GROWTH OF GROUP A HEMOLYTIC STREPTOCOCCUS IN THE STEADY STATE[1]

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The study in static cultures of the synthetic functions of bacteria and their growth is subject to serious limitations. These arise from the inability to specify and to insure constancy of various important cultural conditions such as growth rate, concentration of limiting metabolite and the concentrations of non-limiting metabolites when these are not present in large excess. In the case of acid-forming bacteria maintenance of a constant pH also constitutes a difficulty. This can be readily surmounted by the use of a pH-stat (Jacobsen and Leonis, 1951) but not without alteration of the ionic composition of the culture. The changing environment in static culture also limits the extent to which information derived in this way can be related to the metabolic activities of a microorganism maintained in the less variable environment of an infected host.

The general solution to the limitations of static cultures is to be found in experimental arrangements which allow growth to proceed under steady-state conditions. A few years ago Monod (1950) and Novick and Szilard (1950) demonstrated the efficacy of continuous cultures (continuous dilution at constant volume) as a means of maintaining steady-state growth under controlled conditions. These authors described experimental devices for this purpose and considered some of the theoretical aspects of continuous cultures. They showed that it is possible to maintain a bacterial culture in a steady state in which the bacterial density and composition of medium remain constant as well as the rate of growth. Monod, particularly, emphasized the decisive advantages which steady-state kinetics provides for the investigation of various problems of bacterial physiology. Reference may be made to a recent review by Novick (1955) for a description of other devices used to obtain steady-state cultures.

Since we are concerned with synthetic processes associated with the growth of the hemolytic streptococcus, especially the formation of proteins, it appeared that our investigations would be greatly facilitated if they were carried out with steady-state cultures. We have therefore applied the continuous-culture technique to this organism and found it possible to achieve steady-state growth under a variety of conditions and over a wide range of rate of growth.

In this paper we describe the experimental arrangement, the composition of the medium and the operating procedures which were developed for our purpose. Several of the factors which determine the steady-state conditions as well as the effects of their variation will be illustrated by our experimental results.

THEORETICAL ASPECTS[2]

The continuous-culture technique involves the continuous addition of medium to a culture contained in a growth tube and maintained homogeneous by rapid stirring. The volume of culture in the growth tube is kept constant by an overflow device which removes culture at the same rate that medium is introduced. The rate of growth α is defined by the relation

$$\frac{db}{dt} = \alpha b \qquad (1)$$

where b is the dry weight of bacteria per ml. Let the volume of the culture in the growth tube be

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[2] We are indebted to a referee for pointing out that in this discussion we have not taken into account the possibility of an oscillating steady state. A consideration of this question is given in a recent paper by Spicer (1955). However, with respect to pH-limited and metabolite-limited growth, at least, our theoretical considerations are adequate to account for the observed steady-state behavior to be described below.

V (ml) and the rate at which medium is introduced be given by w (ml/hr). The rate of dilution D is then defined by $D = w/V$ and represents the fraction of the culture volume in the growth tube which is displaced per hr by medium.

It is the essential property of this system that for all values of D which do not exceed a certain maximum value of α the culture will adjust itself in such a way that $\alpha = D$. This adjustment will result from the establishment of a rate-limiting condition of growth, e. g., concentration of metabolite or pH, which will assume such a value that limits the growth rate (α) to the predetermined value D. When α becomes equal to D and remains so the culture is in a steady state and the conditions under which the growth is occurring are steady-state conditions. It is evident that since D can be varied independently this system has the important property that the relation between growth rate and other features of the culture can be studied systematically over a wide range of α. The rate of cellular division (α'), the number of divisions per unit time, is given by the relation $\alpha' = \alpha/\ln 2$. The time-interval between successive divisions, i. e., the generation time (θ), is obtained from $\theta = 1/\alpha' = \ln 2/\alpha$.

In general there are three kinds of rate-limiting conditions of growth which are possible in the case of acid-producing bacteria such as the hemolytic streptococcus. As applied to continuous cultures these are the pH in the growth tube, the steady-state concentration of a limiting metabolite and the steady-state concentration of a growth inhibitor produced by the organism. The particular condition which determines the growth rate in the growth tube depends both on the composition of the medium and the value of D.

Growth limited by pH results, as we shall illustrate, when all metabolites and growth factors are present at sufficiently high concentrations. In this case a bacterial population is established for each value of D such that the rate of acid production per unit volume is just sufficient to reduce the initial pH of the medium, $(pH)_m$, to a steady-state value in the growth tube, $(pH)_g$, at which the growth rate α is equal to D. The rate of acid production per unit volume is given by the product of the bacterial concentration and the rate of glycolysis per unit bacterial mass. The adjustment of acid production for different values of D can be effected by changes in one or both of these factors. The value of $(pH)_g$ is always lower than that for most rapid growth and the reduction in rate represents a pH inhibition effect. By the variation of D over the appropriate range, e. g., 1/hr to 0.1/hr, it is possible to establish the relation between α and $(pH)_g$ over a considerable range of pH.

Under the conditions of pH-limited growth the bacterial concentration depends on the acid-neutralizing capacity of the medium. The greater this capacity the higher is the bacterial density which is required to produce acid at a rate sufficient to maintain $(pH)_g$ at a value determined by D. It is possible therefore to vary the bacterial density by changing the buffer concentration and $(pH)_m$, without any change in the growth rate.

The second type of limiting condition, giving rise to metabolite-limited growth, results when the steady-state concentration in the growth tube (c_g) of an essential metabolite is sufficiently small that the rate of growth becomes dependent on c_g. This situation can be brought about merely by reducing the initial concentration of the metabolite (c_m) in the medium. Although c_m is always larger than c_g, the quantitative relation between them may be expected to vary with different metabolites. The ratio of these quantities may be as large as several hundred, as Monod (1950) has shown for the case of *Escherichia coli* in a defined medium with glucose the limiting metabolite.

In the case of metabolite-limited growth the bacterial density (b) will depend on the amount of the metabolite consumed, i. e.,

$$b = R(c_m - c_g) \qquad (2)$$

and can be varied independently of α by changing c_m. R, the proportionality factor, has been called by Monod (1950) the constant of efficiency. It represents the quantity of bacterial mass formed per unit quantity of metabolite consumed. This factor which may be more appropriately called the efficiency factor will not in general be a constant but may be dependent both on α and c_g. It is of interest to note that this dependence for any particular metabolite can be studied even when the metabolite is not the one limiting growth. For example the variation of the ef-

ficiency factor for glucose may be investigated with an essential amino acid as the limiting metabolite.

The value of $(pH)_g$ for any value of D under conditions of metabolite limitation is no longer a characteristic feature of the bacteria, as it is in pH-limited growth. Although $(pH)_g$ is always higher for metabolite-limited growth than in the case of pH-limitation for the same α, its actual value is highly variable. It depends on the acid-neutralizing capacity of the medium, the bacterial density and the efficiency factor for the acid-producing metabolite, e. g., glucose to lactate.

The third type of limiting condition of growth is that due to inhibitor production by the growing organism. In this case the steady state would be characterized by a concentration of inhibitor which allowed a growth rate α equal to D. A change of D would result in a new steady state in which the bacterial density would be adjusted so that the rate of production of the inhibitor would provide a steady-state concentration appropriate for the new value of α. Here too the value of $(pH)_g$ would be larger than that for pH-limited growth, and would depend on the same factors indicated for the case of metabolite-limited growth.

One of the most useful properties of the continuous-culture technique is that the determination of the rates of various processes associated with bacterial growth is reduced to the measurement of steady-state concentrations. Also, since these measurements are made under constant and defined conditions, it is possible to correlate rates of various reactions, e. g., the synthesis of a particular enzyme, with changes in the steady-state conditions. One of the most interesting of these conditions is the rate of bacterial growth itself, a condition which can be varied, it will be recalled, simply by changing the value of D. In general, the rate of synthesis or utilization of any component in the growth tube will be given by

$$\frac{dc}{dt} = (c_g - c_m)D \qquad (3)$$

In the case of synthetic reactions c_m will usually be equal to zero and measurements of the steady state values of bacterial density and enzyme concentration will give the rates of synthesis of these materials. For substances which are utilized, e. g., glucose and amino acids, the determination of the rates of utilization requires a knowledge of the concentration in the medium as well as the steady-state concentration.

MATERIALS AND METHODS

Organism. The work described here was carried out with Strain H-44 of Group A hemolytic streptococcus, Type 4, which was originally made available through the courtesy of Dr. Karl Meyer. The strain has been maintained in the lyophilized state, with re-cultivation and lyophilization approximately every two years.

Medium. For the fullest exploitation of the continuous-culture technique it is essential to use a medium of known composition. In this way the growth-limiting substances can be recognized and the effect of systematic variations in their concentrations on the growth properties of the organism can be studied in an unequivocal manner. Therefore, pending the development of a completely synthetic medium capable of sustaining high levels of growth, we have employed a medium based on the use of acid-hydrolyzed casein as the source of most of the required amino acids.[3] Aside from the uncertainty regarding the precise composition of this material, and the inability to vary independently amino acid concentrations, the medium is suitable since it contains otherwise only chemically characterized substances of low molecular weight individually available. Wherever possible reagent grade chemicals have been used. In other cases commercially available preparations of the highest purity were selected. The composition of our medium is essentially that described by Bernheimer, Gillman, Hottle and Pappenheimer (1942), with some modifications required by the continuous-culture method.

The medium is made up in 12-L batches from five solutions prepared as follows:

Solution A: 240 g of a vitamin-free acid hydrolyzate of casein (casamino acids, Difco) are dissolved in 1.75 L of distilled water (pH 5.2). To this is added 104.5 g of K_2HPO_4, 85.2 g of Na_2HPO_4, 11 ml of thioglycolic acid (assay 68

[3] Subsequent to the completion of this investigation a completely synthetic medium, employing eighteen amino acids, has been developed in this laboratory by Mr. William Landau.

TABLE 1

Composition of typical medium used in chemostat; final volume = 12.0 liters, pH = 7.4

Component	Concentration in Intermediate Solution		Conc. in Final Medium Moles/Liter
	Grams/liter	Moles/liter	
	Solution A, 2 L, pH 7.2		
Vitamin-free casamino acids*	120	—	(2%)
NaCl (38%)	45.6	0.78	0.130
NH_3	1.11	0.065	0.0108
N (7%)	—	—	—
K_2HPO_4	52.2	0.300	0.0500
Na_2HPO_4	42.6	.300	0.0500
Thioglycolic acid	5.0	0.054	0.0088
L-Cysteine HCl	1.023	6.48×10^{-3}	1.08×10^{-3}
$CuSO_4 \cdot 5H_2O$	0.0060	2.4×10^{-5}	4.0×10^{-6}
$ZnSO_4 \cdot 7H_2O$	0.0060	1.7×10^{-5}	2.8×10^{-6}
$FeSO_4 \cdot 7H_2O$	0.0060	1.8×10^{-5}	3.0×10^{-6}
$MnCl_2 \cdot 4H_2O$	0.0024	1.2×10^{-5}	2.0×10^{-6}
Riboflavin	0.015	4.0×10^{-5}	6.7×10^{-6}
DL-Tryptophan	0.120	5.88×10^{-4}	9.80×10^{-5}
	Solution B, 400 ml†		
$MgSO_4$	15.0	0.126	4.20×10^{-3}
Uracil	0.300	2.70×10^{-3}	9.0×10^{-5}
Adenine SO_4	0.300	1.30×10^{-3}	4.3×10^{-5}
	Solution C, 400 ml†		
Glucose (reagent)	270	1.50	0.0500
$CaCl_2 \cdot 2H_2O$	0.221	1.50×10^{-3}	5.00×10^{-5}
	Solution D, 100 ml‡		
Niacin	0.156	1.26×10^{-3}	1.05×10^{-5}
Pyridoxine HCl	0.156	9.2×10^{-4}	7.7×10^{-6}
Ca Pantothenate	0.600	2.52×10^{-3}	2.10×10^{-5}
Thiamin HCl	0.156	4.62×10^{-4}	3.85×10^{-6}
Riboflavin	0.076	1.91×10^{-4}	1.59×10^{-6}
Biotin	1.2×10^{-4}	4.8×10^{-8}	4×10^{-10}
	Solution E, 240 ml‡		
$NaHCO_3$	105	1.25	0.025

* Purchased from Difco Laboratories, Detroit 1, Michigan.

† Sterilized by autoclaving.

‡ Sterilized by filtration through Seitz EK pads.

per cent), 2.05 g of L-cysteine HCl and 24 ml of a salt mix. The latter contains $CuSO_4$ at 2.4×10^{-5} M, $ZnSO_4$ at 1.7×10^{-5} M, $FeSO_4$ at 1.8×10^{-5} M and $MnCl_2$ at 1.2×10^{-5} M. The above solution is stoppered to minimize exposure to atmospheric oxygen and allowed to stand overnight at room temperature. During this period a small amount of a black precipitate is formed. Its removal before the medium is placed in the chemostat[4] is desirable in order to prevent failure of the ground glass valves of the feeding system to seat properly. Thirty milligrams of riboflavin are added, together with 240 mg of DL-tryptophan previously dissolved in 5 ml of distilled water with 3 drops of concentrated HCl. The pH at this point is usually 7.2 and is adjusted to this value if necessary. The solution, at a volume of 2 L, is then filtered through a Seitz EK pad to render it sterile as well as to remove the black precipitate.

Solutions B, C, D and E are used at volumes of 400 ml, 400 ml, 100 ml and 240 ml respectively. The constituents of these solutions are shown in table 1 along with their concentrations. The last column also shows the molar concentrations in the

[4] This is the name which Novick and Szilard (1950) have used for the kind of device we shall describe.

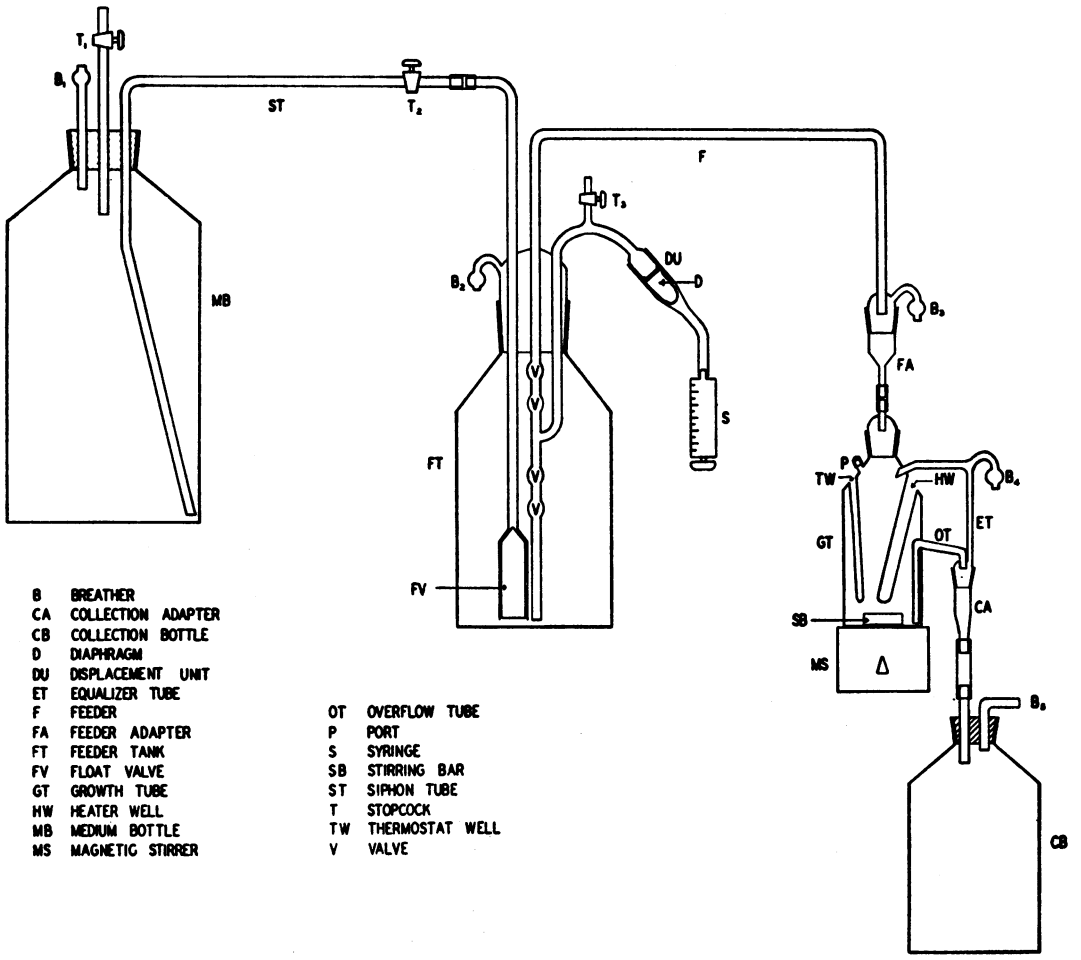

Figure 1. Diagram of the continuous culture apparatus

final medium of all the known components of the medium except the amino acids derived from hydrolyzed casein. The additional riboflavin which is included in Solution A, aside from that in Solution D, was used because during the operation of the chemostat the medium was exposed to daylight and fluorescent light up to periods of one week, and it appeared likely that some of the riboflavin would be destroyed by photochemical action. If adequate precautions are taken to shield the bottle containing the bulk of the medium from exposure to light the additional riboflavin may be unnecessary.

After the five sterile solutions are combined to yield a volume of approximately 3.2 L the mixture is transferred to a 5-gallon pyrex bottle which has been autoclaved with sufficient distilled water to give a final volume of 12 L of medium. Before the medium bottle is connected to the chemostat the solution is covered with 700 ml of mineral oil, sterilized by autoclaving, to reduce the rate of diffusion of atmospheric oxygen into the medium. The oil layer also serves to reduce the rate of escape of CO_2 from the medium although some loss does evidently occur. This is indicated by an increase of about 0.1 of a pH unit after aging one week.

Apparatus. A diagrammatic sketch of the apparatus is shown in figure 1.[5] Bacterial growth takes place in the container at the right, called

[5] The fabrication of the specially-designed glass sections of the chemostat, such as the growth tubes, feeder, float valve, etc., is due to Mr. T. A. Walton, 4583 G St., Philadelphia, Pa. We are greatly indebted to Mr. Walton for his important contributions to the design of the apparatus and for his thoughtful cooperation in its development and construction.

the growth tube (*GT*). We have used growth tubes of different sizes ranging in operating volume from 100 ml to 1500 ml. The culture in the tube is maintained homogeneous by a plastic coated stirring bar (*SB*) actuated by a magnetic stirrer (*MS*). The temperature of the culture is maintained at 38 C by means of a thermostat and a heater which are set into glass wells (*TW* and *HW*) immersed in the culture. More recently the heater well has been removed and more precise temperature control attained by the use of a red-tinted tungsten lamp as the heating unit. The necessity for heat transfer to take place through the glass walls of the wells does not introduce any serious variation in the temperature of the culture. The volume of culture in the growth tube remains constant by virtue of the overflow tube (*OT*). The horizontal section of this tube is tilted slightly downward towards the direction of flow in order to prevent the culture from being sucked back if a small pressure differential between the growth tube and collection adapter (*CA*) should develop. The equalizer tube (*ET*) serves to minimize the occurrence of such pressure differentials. The growth tube also carries a cotton-filled breather (B_4) and a port (*P*) through which sterile solutions can be added directly into the culture.

Medium is continuously supplied to the growth tube by means of a feeding device (*F*), one end of which is connected to the growth tube by an adapter (*FA*) and the other end of which is immersed in medium contained in the feeder tank (*FT*). The feeder adapter consists of two standard taper joints, one male and the other female, connected by a short length of rubber tubing. This tubing can be clamped and medium pulled through the feeder by applying suction to the breather (B_3) attached to the outlet end of the feeder. The medium is pumped through the feeder by a hydraulic system. As indicated in figure 1, the feeder contains four valves which consist of mercury-filled glass floats, ground on the bottom side, that rest on ground glass surfaces. Between the two pairs of valves an auxiliary tube is connected to the feeder tube and terminates outside the feeder tank in the form of a standard taper male joint which has been slightly constricted near the end of the ground surface. The opening of this joint which constitutes part of the displacement unit (*DU*) is covered by a finger cot. The cot provides a sterile liquid-tight rubber diaphragm (*D*) which prevents any mixing of the medium above it and the glycerine below it, but which permits the displacement of the piston in the syringe (*S*) below to be transmitted to the medium. The syringe and the female joint of the displacement unit are filled with glycerine and all air spaces removed when the connection is made. The 10-ml syringe is operated by a synchronous motor at a speed of two cycles per min. The stroke of the piston can be adjusted to provide any displacement between 0 and 10 ml.[6] The feeder tank serves as a medium reservoir for the feeder. It is a 9-liter pyrex bottle with a standard taper mouth. A No. 45 hollow stopper provides mechanical support for the feeder, for a reinforcing tube, not shown, which is connected at the other end to the outlet end of the feeder, for a breather (B_2) and for part of the siphon tube (*ST*). At the end of this tube is a glass float valve which maintains the medium in the feeder tank at a constant level. The medium is supplied from the 5-gal medium bottle (*MB*) through a siphon tube which contains a stopcock (T_2) and which consists of two sections connected by a short piece of rubber tubing. The medium bottle carries a three-hole rubber stopper which serves to support a breather (B_1), a stopcock (T_1) and one section of the siphon tube.

A smaller version of the above apparatus has also been constructed. This is designed to be used with 3-L batches of medium. The feeder tank in this case is a 4-L pyrex bottle but the growth tubes are interchangeable. The smaller apparatus is particularly suitable for studies on the incorporation of labeled amino acids and similar problems.

Operation. Prior to the assembly of the chemostat its several units are separately autoclaved. One unit consists of the feeder mounted in the feeder tank, which contains 200 ml of mineral oil, with the diaphragm in place over the opening of the male joint of the displacement unit. This unit also includes, of course, one section of the siphon tube. The other units are the growth tube, the feeder adapter and the collection adapter. After about 100 ml of culture has been placed in the growth tube the appropriate connections are made with the adapters. The connection with

[6] The motor-driven syringe was designed and constructed by the Drummond Scientific Co., 6011 Media St., Philadelphia, Pennsylvania.

the medium bottle is made by joining the two sections of the siphon tube by means of rubber tubing. This is also the joint which is used when a fresh bottle of medium is to be installed.

The medium bottle is prepared by autoclaving it with 8.8 L of distilled water. During this operation the rubber stopper is kept loose to avoid any pressure differential. After the medium bottle has cooled, the 3.2 L of concentrated medium, contained in a 4-L aspirator bottle, are added through the stopcock (T_1). This step is followed by the addition of 700 ml of mineral oil through the aspirator bottle and the stopcock, and the latter is then closed. The flow of medium into the feeder tank can be started by momentarily applying suction to breather B_2.

To put the feeder into operation the syringe, female joint of the displacement unit and the connecting piece of rubber tubing are filled with glycerine. The connection is then made with the male joint in such a way that no air bubbles are trapped in the glycerine. The air in the feeder is removed either by allowing the syringe to operate and manipulating stopcock T_3 or by carefully applying a small negative pressure to the open stopcock. After the culture in the growth tube has risen slightly above operating level some of it is sucked through the overflow tube to remove air. This precaution is necessary since trapped air will alter the operating level by virtue of the resistance it affords to the flow of culture through the narrow overflow tube. Following this step the feeding rate is adjusted to the desired value. This rate can be measured approximately by counting the drops at the feeder tip. A more accurate measure is found by collecting culture for a period long enough to accumulate 100 to 200 ml. Once the desired rate has been obtained the apparatus will operate automatically with little or no attention.

The emergent culture is collected in a bottle (*CB*) which initially contains a volume of 0.1 M iodoacetate, pH 7, which is 1 per cent of the total volume to be collected, yielding a final concentration of 0.001 M. This inhibitor prevents bacterial growth and glycolysis from continuing outside of the growth tube. As a result the collected culture is a steady-state collection which may be expected to be very close in composition to the culture in the growth tube. For some purposes, which do not require a collection period of more than a few hours, it is preferable to collect the culture in an ice bath. For turbidity and pH determinations small samples of culture may be readily collected by clamping the rubber tube of the collection adapter for a few minutes, momentarily raising the two-hole rubber stopper of the collection bottle and allowing a few ml of culture to run into a small tube.

The protection of the medium in the medium bottle and feeder tank with a layer of mineral oil has been found essential for the maintenance of steady-state growth for periods of several days. The oil serves to reduce the rate of oxidation of the thioglycolic acid. This reducing agent or some other one is evidently continuously necessary for growth, presumably because it maintains one or more essential components, possibly coenzyme A, in the reduced form. The presence of the reducing agent in the culture is periodically checked by the nitroprusside test. It has been found that with an initial thioglycolate concentration of 0.009 M the culture will still show a strong nitroprusside reaction after seven days of continuous operation with one 12-L batch of medium.

The adequacy of our apparatus both with respect to freedom from contamination, although this does occasionally occur, and with respect to its mechanical operation is evidenced by its continuous use for five weeks with the consumption of seven 12-L batches of medium. That the hemolytic streptococcus can grow in this apparatus under steady-state conditions at different rates for long periods of time is shown by the constancy of turbidity and pH which is maintained for as many as 78 generations. The range of generation time which we have found it practical to employ is from 42 min to 166 min. Occasional observation has shown that the generation time can be extended to periods several-fold longer than 166 min.

Quantitative measurements. The course of growth and the establishment of the steady-state conditions were followed by frequent sampling of the culture and measurement of its pH and turbidity. A Klett colorimeter with a 560 filter was used for the turbidity determination. The concentration of the acid formed at each pH was obtained from a titration curve of the original medium and is expressed as equivalents of acid per L of culture. The turbidity values, after correcting for the supernatant contribution, were converted into concentrations in the units of mg

TABLE 2

Summary of results for steady-state growth under various conditions

Batch No.	α (hr^{-1})	(pH)$_g$	T_c (Klett)	b	a	a/b	$\alpha a/b$
0.100 M phosphate, 0.0250 M glucose							
				(mg/ml)	equiv/liter	equiv/g	equiv/g/hr
16	0.98	6.9	136	0.30	0.0267	0.091	0.088
16	0.500	6.5	208	0.49	0.050	0.102	0.051
13	0.513	6.5	216	0.52	0.050	0.098	0.050
14	0.493	6.55	196	0.46	0.045	0.099	0.049
14	0.250	6.35	186	0.43	0.058	0.136	0.034
13	0.250	6.3	192	0.44	0.061	0.137	0.034
0.100 M phosphate, 0.0400 M glucose							
22	1.00	6.9	126	0.27	0.0229	0.085	0.084
22	0.667	6.3	244	0.60	0.062	0.102	0.068
22	0.493	6.2	232	0.56	0.068	0.121	0.059
21	0.500	6.15	246	0.61	0.070	0.115	0.058
21	0.246	6.05	216	0.52	0.076	0.146	0.036
0.100 M phosphate, 0.0500 M glucose							
11	1.00	6.8	141	0.31	0.0308	0.100	0.100
10	0.98	6.8	132	0.28	0.0285	0.100	0.098
11	0.752	6.45	204	0.48	0.052	0.108	0.081
11	0.599	6.2	222	0.54	0.067	0.125	0.075
11	0.526	6.1	218	0.52	0.072	0.137	0.072
10	0.495	6.1	216	0.52	0.072	0.138	0.069
11	0.249	5.9	186	0.43	0.084	0.196	0.049
10	0.252	5.9	192	0.44	0.085	0.192	0.048
0.100 M phosphate, 0.100 M glucose							
15	0.500	6.2	184	0.42	0.067	0.159	0.080
15	0.250	5.85	154	0.34	0.086	0.252	0.063
0.050 M phosphate, 0.055 M glucose							
5	0.944	6.65	148	0.32	0.0270	0.083	0.078
5	0.513	6.1	184	0.42	0.048	0.114	0.059
0.100 M phosphate, 0.0500 M glucose, 1.40×10^{-5} M DL-tryptophan							
19	0.667	6.9	128	0.28	0.0262	0.095	0.063
19	0.500	6.75	134	0.29	0.0339	0.117	0.058
19	0.250	6.55	152	0.34	0.045	0.136	0.034

α is the growth rate in hr^{-1}, T_c is the corrected turbidity (Klett), b is the bacterial concentration in mg dry weight per ml of culture (g/L) and a is the equivalents of acid formed per L of culture. The column headed a/b shows the number of equivalents of acid formed per g of dry weight of bacteria synthesized. The last column ($\alpha a/b$) represents the rate of acid production per g of bacteria per hr.

of dry weight of bacteria per ml of culture (g/L).

RESULTS AND DISCUSSION

Experiments were carried out in which the effect of variations in the medium on the steady state conditions for different growth rates could be evaluated. The results of these experiments are shown in table 2. Since the hemolytic streptococcus ordinarily converts about 95 per cent of the glucose utilized to lactate (Friedemann, 1938; White, Steele and Pierce, 1955), we may

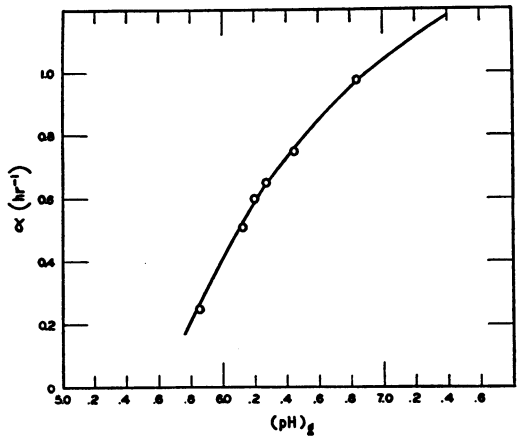

Figure 2. Growth rate (α) as a function of the steady-state pH, $(pH)_g$, under conditions of pH-limited growth.

compute the quantity of glucose metabolized from measurement of the acid formed.

pH-limited growth. An examination of table 2 reveals that with 0.1 M phosphate buffer pH-limited growth for the range of α from 1 hr^{-1} to 0.25 hr^{-1} was obtained only with 0.050 M glucose and above. Thus for α = 0.5 hr^{-1}, the steady-state pH is about the same for 0.05 M and 0.1 M glucose, 6.1–6.2, but increases to 6.5 when the initial glucose concentration is only 0.025 M. A similar situation exists for α = 0.25. It is evident therefore that we may use the values of $(pH)_g$ observed with 0.1 M phosphate and 0.05 M glucose as characterizing pH-limited growth for the several growth rates indicated. The relationship between $(pH)_g$ and α is shown in graphic form in figure 2. The extrapolation of this curve to pH 7.4 yields a growth rate of 1.18 hr^{-1}, corresponding to a generation time of 35 min at this pH.

The dependence of the size of the bacterial population on the initial glucose concentration and the buffer content is shown by the results summarized in table 3. The maximum bacterial density is obtained with 0.040 M glucose and it may be inferred that the optimum glucose concentration is somewhat less than this value. The smaller density observed with 0.025 M glucose is due, of course, to a glucose limitation as shown by the value of 6.5 for $(pH)_g$. At higher glucose concentrations, where growth is pH-limited, the reduction in bacterial density is due to an increase in the rate of glycolysis per unit bacterial mass with increase in the steady-state concentration of glucose in the culture. Thus, as is shown by the values of $\alpha a/b$ (column 7), at constant growth rate (α) and $(pH)_g$ there is a mass action effect of increased glucose, leading to an increase in the specific rate of glycolysis. The minimum value of 0.050 for 0.025 M glucose is consistent with this conclusion but the comparison in this case is rendered somewhat ambiguous by the higher value of $(pH)_g$.

TABLE 3

Effect of glucose and buffer concentrations on bacterial density and glucose utilization
α = 0.5 hr^{-1}

Glucose Conc.	Phosphate Conc.	$(pH)_g$	b	a	a/b	$\alpha a/b$
(M)	(M)		mg/ml	equiv/L	equiv/g	equiv/g/hr
0.025	0.100	6.5	0.49	0.049	0.100	0.050
0.040	0.100	6.2	0.59	0.069	0.118	0.058
0.050	0.100	6.1	0.52	0.072	0.138	0.070
0.055	0.050	6.1	0.42	0.048	0.114	0.058
0.100	0.100	6.2	0.42	0.067	0.159	0.080

See footnote to Table 2 for explanation of symbols.

The reduction in the buffering capacity of the medium, 0.050 M phosphate *vs* 0.100 M phosphate leads to a decrease in bacterial density as expected. The difference between 0.050 M and 0.055 M glucose in table 3 is of little significance for purposes of this comparison. It is of considerable interest to note, however, that in spite of the higher steady-state concentration of glucose which obtains in the 0.050 M phosphate culture compared to the glucose concentration in the 0.100 M phosphate culture, the specific rate of glycolysis is smaller in the former culture than in the latter, 0.058 *vs* 0.070. This result suggests that the rate of glycolysis depends not only on the rate of a glucose-involved reaction but also on the rate of a process or reaction in which inorganic phosphate participates.

The variation in the rate of glycolysis and the efficiency of glucose utilization for growth may also be examined with respect to its relation to growth rate. For this purpose the results summarized in table 2 for 0.100 M phosphate and 0.050 M glucose provide the most useful information. It is evident from the values in the last column ($\alpha a/b$) that the specific rate of glycolysis decreases with decreasing growth rate (and decreasing $(pH)_g$). However, since the growth rate

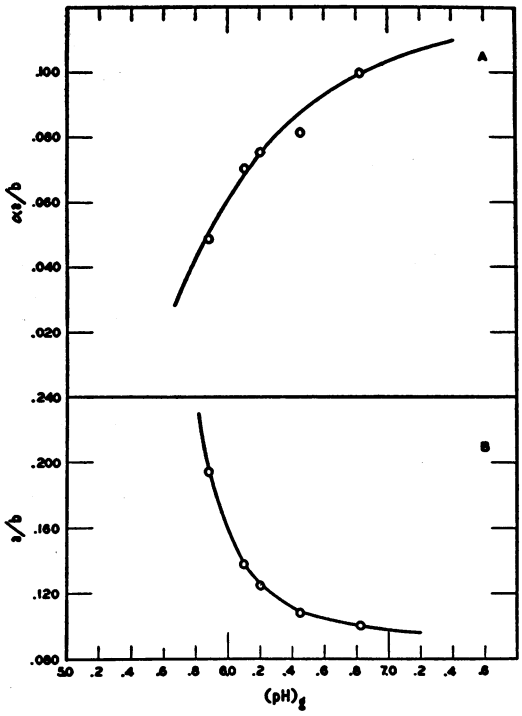

Figure 3. The relations between the steady-state pH, $(pH)_g$, under conditions of pH-limited growth and (*A*) the rate of acid production ($\alpha a/b$) in equivalents of acid per g of dry weight of bacteria per hr and (*B*) acid production (a/b) in equivalents of acid per g of dry weight of bacteria formed.

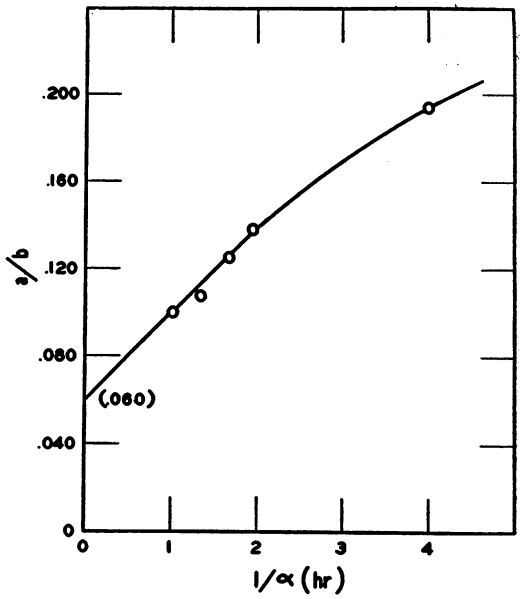

Figure 4. Acid production (a/b) in equivalents of acid per g of dry weight of bacteria formed as a function of the reciprocal of the growth rate ($1/\alpha$) under conditions of pH-limited growth.

decreases more rapidly with decrease of $(pH)_g$ than does the glycolytic rate the acid produced per unit bacterial mass formed (a/b) increases as the steady-state pH declines. The calculated values of a/b are included in table 2 and plots of $\alpha a/b$ *vs* $(pH)_g$ and a/b *vs* $(pH)_g$ are shown in figures 3*A* and 3*B* respectively. Thus the efficiency of utilization of glucose for bacterial synthesis, which may be taken as equal to b/a, is highly variable and increases with increasing growth rate. Perhaps the simplest explanation of this relationship is to be found in the view that the adenosinetriphosphate (ATP) resulting from glycolysis may either be coupled to synthetic processes associated with bacterial growth or may be hydrolyzed by an apyrase (Clark and MacLeod, 1954). The extent to which the ATP is utilized for growth, i. e., the efficiency of glucose utilization, would depend on the relative rates of the synthetic and dissipative processes.

With the data available it is possible to calculate the minimum quantity of glucose which must be metabolized per unit of bacterial mass produced. This calculation is based on the assumption that the observed value of a/b is the sum of this minimum quantity plus a contribution which decreases with increasing growth rate. This formulation is consistent with the interpretation of the utilization of ATP we have suggested above and is supported by the observed relation between α and a/b. The quantity sought is obtained from a plot of a/b *vs* $1/\alpha$ by extrapolation of the experimental curve to the vertical axis. This procedure is illustrated in figure 4 from which it can be seen that a reliable extrapolation is possible. In this way the minimum value of a/b is found to be 0.060 equivalents of acid per g of dry weight of bacteria or 5.4 g of glucose per g of bacteria. This figure may be compared with the results found by Monod (1942) for the growth of *E. coli* on a synthetic medium plus glucose and for *Bacillus subtilis* grown on a synthetic medium plus sucrose. In the first case 4.3 g of glucose were utilized per g of dry weight of bacterial mass formed and in the second 4.6 g of sucrose per g of bacterial mass. The similarity of these figures to that for the streptococcus is striking in view of the wide differences in the metabolic patterns involved.

Glucose-limited growth. When the initial glucose concentration is 0.025 M the growth rate becomes limited by the steady-state concentration of the glucose in the growth tube for α equal to 0.5 and 0.25 hr^{-1}. This is shown by the results listed in table 2 for 0.100 M PO_4 and 0.025 M glucose. For $\alpha = 1$ hr^{-1} the value of $(pH)_g$ is about the same as that observed with 0.05 M glucose, indicating that in this case the system is pH-limited. For the smaller values of α, however, the values of $(pH)_g$ are substantially higher than those observed with higher concentrations of glucose: 6.5 *vs* 6.1 and 6.3 *vs* 5.9.

The shift from pH-limited to glucose-limited growth is associated with the practically complete utilization of the available glucose. This conclusion is based on the observation, as can be seen from the values of a, that for $\alpha = 0.5$ hr^{-1}, the amount of acid is equal, within experimental error, to that expected for the complete conversion of the glucose to lactic acid. Unfortunately it has not yet been possible to determine the steady-state concentration of glucose at this growth rate. It is clear, however, that it must constitute only a small percentage of the initial glucose concentration. At $\alpha = 1$ hr^{-1}, on the other hand, only about 50 per cent of the glucose is converted to acid with a resulting high steady-state concentration of the sugar. This condition is consistent with the conclusion that the culture was pH-limited at this growth rate.

At $\alpha = 0.25$ hr^{-1} the interesting result has been obtained that more acid is produced than can be accounted for by the conversion of one mole of glucose to two moles of acid. Since the hemolytic streptococcus is known to produce formic and acetic acids in addition to lactic acid (Friedemann, 1938; White, Steele and Pierce, 1955), this additional acidity is undoubtedly due to the formation of these one-carbon and two-carbon acids. To account quantitatively for this increment at least 20 per cent of the glucose must have been converted into these acids. Since only about 5 per cent of the glucose ordinarily appears in these forms it seems that the steady-state conditions associated with a small growth rate ($\alpha = 0.25$ hr^{-1}) facilitate the breakdown of pyruvate to acetate and formate.

Comparison of the values of a/b for 0.025 M glucose and 0.050 M glucose shows that the acid produced per unit of bacterial mass formed is consistently smaller for lower initial sugar concentration at corresponding growth rates. This relationship is evidently due to the lower steady-state concentrations of glucose which obtain in the cultures from the 0.025 M glucose medium. It may be noted that this relationship appears in spite of the consideration that the difference in the $(pH)_g$ values observed would be expected to effect a relative increase in the rate of glycolysis with this medium leading to an increase of a/b. This factor may indeed be operative but it is evidently not the decisive one.

Tryptophan-limited growth. The effect of an amino acid limitation on growth was studied by the use of tryptophan as the limiting metabolite. It was established from a titration in static cultures that initial concentrations of tryptophan of the magnitude of 1×10^{-5} M yielded bacterial densities which varied with the initial concentration. On this basis a value of 1.4×10^{-5} M DL-tryptophan was chosen for the concentration of the amino acid in the Chemostat medium. The steady-state conditions observed with such a medium at three growth rates are summarized in table 2. It will be noted that the $(pH)_g$ values are higher and the bacterial densities (b) lower than those for pH-limited growth at corresponding growth rates. These relations demonstrate that for each growth rate the steady-state concentration of tryptophan outside of the cells was such as to limit the growth to this rate.

It is not possible with the information on hand to calculate these concentrations but it is evident from the small rate of increase of bacterial density with increase of growth rate that most of the L-tryptophan supplied is assimilated at all rates. It may be estimated, however, that the order of magnitude of the steady-state concentration of extracellular L-tryptophan is 1×10^{-6} M based on the observation that the bacterial density does change significantly with growth rate. This estimate involves the assumption that there is no variable destruction of tryptophan by the streptococcal cells.

Examination of the values of a/b and $\alpha a/b$ in relation to the variation of growth rate reveals the same qualitative pattern previously noted. Thus the amount of acid produced per unit bacterial mass formed increases with slower growth. As before, this result depends on the fact that, although the specific rate of glycolysis decreases as a is decreased, it decreases less rapidly than does the growth rate.

On the other hand, a quantitative comparison of pH-limited growth and tryptophan-limited growth points up a very significant difference between the two situations. The rates of glycolysis are consistently and significantly smaller in the latter case as shown by the pairs of values of $\alpha a/b$ for $\alpha = 0.67, 0.50$ and $0.25\ hr^{-1}$ respectively: 0.063 *vs* 0.080, 0.058 *vs* 0.070 and 0.034 *vs* 0.048. The figure 0.080 was calculated with the aid of figure 4. It should be noted that this difference appears in spite of the tendency of the higher steady-state concentrations of glucose in the amino acid-limited cultures to produce the opposite result. We interpret this effect to mean that the tryptophan limitation results in a reduced bacterial concentration of one or more of the glycolytic enzymes involved in a rate-limiting reaction. This conclusion serves to emphasize the general point that the enzymatic composition of the bacterial cell is variable and dependent on the composition of the medium. With regard to the relation between rate of bacterial growth and rate of bacterial synthesis of protein we may remark that in the steady state the former does not evidently uniquely determine the latter.

SUMMARY

The steady-state growth of group A hemolytic streptococcus has been investigated using the continuous-culture technique, i. e., continuous dilution at constant volume. The apparatus and medium developed for this purpose together with the associated operating procedures are described in detail. It has been possible to establish steady-state growth over a wide range of growth rate, 0.25 to 1 hr^{-1}, and to maintain the steady-state conditions for at least 78 generations.

Some features of the steady-state cultures have been studied for pH-limited growth, glucose-limited growth and tryptophan-limited growth. In the first case there is a characteristic relation between the growth rate and the steady-state pH. The rate of glycolysis has been found to decrease less rapidly with pH than does the growth rate with the result that the efficiency of utilization of glucose increases with increasing growth rate. The metabolite-limited cultures have higher, and non-characteristic, pH values than the pH-limited cultures. The metabolite-limited cultures reveal several distinctive properties which can be related to the low steady-state concentration of the limiting metabolite.

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