this second cycle, the $(NH_4)_2SO_4$ level was raised to 0.05% instead of 0.1%. On reinoculation and incubation, a small amount of growth (140 million cells per ml) occurred in the unsupplemented filtrate (Table 2, growth cycle 2). This is understandable because the chemical analyses of the filtrate (Table 1) had shown the presence of very small but apparently significant amounts of residual glucose and NH_4^+-N. These small quantities may not have been adequate for additional growth of the original dense culture because the nutrient concentrations per cell were too low. But they could support a small secondary crop in the second cycle where the nutrient concentrations per cell were much higher.

The filtrate supplemented with glucose gave somewhat more growth (240 million cells per ml) than the untreated filtrate but the one which received additional $(NH_4)_2SO_4$ supported considerably more growth (1.3 billion cells per ml). The nitrogen supply in the filtrate, therefore, was at a more critical level than that of carbon and energy. In the filtrate which had received both glucose and $(NH_4)_2SO_4$, excellent growth occurred. A maximal level of 3.1 billion cells per ml was obtained, which is close to that in the original culture. Undoubtedly the cell crop would have been greater if 1% rather than 0.5% glucose had been used. These results indicate also that there is no accumulation of toxic metabolic products in the *P. fluorescens* cultures and that the only factor which limits growth is the supply of nutrients.

When the process of filtration, supplementation, and reinoculation was repeated, essentially the same results were obtained (Table 2, growth cycle 3). The principal difference was a much smaller cell population (32 million cells per ml) in the filtrate supplemented with $(NH_4)_2SO_4$, owing to the further reduction of the small amount of residual glucose in the filtrate to 0.09 mg per ml in the previous growth cycle. Thus, both glucose and $(NH_4)_2SO_4$ were at critically low levels after the second cycle.

The results remained the same through cycles 4 and 5. A decrease in maximal growth, however, was noted in cycle 6, in the filtrate enriched with both glucose and $(NH_4)_2SO_4$, and a further decrease occurred in cycle 7 (Fig. 2). Either toxic metabolites had accumulated to inhibitory levels in the filtrates during the long series of cycles or deficiencies in essential minerals had developed. Spectroscopic analysis for mineral elements was made of the filtrate from the seventh cycle and also of the original culture medium (Table 3).

There were sharp decreases in the P, Mg, K, and Fe levels (67, 80, 52, and 38%, respectively) as a result of the growth cycles. Al and Ti also were depleted appreciably. There was little change, relatively, in the Si, Ca, Ag, and Pb levels. Cu increased somewhat and Na increased greatly, owing to the constant addition of Na when each spent culture was neutralized with NaOH prior to reinoculation.

On the basis of the spectroscopic analyses, mineral salts, as well as glucose and $(NH_4)_2SO_4$, were added back to the filtrate of the 7th cycle; i.e., the complete medium in dry form was added to the filtrate. This resulted in full growth (505 Klett units) in the 8th cycle. It can be concluded, therefore, that deficiencies in minerals, rather than accumulation of toxic waste products, was responsible for the decreased growth in cycles 6 and 7.

Effect of nutrient and buffer concentration and O_2 supply. If the level of glucose is raised from 1 to 3% without a concomitant increase in $(NH_4)_2SO_4$ concentration, there is no increase in the maximal population (Table 4). This could be anticipated, since $(NH_4)_2SO_4$ would be limiting at 0.1%. The additional glucose merely leads to a marked drop in the final pH of the culture (to pH 4.9) and to a large amount of residual glucose. If glucose is kept at the 1% level and $(NH_4)_2SO_4$ is increased from 0.1 to 0.3%, maximal growth increases from 505 to 578 units and a final pH of 6.4 is attained. If both glucose and $(NH_4)_2SO_4$ are increased threefold, the maximal growth is 583 units and the final pH is 4.4. Since considerable amounts of both glucose and NH_4^+-N remain in the culture filtrate, factors other than nutrient supply must be limiting growth. An obvious factor is the low pH.

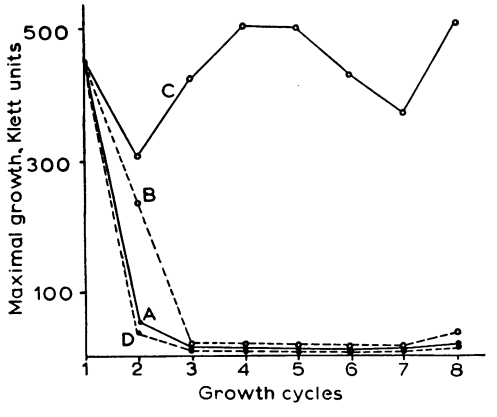

FIG. 2. *Aerobic growth of Pseudomonas fluorescens on serial recycling in its own culture filtrate.* (A) *Glucose added;* (B) *$(NH_4)_2SO_4$ added;* (C) *glucose and $(NH_4)_2SO_4$ added;* (D) *nothing added.*

TABLE 3. *Spectroscopic analysis of mineral elements in the culture medium and filtrate of Pseudomonas fluorescens*

Element	Initial culture medium	Seventh growth-cycle filtrate
	μg/ml	*μg/ml*
P	1,250	410
K	6,000	2,900
Na	1,050	4,200
Mg	50	10
Fe	21	13
Al	25	10
Si	42	38
Pb	19	14
Ca	1.9	1.8
Ti	2.5	0.5
Cu	0.5	1.0
Ag	0.3	0.25

TABLE 4. *Effect of glucose and ammonium sulfate concentration on maximal growth of Pseudomonas fluorescens*

Initial		Final			
Glucose	$NH_4^+ - N$	Glucose	$NH_4^+ - N$	Maximal growth	pH
mg/ml	*mg/ml*	*mg/ml*	*mg/ml*	*Klett units*	
10.8	0.22	0.5	0.02	505	6.3
32.5	0.23	18.6	0.04	490	4.9
11.0	0.67	0.4	0.20	578	6.4
31.3	0.67	15.5	0.20	583	4.4

To test this possibility, the buffer content of the medium with the threefold concentration of glucose and ammonium sulfate was increased from the usual 1.4 to 2.8%. Also, the effect of increased aeration was determined, since O_2 might also be limiting. At the standard shaking rate of 6.5, doubling of the buffer concentration increased growth and prevented the pH from falling below pH 6.1 (Table 5). When the shakind rate was increased, the greater aeration permitteg almost as much growth, with the standard buffer concentration as with the higher concentration. But the most growth was obtained when both the buffer concentration and aeration were increased.

It is apparent, therefore, that when ordinary

TABLE 5. *Effect of aeration and phosphate buffer concentration on maximal growth of Pseudomonas fluorescens*

Shaking rate*	Per cent buffer	Maximal growth†	Minimal pH
6.5	1.4	580	4.4
	2.8	680	6.1
8.0	1.4	660	4.2
	2.8	735	5.8

* New Brunswick shaker units (Model G25).
† Klett units.

TABLE 6. *Aerobic growth and utilization of glucose and ammonium-nitrogen by Escherichia coli*

Determination	Initial	Final
Turbidity, Klett units	6	355
Viable cells, millions per ml	1.3	4,800
pH	7.0	5.7
Glucose, mg per ml	10.0	2.6
NH_4^+ – N, mg per ml	0.27	0.009

nutrient levels are raised, maximal growth also is increased. But this increase, in turn, becomes limited, not by nutrient supply but by the new factors of insufficient oxygen and low pH.

Complex nitrogenous media. The early investigators who had reported the presence of toxic products in bacterial cultures had used nutrient broth and similar complex nitrogenous media. For example, Rahn (1906) used bouillon with *P. fluorescens*. An attempt was made, therefore, to induce the accumulation of toxic metabolites by growing *P. fluorescens* for several cycles in Trypticase soy broth. Even after three cycles, full growth was obtained when the dehydrated medium was added to the culture filtrate at the level used in the initial culture. Virtually no growth occurred in the unsupplemented filtrate even after the first cycle. Thus, energy or nitrogen or both became critically limiting after a single culture cycle in a typical complex nitrogenous medium, but there was no evidence of the formation of toxic products in the cultures.

Aerobic growth of E. coli. In the first few growth cycles, the results were similar to those with *P. fluorescens*. In the first cycle, maximal growth of 4.8×10^9 viable cells per ml was obtained in about 24 hr; virtually all of the NH_4^+-N and most of the glucose was consumed, although about 25% of the glucose remained (Table 6). The latter was sufficient to support full growth in the second cycle on addition of only $(NH_4)_2SO_4$ to the culture filtrate from the first cycle (Fig. 3). Full growth was obtained, also, when the culture filtrate was enriched with both glucose and $(NH_4)_2SO_4$. During the second cycle, residual glucose was further reduced to 0.1 mg per ml and was insufficient, therefore, to support good growth during the third growth cycle. But full growth was obtained in the latter when both glucose and $(NH_4)_2SO_4$ were added to the culture filtrate of the second cycle.

Beginning with the fourth cycle, however, maximal growth began to decrease gradually despite additions of glucose and $(NH_4)_2SO_4$. The decrease became abrupt after the fifth cycle and at the ninth cycle no growth occurred. This decrease does not appear to be owing to an earlier development of an inhibitory pH level in these later cycles as the buffering capacity of the filtrates decreases. The final pH values in cycles 1, 2, 3, 4, 5, and 6 were 5.2, 5.2, 5.5, 5.8, 5.4, and 6.3, respectively. Thus, the pH levels were lower in the initial cycles.

To test the possibility that the reduction in maximal growth was owing to mineral element deficiencies, as had been observed previously with *P. fluorescens*, the complete basal medium including the salts was added back to the filtrates from cycles 6 and 7. On inoculation and incuba-

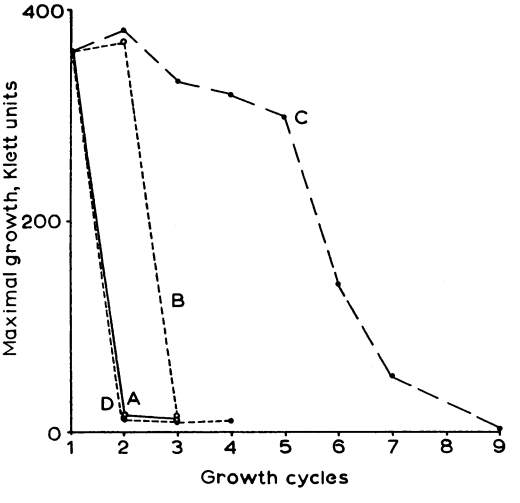

FIG. 3. *Aerobic growth of Escherichia coli on serial recycling in its own culture filtrate.* (A) *Glucose added;* (B) *$(NH_4)_2SO_4$ added;* (C) *glucose and $(NH_4)_2SO_4$ added;* (D) *nothing added.*

tion, it appeared that little or no growth would take place. But after a long lag period of 2 days, full growth eventually occurred. There had been no such lag period with *P. fluorescens* when the complete medium had been added back to the seventh cycle. It appears, therefore, that some inhibitory material accumulated in the *E. coli* cultures after several cycles. It did not prevent growth but delayed it. At least 25% of the inhibitory activity remained after autoclaving. Also, when the inhibitory filtrate was diluted with 25% or more of fresh medium, the inhibitory activity disappeared. These results are remarkably similar to those obtained by Hajos (1922). Employing a growth-recycling technique similar to ours, he noted the gradual accumulation of an inhibitory metabolic product in bouillon cultures of coli-typhoid bacteria. The inhibitory substance delayed growth for 72 hr, was heat stable at 100 C, and its effect disappeared when the toxic culture filtrate was diluted with two parts of fresh broth. However, since the inhibitory effect in Hajos' cultures and ours is evident only after several growth cycles, it does not appear to be significant in limiting growth in normal cultures.

The inhibition may be owing to the accumulation of organic acids, since the pH at the end of each cycle was below pH 6.0 and as low as pH 5.2. Roberts et al. (1955) have shown that as much as 15% of glucose carbon can be converted to acetic acid during aerobic growth of *E. coli*. Also, according to Dagley, Dawes, and Foster (1953), the growth of the closely related organism, *Aerobacter aerogenes*, is inhibited by acetate at a level of 3.46 g per liter even in the pH range of 6.2 to 7.1.

In general, the results with *E. coli* are similar to those with *P. fluorescens*. In a 1% glucose, 0.1% $(NH_4)_2SO_4$ medium, the nitrogen is almost completely consumed by the cells during the first growth cycle, and the glucose by the end of the second cycle. After the third cycle, mineral deficiencies, possibly of trace elements, manifest themselves. But in contrast to *P. fluorescens*, growth-inhibitory substances, probably organic acids, accumulate.

As with *P. fluorescens*, full growth of *E. coli* was obtained in Trypticase soy broth through three growth cycles when the culture filtrates were enriched with the dehydrated medium to the level used in the original culture. There was virtually no growth in the unenriched filtrate

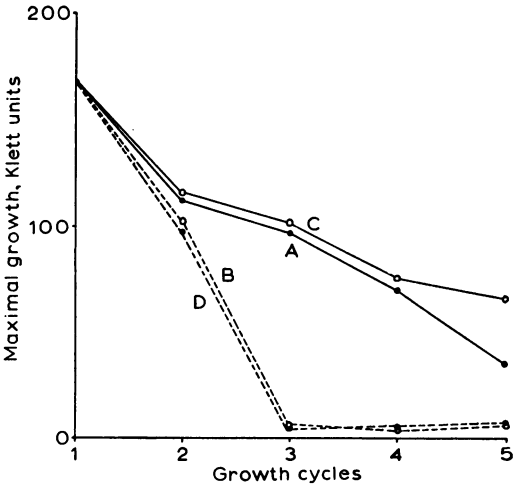

FIG. 4. *Anaerobic growth of Escherichia coli on serial recycling in its own culture filtrate.* (A) *Glucose added;* (B) *$(NH_4)_2SO_4$ added;* (C) *glucose and $(NH_4)_2SO_4$ added;* (D) *nothing added.*

even after the first cycle. Thus, the energy or nitrogen sources or both are exhausted after one growth cycle. Also, there is no evidence that toxic metabolites accumulate during at least three cycles.

Anaerobic growth of E. coli. When the organism is grown in an atmosphere of 95% N_2 and 5% CO_2, maximal growth is only about one-half that obtained aerobically (Fig. 4). Also, in contrast to aerobic growth, glucose is more rapidly depleted than nitrogen. The former becomes limiting after the third cycle and the latter after the fourth cycle. This rapid depletion of glucose anaerobically is undoubtedly owing to the loss of much of the energy of the glucose in the fermentation end products and the consequent need of the cells to metabolize larger amounts of glucose to supply sufficient energy for growth. Maximal growth progressively decreases with each cycle despite the addition of glucose and $(NH_4)_2SO_4$. This indicates that inhibitory substances may accumulate, as in the aerobic cultures, but more rapidly. If the inhibitory substances are indeed organic acids, they should accumulate more rapidly under fermentative conditions (Stokes, 1949). Almost full growth was obtained after the fifth cycle by the addition of the mineral salts as well as glucose and $(NH_4)_2SO_4$, but only after an extended lag period of 3 days. These results are similar to those obtained under aerobic conditions.

DISCUSSION

Bail's hypothesis that excessive crowding of microbial cells in cultures causes the cells to stop multiplying does not seem to merit serious consideration. A great deal of data in the literature and our results refute Bail's contention. Thus, in the *P. fluorescens* experiments, growth ceased not because of crowding but because essential nutrients were exhausted. And the cell population could be raised to ever higher levels by increasing the nutrient content of the medium, its buffering capacity, and the supply of oxygen.

The concept of autoinhibition of growth by toxic metabolic products, however, is more firmly based on experimental data. Metabolites such as H_2O_2 and undissociated organic acids may limit maximal growth in some cultures. Formation of acid was one of the limiting factors in the *S. lactis* cultures of Rogers and Whittier (1928) and in our *P. fluorescens* cultures grown with relatively high concentrations of glucose, and probably also in our *E. coli* cultures. Whether specific autoinhibitory substances, however, are produced by microorganisms is not certain. Rahn (1906) reported the occurrence of a heat-labile autoinhibitory substance in broth cultures of *P. fluorescens*. Yet, in our experiments with this organism, there was no evidence of the formation of such a toxic material, even after many growth cycles in chemically defined and complex nitrogenous media. The phenomenon of "staling," which refers to the failure of a bacterial culture to grow when streaked on nutrient gelatin or agar heavily seeded with its own viable cells, has been ascribed to the formation of specific autoinhibitory substances (Eijkman, 1904; Powers and Levine, 1937). However, the investigations of Lockhart and Powelson (1953) and of Levine (1953) indicate that growth inhibition on staled agar is owing to an insufficiency of nutrients; i.e., the nutrient concentration per cell in the heavily seeded agar may be too low to permit growth of the freshly inoculated cells.

Undoubtedly, the major factor which limits bacterial growth is exhaustion of essential nutrients from the culture medium (Dagley and Hinshelwood, 1938; Monod, 1942; van Niel, 1949; Ecker and Lockhart, 1961*b*). Our experiments have shown, by means of chemical analyses for residual carbon, energy, and nitrogen sources in the cultures after maximal growth and by replenishment experiments, that this is the case with *P. fluorescens* and *E. coli*. In media containing 1% glucose and 0.1% $(NH_4)_2SO_4$, quantities which are used frequently in culture media, both of these nutrients are virtually exhausted in a single growth cycle. Because of this, the unenriched spent culture filtrate permits only slight growth when reinoculated but full growth when enriched with adequate amounts of nutrients. When relatively high concentrations of nutrients are used, e.g., 3% glucose and 0.3% $(NH_4)_2SO_4$, aerobic growth is increased. But now the maximal growth level is limited not by the supply of nutrients but rather by new factors, namely, insufficiency of O_2 and low pH.

ACKNOWLEDGMENT

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