THE GROWTH OF BACTERIA WITH A CONSTANT FOOD SUPPLY

I. Preliminary Observations on Bacterium Coli

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INTRODUCTION

The literature dealing with the growth phases through which developing bacterial cultures pass is already extensive. The succession of phases is well established, but the underlying reasons for their existence are still not fully understood. In particular, it remains a problem why the rate of growth should fall away so rapidly when a certain density of living cells has been reached. Growth does not then actually cease, as is shown by the continually increasing total cell numbers, but it is balanced by death so that a constant population of viable cells is established. This condition often occurs long before there is a shortage of food since, if the majority of the cells are removed (Penfold, 1914) or killed (Bail, 1929), fresh growth may occur. In the case of the pneumococcus, toxic metabolic products cause the retardation of growth (Chesney, 1916), but this explanation is not a general one. Lodge and Hinshelwood (1939) found that exhaustion of food, toxic products of metabolism, and adverse pH might all be the cause of the cessation of growth of *Bacterium lactis-aerogenes* in synthetic media. Bail (1929) introduced the concept that in any fluid culture there is a maximum population density that cannot be exceeded. Topley and Wilson (1936, p. 81) suggested that, in so far as aerobic and facultatively anaerobic bacteria are concerned, a lack of oxygen may be the cause of the retardation in growth since aeration resulted in a considerably increased maximum population of viable cells. The view was taken that, in ordinary broth cultures incubated aerobically, growth continues until it becomes impossible for each cell to obtain sufficient oxygen for its requirements. A further suggestion was that the oxygen required depends on the nature of the food available, i.e., that the more complex foodstuffs left after the simpler and more easily available ones have been consumed may require more abundant oxygen for their utilisation. Regarded in this way it is seen that the retardation of growth in an ordinary broth culture may really be due to starvation, since the food left may be utilisable only very slowly under the prevailing conditions.

Whatever the true position, it is clear that in much of the work on bacterial growth curves there have been several varying factors. Usually the oxygen supply has not been controlled and, linked with that, the carbon dioxide concentration seems to have varied considerably. Often no attempt has been made to eliminate changes in the reaction of the cultures. Important, also, is the

fact that, as the general method of experimentation has been to inoculate a few organisms into a large amount of food and to follow the subsequent cell proliferation, the exact condition of the cells with respect to the food supply at any time has been unknown.

It seemed, therefore, that a fresh approach to the problem of bacterial growth might profitably be made by subjecting a population of cells, held in a rigidly controlled environment, to a constant food supply over a relatively long period. A few experiments along these lines have been made by Cleary, Beard, and Clifton (1935), who concluded that growth ceases because of changes in the availability of nutrient material and in the energy demand of the cells, but the value of their work is greatly diminished by the absence of any total cell counts.

The methods described below have enabled the original aim to be achieved. The results obtained have been accumulated over a period of two years during which minor adjustments in technique have been made as they were seen to be desirable for the attainment of greater accuracy. The figures are, therefore, to be regarded as of qualitative rather than of quantitative significance. Nevertheless, statistical analysis has shown that satisfactory agreement between replicate experiments has usually been obtained, and so the calculations based on the data, although approximate, are considered to be justified.

APPARATUS AND METHODS

The automatic syringe mechanism used for the continuous addition of the nutrient solution to the bacterial culture is described in detail elsewhere (Sims and Jordan, 1942). It consists essentially of a 1 ml pyrex "tuberculin" syringe the plunger of which is operated automatically at predetermined intervals by a compact series of levers the function of which is to convert the rotatory motion of a synchronous motor into a to and fro linear movement. By the action of a pair of mercury-weighted glass valves a known volume of sterile nutrient solution is transferred from a calibrated burette to the culture flask at each complete operation of the syringe. The interval between successive movements of the syringe plunger and the extent of its excursion at each operation are capable of wide and rapid variation. The food solution comes into contact with no surface other than glass and all parts of the apparatus which require it can be steam sterilised and assembled aseptically.

The culture flask used is illustrated in figure 1. It consists of a 5 litre, pyrex, round bottom flask with a ground joint A on a central, wide, short neck around which are arranged three additional necks, two vertical and one normal to the surface, each having a ground joint. The joint in the central wide neck is sealed by a glass tube, of approximately 28 mm in diameter, which extends to within 1 cm of the bottom of the flask and which has a narrow ground joint B at its upper end and another, C, on a horizontal side tube. Through the dustproof and flexible glass joint B passes the jet by which nutrient solution is added to the culture, and a stream of dry, sterile air from Dinton aerators enters through the side tube C (plugged with cotton wool), bubbles through the culture, and escapes from the flask by the neck D. By thus surrounding the food jet with a glass

guard tube and air stream, contamination of the ingoing broth by bacteria-laden spray from bursting froth bubbles was completely prevented—a condition upon which the success of the technique was largely dependent.

The air before entering the culture is dried by sulphuric acid and sterilised by passage through numerous cotton wool plugs before being warmed in glass coils immersed in the water bath beneath the culture flask. The functions of the air stream are to stir the culture efficiently and to distribute rapidly the added nutrient solution throughout the culture, to maintain gaseous equilibrium, and to evaporate water from the culture at the same rate as the broth is added, thus preserving a constant volume. That the rate of air flow through a bacterial cul-

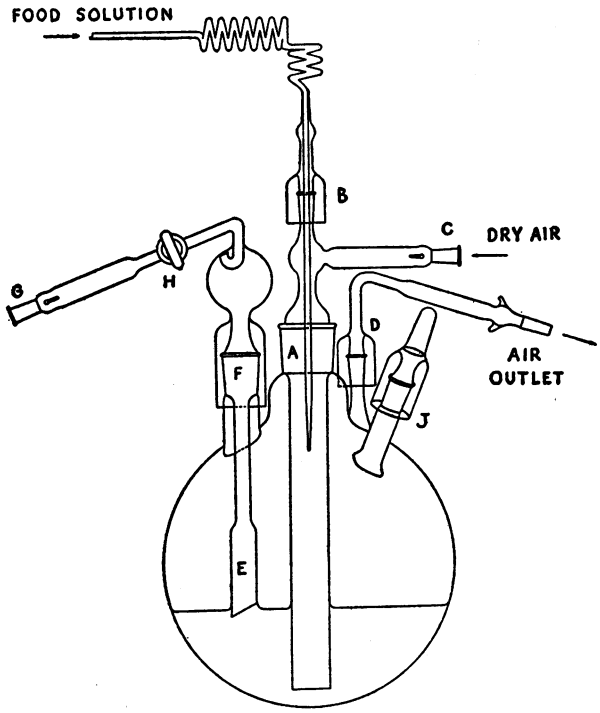

FIG. 1. CULTURE FLASK (FOR DESCRIPTION SEE TEXT)

ture does not affect, within wide limits, the rate of growth of the organisms was shown by Dagley and Hinshelwood (1938b) working with *Bacterium lactis-aerogenes*.

To check the culture volume periodically a modified dipstick device is used. Direct visual observation of the liquid level is impossible owing to the considerable agitation and froth at the surface and to the immersion of the culture flask in a bath containing water in constant movement. The tube E, inserted through a dustproof joint in the neck F, is ground to such a length that when in contact with 1450 ml of culture in the flask (the initial volume in each experiment) the meniscus formed at the interface, as shown in figure 1, is broken when, as a re-

sult of slight suction applied to the cotton-wool-filled tube G, the liquid rises in E to the level of the top of the neck F. At intervals during each experiment the agitation of the culture caused by the air stream is stopped momentarily and, by reference to the height to which the column in E rises above or below the zero mark when the meniscus breaks, any change in culture volume is detected and, if necessary, adjustment of the rate of air flow to regulate the evaporation is made. By this simple means the culture volume can be maintained constant to within less than ± 2 per cent.

Samples are removed from the culture periodically with a sterile pipette via the neck J which is attached normally to the surface of the flask to enable samples to be taken from the centre of the culture.

The culture flask is immersed in a thermostatic water bath at 35 C ± 0.1 up to the unions of the side tubes with the body of the flask and maintained in position by a special clamping device fastened to a supporting framework fixed to the inside of the bath.

The choice of a suitable organism was restricted by several desiderata, e.g., it should be aerobic, forming individually separated cells evenly distributed throughout the medium, and preferably of low pathogenicity. *Bacterium coli* satisfied these requirements and proved to be eminently suitable for the work.

Since, from preliminary determinations in weakly buffered media, it appeared that increase in pH of the medium owing to accumulation of basic endproducts had a potent inhibitory influence on the growth of this organism under the conditions of semistarvation to be employed, the cultures were buffered at pH 7.0 with phosphate. Even at the end of experiments lasting over 800 hours at the higher of the two rates of food addition used, the reaction of the medium never exceeded pH 7.4. Later work has shown that pH changes of this magnitude have little effect on the cultures.

The viable population was determined by the usual plating method, using as diluent a sterile, phosphate buffer solution equimolar with that in the culture flask, but making the modification of placing a thin layer of nutrient agar in each petri dish and allowing it to set before plating out the diluted culture. This procedure was found to give more evenly spaced colonies with a minimum of spreading ones. Eight plates were made from each sample, and after incubation at 35 C for 48 hours the colonies were counted, using the automatic counting device of Sims and Jordan (1941). Statistical analysis (Fisher, 1938) of the data from numerous plate counts showed the methods employed to be satisfactory.

For the determination of the concentration of total cells present the direct microscopic count was at first employed. A Thoma counting chamber (0.02 mm deep) was used in conjunction with a $\frac{1}{6}$ inch objective and an 18× compensating ocular. The organisms were usually stained and prevented from further growth by the addition of acriflavine (0.5 ml of 0.1 per cent solution to 5 ml of culture), but occasionally methylene blue or gentian violet was substituted. When necessary, the culture-dye mixtures were diluted to a suitable cell density before counting. As a result of many counts spread over several experiments, one of

which lasted for 900 hours, it was established that so long as food was being added agglutination of the cells did not occur to any significant extent. The relatively few paired cells seen were counted as one unless obviously on the point of separation. This practice was adopted owing to the difficulty of estimating, under the conditions in which the cells were examined, exactly when division was complete. Such few small clumps as were seen were counted as one cell. A considerable number of satisfactory counts was obtained in this way. Their standard error varied from ± 2 per cent to ± 6 per cent according to the number of organisms counted (Fisher, 1938). Simultaneously, the turbidity of the culture was measured with a Hilger-Spekker photoelectric absorptiometer. Two long experiments were performed, and it was found that the ratios of the direct counts to the corrected absorptiometer readings were scattered about a mean value which was unaltered when the food supply was doubled. The absorptiometer was thereafter relied upon exclusively for the total cell determinations. One absorptiometer unit was equivalent to a total cell population of 13.62×10^6 organisms per ml under the conditions employed. The standard error of this constant was $\pm 0.26 \times 10^6$, i.e., approximately 2 per cent.

Preliminary experiments revealed that a rate of addition of 0.066 ml per 100 sec of a nutrient solution containing 6.0 g dehydrated Difco broth per L (before autoclaving) gave convenient levels of total and viable organisms and that when this rate was doubled satisfactory results were still obtained.

The standard technique employed at the commencement of each experiment is to place in the culture flask 250 ml stock pH 7.0 buffer solution (27.2 g KH_2PO_4 Analar + 4.55 g NaOH Analar per L) and 1300 ml distilled water. With suitable precautions, the flask containing this mixture is sterilised in steam at 20 lb/sq in pressure for 45 min. During autoclaving the volume becomes reduced to 1450 ml. The food jet, guard tube, level indicator, etc., are all wrapped separately in paper and are sterilised, together with the syringe mechanism (Sims and Jordan, 1942), in steam at 20 lb/sq in for 20 min and then dried at 100 C for 30 min in a suitable autoclave. It was found advisable to lubricate slightly the outer end of the syringe plunger with a drop of sterile, liquid, paraffin-vaseline mixture to prevent seepage of broth along the barrel towards the end of long periods of operation.

The nutrient solution is made up in the 3 litre pyrex reservoir flask (Sims and Jordan, 1942) and sterilised in steam for 45 min at 20 lb/sq in pressure.

When cool, the culture flask is placed in position in the water bath, the various parts of the apparatus fitted together aseptically, the air stream and food supply commenced, and the solution in the culture flask inoculated. No trouble has been experienced from contamination of the culture by air-borne organisms either during assembly of the apparatus or during the long experiments, since each joint of the flask except A is covered by a dust shield. Around A is placed a little sterile liquid paraffin.

It was not at first appreciated that absolute standardisation of the inoculum is essential in order to obtain reproducible results in the early stages of the experiments but in later work the standard inoculum used is as follows: A little

growth of *Bacterium coli* from a stock agar slant is transferred to 150 ml broth (8 g dehydrated Difco broth per L) and incubated at 35 C for 24 hours. From this culture 1 ml is diluted in sterile water to a population of approximately 1×10^5 organisms per ml. Of this suspension 5 ml is used as inoculum into the 1450 ml sterile, dilute, buffer solution in the experimental culture flask, giving an initial population of 300 to 400 *B. coli* per ml.

As required, a sample of 2.5 ml of the culture, removed through side neck J with a sterile pipette, is diluted to 10 ml with sterile, distilled water and this suspension placed in a sterile 1 cm cell for the determination of the turbidity in the Spekker absorptiometer using distilled water as control "turbidity." A correction is made when necessary during long experiments for the increasing yellow colour of the culture owing to the added broths. Five ml of this suspension are then transferred from the cell to 100 ml sterile, dilute, buffer solution and successive dilutions made in the usual way preparatory to plating on nutrient agar.

RESULTS

The results of 14 experiments in which food was supplied at the rate of 15.2 mg dehydrated Difco broth per hour are recorded in tables 1 and 2 and those of 6 experiments at double this rate in table 3. For the sake of clarity the data have been simplified in that the counts are recorded as having been made at intervals of exactly 24 hours whereas, in fact, samples were taken within 1 or 2 hours of midday on each occasion, the exact time being noted. The error introduced by this simplification is insignificant.

It will be seen that in their very early stages replicate experiments gave extremely divergent results. Some of the divergencies may have been due to variations in the lag phase induced by transference of the cells of the inoculum to the new environment. Possibly a more careful standardisation of the amount of inoculum, or of its method of preparation, would go far to obviate this trouble. Small differences in the nutritive value of the food solutions made from different samples of the dehydrated broth may not only have contributed to this lack of uniformity in the early stages but also have caused the cell population in certain experiments to be consistently above or below those in the majority. The use of accurately controllable synthetic media is envisaged as a means of obtaining more consistent results. However, from about 50 hours onwards, replicate experiments showed a satisfactory agreement amongst themselves. At the lower food level the mean coefficients of variation between replicate values at each 24-hr interval are 10 per cent for the viable counts and 8.8 per cent for the total counts. In view of the difficulties involved in the technique such variations are not considered excessive in these preliminary experiments.

The agreement between replicate experiments has also been tested in another way. Consideration of the results for individual experiments reveals that from 40 hours onwards the total population tended to increase in a stepwise fashion. The phenomenon is believed to be a real one, though no explanation is offered at present. However, broadly speaking, the trend was for the total counts to bear a linear relationship to time. Accordingly, the regression coefficients of

these lines were calculated, and the significance of the differences between pairs of coefficients tested (Fisher, 1938). In these calculations the actual times at which the counts were made were employed, not the approximate times given in

TABLE 1

Results of experiments on constant food supply of 15.2 mg dehydrated Difco broth per hr at 35 C

Viable Population

Millions *Bacterium coli* per ml

TIME	SERIAL NUMBER OF EXPERIMENT														MEAN	
	44	45	46	47	48	49	50	51	53	54	61	62	63	67		
hr																
20	60	72										0.02	0.02	74	(41)	
44		138										0.6	194	144	(119)	
68	106	177									242	222	222		194	
92	140	212	254				213	218			276	336	271	267	243	
116		232				235	232	247	242		292	320		274	259	
140	159	248		322		262	268	258	268	210	340	396	364		281	
164				360	281	272	275	279	283	226	358				291	
188			310	377	291	301	390	266	286	260	329				301	
212			308		304	312	311			252					297	
236					320			316	292	252	354				307	
260					330	328	322	330	274		384				328	
284					333	328	303	342	270	275	345				314	
308					316	338	314	370	263	263	354				317	
332					320	330	318	364	267	263	334				314	
356					321	312	285	331	239	260	356				301	
380					297	314	280			245					284	
404					287			330	236	248	342				289	
428					299	300	260	331	*	*291	*399				298	*345
452					293	299	276	318	370	345	460				297	392
476					292	302	271	320	425	352	460				297	412
500					301		246	302	493	391	464				283	449
524					290		255	286	518	417	530				277	488
548					309		255			460					282	(460)
572					304			280		455	484				292	470
596					302		247	262							270	
620							234	267		438	506				250	472
644							243	249		416	530				246	473
668							258	235							247	
692							242	243							242	
716							246								(246)	
740								258							(258)	
764							260								(260)	
788							255								(255)	

* Food supply doubled.

the tables. At the lower food level 7 coefficients were available, the data used in their calculation having extended over approximately the same time range in each case. Of the 21 differences between pairs of coefficients 17 were not signif-

icant, 1 was on the borderline of significance (P being slightly below 0.5) and the remaining 3 were significant. Each of these 3 involved the same experiment (no. 49). It may, therefore, be claimed that the agreement on the whole is

TABLE 2

Results of experiments on constant food supply of 15.2 mg dehydrated Difco broth per hr at 35 C

Total Population

Millions *Bacterium coli* per ml

Time	SERIAL NUMBER OF EXPERIMENT														MEAN	
	44	45	46	47	48	49	50	51	53	54	61	62	63	67		
hr																
20	107	86										14	14	192	(70)	
44		218										14	354	286	(170)	
68	263	314									436	450	517		396	
92	350	361	433				454	490			545	654	654	681	514	
116	379	402				532	613	518	532		600	720		600	544	
140		470		653		615	558	668	606	552	681	804			623	
164				680	635	636	600	613	776	600	722				658	
188			660	674	606	640	640	708	735	552	770				665	
212			763		597	660	626			600					649	
236					690			620	796	728	858				738	
260					693	805	722	695	913		974				800	
284					800	740	702	850	865	784	885				804	
308					796	773	858	858	913	817	980				856	
332					797	783	838	892	975	892	974				879	
356					815	768	1022	912	980	885	1064				921	
380					876	783	1042			920					905	
404					834			968	1090	995	1078				993	
428					872	956	1040	885	*	*1160	*1172				938	*1162
452					1060	992	975	1015	1398	1322	1376				1010	1365
476					1085	1005	1170	968	1465	1338	1430				1057	1411
500					1136		1082	1138	1614	1442	1460				1119	1505
524					1182		1146	1050	1725	1595	1500				1126	1607
548					1202		1125			1678					1163	(1678)
572					1272			1100		1690	1540				1186	1615
596					1290		1190	1187							1222	
620							1300	1208		1868	1635				1254	1752
644							1310	1217		1962	1710				1263	1836
668							1300	1210							1255	
692							1285	1220							1252	
716							1348								(1348)	
740								1480							(1480)	
764							1535								(1535)	
788							1588								(1588)	

* Food supply doubled.

satisfactory. At the higher food level only 3 coefficients were available and the agreement is much less satisfactory as all 3 differences were significant. It must be remembered that the indications or systematic divergencies from a linear rela-

tionship between total count and time should cause this statistical treatment to be regarded with reserve. Nevertheless, it is evident that a considerable degree of success has attended these efforts to obtain reproducible results. For convenience in presenting and interpreting the data, only the mean values of the

TABLE 3

Results of experiments on constant food supply of 30.4 mg dehydrated Difco broth per hr at 35 C

Time	VIABLE POPULATION — Millions *Bacterum coli* per ml								TOTAL POPULATION — Millions *Bacterium coli* per ml							
	Serial number of experiment						Mean		Serial number of experiment						Mean	
	52	55	60	64	65	66			52	55	60	64	65	66		
hr																
20				0.2	74	152	(75)					136	286	272	(231)	
44				321	363	328	334					626	654	586	622	
68				484		415	450					893		872	882	
92				538	560		549					1146	1146		1146	
116		392			595	630	539			1014			1252	1310	1192	
140	474	467	620				522		1158	1130	1268				1189	
164	510	498	658				555		1268	1120	1580				1323	
188	525	560	698				594		1340	1212	1548				1367	
212	570	515	719				601		1430	1363	1540				1444	
236			778				(778)				1675				(1675)	
260	552	513	748				604		1565	1594	1635				1598	
284	521	482					(502)		1690	1662					(1676)	
308	514	482	720				572		1730	1800	1800				1777	
332	460	475	700				545		1800	1770	1890				1820	
356	474	516	633				541		1910	1948	1880				1913	
380	467	530	632				543		1980	2162	2100				2081	
404			558				(558)				1990				(1990)	
428	444	584	676				535		2170	2560	2030				2253	
452		*304	*					*(304)		*2640	*					*(2640)
476	468	194	196				(486)	(195)	2330	2620	1810				(2330)	2215
500	435	149	157				(435)	153	2422	2670	1772				(2422)	2221
524	416	126	129				(416)	127	2518	2580	1800				(2518)	2190
548	428	105	104				(428)	105	2630	2560	1870				(2630)	2215
572			104					(104)			1870					(1870)
596	391	75	123				(391)	99	2920	2600	1935				(2920)	2268
620																
644		61	132					96		2670	1950					2310
668																
692																
716			117					(117)			2030					(2030)

* Food supply stopped.

bacterial populations at comparable times have been considered (figures 2 and 3). It must be pointed out that the scatter of points is greater in these composite curves than when each experiment is plotted separately, for the reason that the mean values have not always been derived from the same number of replicate

values and at times only one value has been available. When this has come from an experiment which was running consistently at higher or lower values than the rest, the effect has inevitably been to exaggerate the scatter of points on the composite diagram. This is especially true of the values for the total population after the food supply had been stopped. The less satisfactory values in the tables and curves have, therefore, been indicated in parentheses. The straight lines representing the relationship between the total cell population and time are the regression lines calculated from the mean populations and approximate times given in the tables.

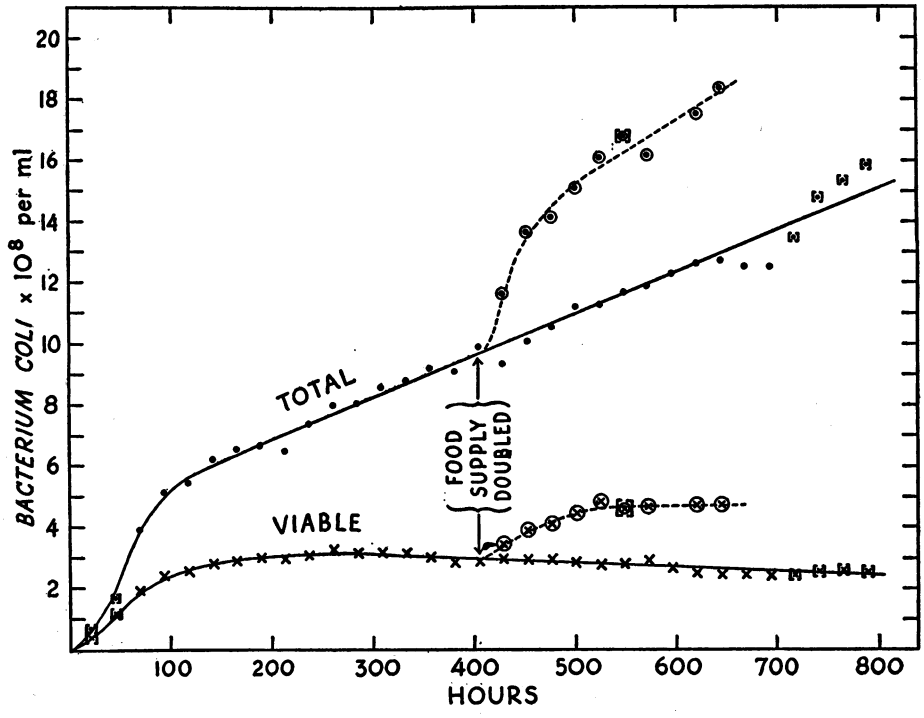

FIG. 2. MEAN GROWTH CURVES FOR BACTERIUM COLI ON CONSTANT FOOD SUPPLY OF 15.2 MG DIFCO BROTH PER HR AT 35 C

DISCUSSION

It is evident that, in the particular conditions of these experiments, the development of each culture fell into two phases, one in which the viable count was rising and another in which this count was either stationary or diminishing. In the following discussion the former is referred to as the initial phase and the latter as the steady phase. During this steady phase the total count will be treated as if it had increased at a constant average rate, instead of in a stepwise manner. The observed rates of new cell formation are, naturally, net rates compounded of the true rates less the rates of cell disappearance through autolysis, but it is considered that in these experiments autolysis was slight and that the observed

rates approximate very closely the true rates. Autolysis may be regarded as endogenous catabolism unbalanced by synthesis and, therefore, the food supply must be one factor governing its occurrence. Since food was added continuously,

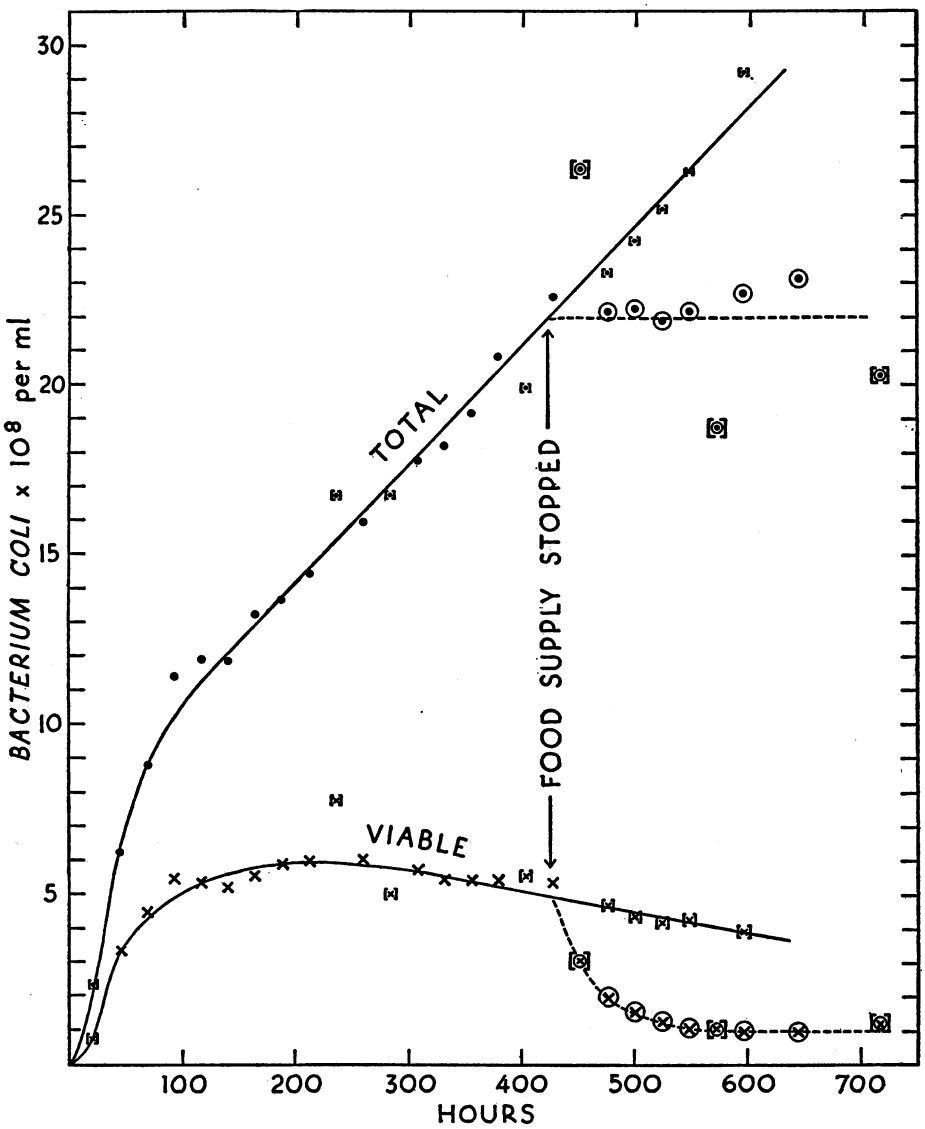

FIG. 3. MEAN GROWTH CURVES FOR BACTERIUM COLI ON CONSTANT FOOD SUPPLY OF 30.4 MG DIFCO BROTH PER HR AT 35 C

autolysis may well have been very slight. Other reasons for this belief are that very few degenerate cells were seen during the many direct microscopic counts made during calibration of the absorptiometer, and Sturges and Rettger (1922) have shown that *Bacterium coli* is an organism which does not autolyse

readily. It is not contended that no autolysis occurred during these long experiments, but only that so long as food was still being added its magnitude was small. Its effects have, therefore, been neglected.

From the earliest stages of each experiment there was always a considerable difference between the total and viable counts. Wilson (1922) showed that in ordinary broth cultures the total count generally exceeds the viable even during the logarithmic phase of growth, i.e., that in the period of most active multiplication some of the cells formed are incapable of division. These cells may, however, possess considerable powers of utilising food. Such cells will be referred to here as "nonviable," and the expression "nonviability index" (*n.v.i.*) will be used to denote that proportion of the cells formed in each generation which is nonviable.

Since Kelly and Rahn (1932) obtained evidence that in favorable conditions and for short periods all cells continue to multiply if they have once started to do so, the difference between the two counts recorded by Wilson may be regarded as a result of the medium or conditions not being ideally suited to the organism. This unsuitability could arise in a number of ways; e.g., insufficiency of food or unsuitable type of food, lack of oxygen, incorrect or varying pH, accumulation of toxic metabolic products, and so on. In the present experiments lack of oxygen and varying pH can be ruled out, but an investigation of the effects of various pH levels on the growth of *Bacterium coli* under these conditions of constant food supply is to be made, since the pH employed may not have been ideal for the organism. Also, an accumulation of toxic products is unlikely to have occurred, as the maintenance of the steady average rate of cell formation in cultures over 30 days old, and the immediate response of cultures some 16 days old to an increased food supply, would seem to preclude this possibility. Thus the food supply would appear to be the main factor leading to the big difference between the total and viable populations in these experiments. The aspects of quality and quantity of food cannot entirely be separated since a deficiency in quality might conceivably be made up by an increase in quantity, although, as shown below, doubling the quantity of food made no appreciable difference to the *n.v.i.*

The *n.v.i.* in the initial phase may be calculated quite simply by taking advantage of the fact that at each rate of food addition the total counts were approximately twice the corresponding viable counts. If at any instant there were n viable cells, then there were $2n$ total cells and, therefore, n nonviable. At the end of a generation time $2n$ cells would have been formed from the n viable cells so that with the original n nonviables there would be $3n$ total cells. If p represents the *n.v.i.*, the final number of viable cells would have been $2n(1-p)$. But the total cells were still twice the viable so that $2[2n(1-p)] = 3n$, from which $p = 0.25$. So long as the total count remained twice the viable, the *n.v.i.* must have remained constant at this figure.

Penfold and Norris (1912) and, more recently, Dagley and Hinshelwood (1938a) have shown that the mean generation time (*m.g.t.*) of bacteria is independent of the food supply as long as this is above a certain critical value. Be-

low this value the *m.g.t.* depends on the food available. In the very early part of the initial phase in the present work even the lower rate of food addition probably exceeded the rate of utilisation, and possibly the concentration of food was for a time raised above the critical level. This condition could not have lasted long, however, since it can be shown that the *m.g.t.* was steadily increasing during the greater part of the initial phase. The actual values of the *m.g.t.* in this period cannot be determined, but relative values for successive generations can be obtained from the curves of viable cells, since as the *n.v.i.* has been shown to be 0.25, the increase in viable cells in each generation must be half the number initially present. At both food levels the *m.g.t.* in the initial phase increased from the earliest moment at which the curves could be regarded as at all accurate. Table 4 gives an illustration of the results obtained from the viable curve for the higher rate of food addition. In this instance the results were calculated on the assumption that a population of 600×10^6 cells per ml was reached at the end of a generation time. The data are insufficient to decide whether the

TABLE 4

Relative mean generation times in the initial phase

Food supply, 760 mg dehydrated Difco broth per 25 hr

NUMBER OF VIABLE CELLS AT END OF SUCCESSIVE GENERATIONS	TIME (FROM VIABLE COUNT-TIME CURVE, FIGURE 2)	RELATIVE MEAN GENERATION TIME
Millions/ ml	*hr*	*hr*
79	20	
118	24	4
178	30	6
267	39	9
400	54	15
600	150	96

m.g.t. remained constant at a minimum value for any length of time during the early initial phase, but evidently the *m.g.t.* was increasing by the end of the first 24 hours. Hence, except possibly for a very short time, the food supply per cell was always below the critical level, i.e., the development of the cultures was throughout dependent on the food supply as a limiting factor and the results must be interpreted in that light.

Bearing this in mind, it is interesting to note that at both rates of food addition the cultures took approximately the same time to reach the steady phase and that throughout the initial phase the total and viable counts at the double rate of food addition were approximately twice those at the single rate. There was a tendency for these ratios to be rather greater than 2:1 in the middle part of the initial phase, but it is doubtful whether this can be regarded as significant. However, such a deviation from the 2:1 ratio might be expected since at the higher rate there should have been a greater accumulation of food awaiting the organisms when they emerged from the lag phase and consequently a more rapid multiplication. By the end of the initial phase the cultures at both food levels

were in the same condition in that the amount of food per cell was the same, although the concentrations were different. As the cultures then entered a steady phase, characterised by a roughly constant viable count together with a constant average increase in total cells, it would seem that by then any initial accumulation of food had been used up and the ingoing food was being utilised as fast as it was added. It would appear from work in progress that only at 35 C is a steady state with constant viable counts established, and we may here be dealing with a special case. During the steady phase the value of the *n.v.i.* must have been 0.5 since the viable count remained constant. Also, the *m.g.t.* must have been constant owing to the fixed rate of increase in total cells. Evidently, towards the end of the initial phase the *n.v.i.*, which had hitherto been constant, increased rapidly, and this suggests that its value rose when the food available to each cell had fallen below a critical level. When this occurred, the value of the *n.v.i.* may have been proportional to the amount of food available. If so, since the *n.v.i.* remained constant at its new level, the food consumed by the viable cells must have been constant. This conclusion is also reached from the fact that the *m.g.t.* was constant. It follows that in spite of their increasing numbers, the nonviable cells as a group consumed a constant amount of food. Each cell may have disposed of food at the same diminishing rate, but this is unlikely as the cells varied greatly in age. Probably the metabolism of the older nonviable cells was considerably less active than that of the younger cells, and the constancy of the food consumption of the group of nonviable cells came about through this cause. If the adjustment were not perfect and the food consumed by the nonviable cells had tended to rise slightly, the decline in the viable cell population in the later stages of the steady phase is explicable. Less food would have been consumed by the viable cells, and the *n.v.i.* must therefore have increased to a value greater than 0.5. An anomaly is that the total cell population apparently continued to increase steadily, despite the falling viable count, and this would seem to imply that the *m.g.t.* was diminishing. In view of the improbability of this it is felt that a decreasing rate of increase in total cells must have been obscured in some way, for example, by an increase in turbidity due to slight agglutination. Further work on this point is obviously desirable.

It has been made clear that at the onset of the steady phase the cultures at both food levels were in the same state, since in the culture receiving twice the food supply the numbers of both total and viable cells were double those at the lower food level, so that the food supply per cell was the same. Despite this similarity in conditions the ensuing steady phases differed in that the total cell counts in the culture with the higher food level were more than double those with the lower. The respective rates of new cell formation were 3.49 ± 0.114 and 1.37 ± 0.043 millions of cells per ml per hour. In view of the magnitude of the standard errors it is probable that the higher rate is significantly more than twice the lower, but it would be unwise to stress this point unduly since the experiments at the higher food level were not statistically complete replicates. Attention may, however, be called to the fact that immediately following each addition of food solution, the rate at which the viable cells were able

to absorb food could have been twice as high in cultures receiving the double food supply. This may have had a slight accelerating effect on the rate of growth.

As these experiments were roughly quantitative, the results can be used to calculate the approximate consumption of dehydrated Difco broth by a *Bacterium coli* cell under the given conditions. For convenience the food requirement of a cell has been divided into two fractions : (1) the amount x used in the maintenance of the full activity of the cell in unit time apart from reproduction, and (2) the amount y used in the production of a new cell. The quantity x must include not only the actual material used in the replacement of cell substance lost through endogenous catabolism but also food used in supplying energy for the synthesis of this replacement cell substance. The amount y will be made up of two similar portions except that the cellular material synthesised will be that of a new cell. Probably the values of x and y change with the food available to the cell. For example, abundant food might lead to the formation of reserve material in the cells and tend to increase the food wasted. In these experiments, however, the cells were for most of the time limited in their growth by the food supply so that it is unlikely that the deposition of reserves occurred to any significant extent. Changes in over-all efficiency with varying food supply cannot readily be assessed, and in the calculation described below x and y have been assumed to remain constant throughout the whole experiment.

Changes in the cultures were naturally continuous. New cells were being formed, some of them viable, and it is assumed that these retained their full activity and consumed their full quota of food so long as they remained viable. Others were nonviable and their food consumption is regarded as having decreased continuously as they aged. Also, cells once viable may have passed into the nonviable state. It is not easy to assess the food requirements of the cultures on this basis of continuous change and so, for simplicity, the experiments have been divided into intervals of 25 hours. The amount x then becomes the food requirement for a fully active cell, apart from reproduction, for 25 hours. From the composite curves values for the total and viable populations at the end of each 25-hr interval can be obtained. Consequently, the numbers of nonviable cells can readily be calculated and the numbers of new cells formed in each interval determined.

In any interval the food consumed by the culture was the total of the following amounts: (1) that used in the formation and maintenance of the new cells which appeared in the interval; (2) that used in maintenance by the viable cells present at the beginning of the interval; and (3) that used by the nonviable cells present at the beginning of the interval.

Under heading (1) each new cell, whether viable or not, is assumed to have required an amount y for its formation and also food for maintenance at the full rate. The cells were obviously not formed simultaneously at the beginning of the interval but continuously throughout it, and an estimate of the food used by them in maintenance will be obtained by assuming each to have had an average life of half the period. The food used by the newly formed cells will thus

be equal to their number multiplied by $(y + \frac{1}{2}x)$. Under heading (2) the initial number of viable cells required maintenance at the full rate throughout the period and the food used by them will be their number times x. It is assumed that a viable cell which became nonviable continued to use food for maintenance at the full rate until the end of the period in which the change occurred. No different method of estimation is, therefore, necessary even when the viable cells decreased during an interval. Under heading (3) the food used varied according to the proportions present of cells of different ages. Estimation of its amount is based on the assumption that the nonviable cells formed in any interval behaved, during the succeeding interval, as if they possessed a certain proportion of their original activity and that in successive intervals their activity continued to decrease in the same proportion. The diminution of activity with increasing age of an individual nonviable cell probably approximates to the compound interest law ($Q_t = Q_o e^{-kt}$) but, since there was a continuous formation of these cells, and during the initial phase the rate of formation of nonviable cells was not constant, this process of estimating stepwise the diminishing food consumption of ageing cells has been used as a convenient simplification. Thus if N nonviable cells were formed during an interval, in successive intervals these would require FNx, F^2Nx, F^3Nx . . . food for maintenance if F is the fraction of their full activity which they retained. On this basis, as on that of the compound interest law, a cell never loses all activity but there must surely be some limit to the age of a cell over which it ceases to use any food. An approximation to the true state of affairs has been aimed at. On the basis of the above assumptions it became possible to deduce expressions relating the amounts of food used under each of the three headings in terms of F, x, and y and by summation the food used by the whole culture in each interval. To evaluate x and y great simplification was afforded by assigning arbitrary values to F as shown below.

The food available to the culture is known accurately since, for the reasons given earlier, no allowance has been made for autolysis. Whether any of the products of endogenous catabolism can be re-utilised is debatable. In any case the amount of such products could only have been very small in comparison with the food added, and so this source of food has also been neglected. As already shown, during the steady phase the food was being used as fast as it was added, and, therefore, each expression for the food used in any interval during that phase can be equated to the food added during that interval; and that amount was constant. Each expression has a term in x and a term in y. The latter term must become constant during the steady phase because of the constant average rate of formation of new cells. Consequently, the term in x must also be constant in successive expressions. But the value of the x term depends on the value chosen for F, so that criterion gives a definite indication of the proper value of F. Actually, when the expressions corresponding to various values of F are examined, it appears that no value will lead to absolute constancy of the x term. This is hardly surprising in view of the simplifications and assumptions that have been made. Nevertheless, within the range 0.6 to 0.9 a very fair degree of constancy is obtained. Constancy of the x term, however, is not

the only consideration. The best value for F must lead to such values of x and y that during the steady phase the estimates of the food consumed as nearly as possible equal the amount of food added. Also, from analysis of the population-time curves it would seem probable that before stability was reached the food consumed in successive intervals varied widely. At first less food would be used than was supplied and the excess would be available for use in later periods to support a rate of cell increase which could not be maintained. The food consumed in each interval must then have fallen to the actual amount supplied. Accordingly, the estimates of the food used in successive intervals should conform to this pattern and, obviously, at no stage should the estimated total consumption exceed the total amount supplied. A final choice of the value of F cannot, therefore, be made without calculating x and y for various values of F within the range previously indicated. These calculations each require two equations. One was obtained by postulating that by the end of the sixth period (150 hr), when the steady phase had been reached, all the food supplied had been used up, so that the sum of the first six expressions for food utilisation could be equated to the total food supplied during that time. Another equation was obtained by totalling the next six expressions and equating to the food supplied in these periods. The equations were then solved for x and y and the values of the separate expressions calculated. On all counts at the lower food level a value of 0.8 for F appears to be the best. The corresponding value of x is 0.474×10^{-9} mg per cell per 25 hours and of y, 1.083×10^{-9} mg. Table 5 gives, for the lower rate of food addition, the estimated consumption by the whole culture in successive intervals and a comparison with the food supplied. The agreement is considered to be satisfactory. At the higher rate of food addition, also, 0.8 appears to be the best value for F. Here the corresponding value for x is 0.440×10^{-9} mg per cell per 25 hours, and for y, 1.063×10^{-9} mg.

When these values for F, x, and y were applied to the data obtained following the sudden doubling of the food supply after 404 hours, the estimates of the food used were found to be low. Apparently about 10 per cent of the total food added during the 150 hours following the increase (by the end of which time a new steady phase had been entered) was unused, and during the steady phase about 20 per cent of the food added in each interval was not utilised. Naturally this cannot represent the true state of the culture, but the discrepancies can be understood when it is realised that a sudden doubling of the food supply cannot be expected to produce the same result as when the extra food is given from the start. When the food concentration around the already existing cells was suddenly doubled, all the cells, viable and nonviable, young and old, obtained more food than before. This is equivalent to an increase in x when more food is obtained by the young cells and to an increase in F when more food is obtained by the older cells. It is also possible that y might increase slightly for a time owing to the deposition of reserve food materials.

When the food supply was completely stopped there was an immediate cessation of cell formation as judged by the absorptiometer readings, which became roughly constant. The viable cells decreased rapidly but not to zero, a constant

population of about 100×10^6 cells per ml being finally established. Difco broth no doubt contains substances which are not readily available to *Bacterium coli* and it is possible that after the food supply was stopped the cells were utilising an accumulation of this material. But such an accumulation is unlikely since the cultures were always short of easily assimilable food. Alternatively, autolysis may have provided the food necessary to maintain this residual viable population. The constancy of the absorptiometer readings during this phase of the experiments may have been the resultant of opposing tendencies—growth, agglutination, autolysis, and sedimentation, of which the latter was observed to

TABLE 5

Comparison of the estimated food used by the whole culture with that actually supplied

Rate of food supply, 380 mg dehydrated Difco broth per 25 hr

$F = 0.8$

TIME INTERVAL	ESTIMATED FOOD USED IN EACH INTERVAL	ESTIMATED TOTAL FOOD USED BY END OF EACH INTERVAL	TOTAL FOOD SUPPLIED	FOOD LEFT IN CULTURE
hr	*mg*	*mg*	*mg*	*mg*
0–25	158	158	380	222
25–50	369	527	760	233
50–75	509	1036	1140	104
75–100	451	1487	1520	33
100–125	413	1900	1900	0
125–150	380	2280	2280	0
150–175	378	2658	2660	2
175–200	377	3035	3040	5
200–225	378	3413	3420	7
225–250	382	3795	3800	5
250–275	382	4177	4180	3
275–300	383	4560	4560	0
300–325	383	4943	4940	−3
325–350	385	5328	5320	−8
350–375	383	5711	5700	−11
375–400	383	6094	6080	−14
400–425	381	6475	6460	−15
425–450	382	6857	6840	−17
450–475	379	7236	7220	−16
475–500	377	7613	7600	−13

occur. Whatever the true state of affairs, the constancy of the absorptiometer readings suggests that autolysis was very slow, a view which accords with the findings of Sturges and Rettger (1922) quoted above. If autolysis were the source of food for the maintenance of the final steady population of viable cells, the question arises of how much food was likely to have been available from this source. Assuming that during the final steady phase all nonviable cells were available for autolysis, we find that the total of such cells was about 315×10^{10}. The size and dry weight of bacteria vary greatly according to the conditions of cultivation, but Guilleman and Larson (1922) give for *Bacterium coli* a mean volume of $1.0\mu^3$ and an organic matter content of 10 per cent. If the density of the cells be 1.0, this gives a dry weight of 1×10^{-10} mg per cell. Rahn (1932)

gives the dry weight as 2.2×10^{-10} mg per cell. With the larger figure, it appears that the bacterial solids in the whole culture when the food was stopped may have amounted to 693 mg. Now the 100×10^6 viable cells per ml would have required, for maintenance at the full rate, 69 mg food. Autolysis could not, therefore, have provided even maintenance food for the surviving organisms for more than about 250 hours if the previous rate of food consumption were maintained. Actually, some 300 hours after the food had been stopped the number of viable cells was still unchanged at 100×10^6 per ml. From this fact, together with the evidence that *Bacterium coli* autolyses slowly, it is indicated that the value of x in these foodless cultures was considerably decreased. This is in harmony with the previous suggestion that a sudden increase in food available simply leads to an increase in the value of x. A complicating factor, if autolysis does in fact occur, is that the nature of the food had changed from Difco broth to autolytic products, and it is possible that cell substance may be formed more readily from the latter than the former. Here it may be noted that since the dry weight per cell is 2.2×10^{-10} mg and the amount of dehydrated Difco broth used in the formation of a new cell is approximately 1.1×10^{-9} mg, about 20 per cent of the material of the broth was actually converted to cell substance.

The maintenance ration of a fully active cell, under the conditions of these experiments, has been estimated at 0.474×10^{-9} mg per 25 hours, i.e., approximately 2×10^{-14} g per hour. Taking the calorific value of Difco dehydrated broth to be approximately that of dry protein (5000 cal per g) the heat equivalent of the maintenance food ration is 1×10^{-10} cal per cell per hour. It is interesting to note that by direct temperature measurements, but under very different conditions of growth, Bayne-Jones and Rhees (1929) showed that the caloric output of *Bacterium coli* cultures in which multiplication had nearly ceased was falling and approaching 1×10^{-10} cal per cell per hour. Although this figure is a mean for all cells irrespective of age, it was obtained from cultures only 6 hours old in which practically all the cells might be expected to be fully active.

At the present stage in this investigation, it is not proposed to hazard an explanation of the significance of the period of 140 hours occupied by the initial phases in both cultures in reaching a new steady state after the food supply had been doubled, and in attaining the constant "residual" viable population when the food supply was stopped. The effects of variations in temperature and pH of culture on these time intervals, as well as their duration when broth is replaced by pure synthetic media, are being determined before an attempt at an explanation can be advanced.

SUMMARY

Bacterium coli has been cultivated in an apparatus which permits rigid control of temperature, pH, aeration, and culture volume, and also allows food to be supplied at any desired rate by means of an automatic syringe mechanism.

From a number of experiments, determinations of total and viable cell populations were made and growth curves constructed. At each of the two rates of constant food supply used, an initial period in which the total and viable counts

were both increasing was followed by a steady phase in which the viable counts remained constant or decreased slightly while the total cell counts steadily increased. When, in experiments which had reached the steady phase, the food supply was suddenly doubled, a second phase of cell multiplication similar to that in the initial period began and was succeeded by a second steady phase. When during the first steady phase the food supply was suddenly stopped, the total cell population remained constant but the viable cells decreased to a constant low level.

The significance of the growth curves is discussed and approximate calculations made of the amount of food used in the formation of a new *Bacterium coli* cell and in maintenance of a cell apart from reproduction. These amounts of Difco dehydrated broth are found to be 1.1×10^{-9} mg and 0.4 to 0.5×10^{-9} mg per 25 hours respectively.

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