THE GROWTH OF BACTERIAL COLONIES AND THEIR VIABLE POPULATION

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(With 2 Figures in the Text)

INTRODUCTION AND HISTORICAL REVIEW

The numerical aspects of bacterial growth have hitherto been investigated chiefly in fluid media and the laws governing the development of bacterial populations in fluid cultures are known in some detail.

Buchner, Longard & Riedlin (1887) were the first to determine the generation time of an organism, *V. cholerae*, in fluid cultures. Later the conditions influencing the development of a fluid culture were investigated by a number of workers, and bacterial growth phases, viz. lag phase, logarithmic phase, plateau and phase of decline, were demonstrated in fluid bacterial cultures.

Penfold & Ledingham (1914) undertook a mathematical analysis of these phenomena and found that, during the lag phase and the logarithmic phase, the time and the numbers of organisms were connected by relationships which they expressed by mathematical formulae. The influences affecting the phases were also studied extensively by Penfold (1914) and by a number of other workers.

Bail (1929), in a theoretical discussion, summarized the results of much experimental work carried out in his laboratory. He developed the concept that in liquid cultures the maximum number of viable organisms attained in a given volume was a constant, independent of the cultural conditions. Such changes as removal of organisms by centrifuging, dilution of cultures by distilled water, addition of large numbers of organisms, destroying organisms by heat and reinoculating and incubating, adding enriching foodstuffs such as glucose, although they may alter the rate at which maximal population is attained, eventually the organisms reachieve the same concentration as was attained in the original media—called the '*M* concentration'. Many of these remarkable findings have been confirmed by other workers in specific instances, though some are only true for particular organisms, and within certain limits of cultural conditions.

Graham-Smith (1920), using weak media, found the maximal population which was viable was roughly proportional to the dilution of the media. The concentration of some constituent of the media was evidently, in this case, the limiting condition of growth. In Bail's case the attainment of the maximal viable population was not limited by this, as removal of the organisms was followed by regrowth until the same viable density was achieved. Dilution up to 25 times with distilled water was followed by the attainment of the same maximal viable count, though the total amount of bacterial substance present in unit volume was much reduced. In Graham-Smith's media, however, dilution from 2 to 10 times caused a similar reduction in viable count. Analogous results have been more recently obtained in carefully controlled quantitative experiments by Jordan & Jacobs (1944), but in Bail's examples there is still some unknown limitation to the survival of living bacteria. That this limiting factor is not the accumulation of toxic material is shown by the regrowth of organisms when they are removed by centrifugalization. The limitation Bail calls the 'biological space' necessary for survival.

Topley & Wilson (1936) failed to confirm Bail's finding that the addition of glucose did not increase the viable maximal population, though they agree that their suggestion, that available oxygen is a limiting condition causing '*M* concentration', does not explain all of Bail's facts.

The growth of bacteria on solid media has been much less investigated. Henrici (1925) has shown that the development of bacterial growth on agar slopes follows a similar growth curve to that of liquid cultures. Wilson (1922) compared the total and viable counts on agar slopes, and his results, together with Henrici's (1925), show that the initial lag phase, the logarithmic increase which follows, the decline in rate of multiplication as a maximal viable population is reached, and the final decrease in the number of viable organisms are all present. The capacity of a few organisms in most bacterial colonies to survive for long periods of time, especially at low temperatures, is well known. Two publications regarding bacterial populations on solid media are both inaccessible to me except by quotation (Gotschlich, 1929). In one of these, Gotschlich & Weigang (1895) reported an investigation into the

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total number of living organisms (*V. cholerae*) on agar slopes maintained at different temperatures; at 37° C. the maximum number of living organisms was attained after 12 hr. of growth, whilst at 22° C. a considerably higher maximum was reached after 48 hr., but by this time many dead organisms were present in addition. The other paper dealing with the number of individuals in colonies is by Ficker (1895) but, unfortunately, no details of his methods are given in the quotation and his results are not clearly described. The fact that much higher populations are achieved on solid surfaces than in liquid media is now well established, even when those surfaces are inert material such as glass slides, particles of sand or talc. Bigger & Nelson's demonstration (1943) of the multiplication of *B. coli* on powders with CO_2 and NH_3 as the sole sources of nourishment proves the importance of the absorbed layers of chemicals on surfaces. It follows that the use of gels for the cultivation of bacteria may have important effects, which result in different optimal conditions of growth to those found in liquid media. Bigger found that a concentration of ammonium carbonate incapable of supporting growth in liquid media was sufficient for this purpose when absorbed from it on to a solid surface. On the other hand, Graham-Smith (1919) showed that many dyes had to be present in much higher concentration in agar plates than in meat extract to inhibit bacterial growth. The minimum concentration of penicillin which inhibits *Staphylococcus aureus* on agar is greater than the concentration necessary in liquid media.

The necessity for the diffusion of nutrient substances through the agar gel to a growing colony, and for the diffusion away from the colony of the products of growth, is another differing condition of growth on agar plates from the ready convection of materials which takes place in liquid cultures. An investigation of the viable counts of organisms in colonies is therefore of some interest and importance, though the experimental difficulties involved may be a partial explanation of the scantiness of the literature so far accumulated on the subject.

PLAN OF PRESENT INVESTIGATION

In working with colonies on a solid medium a number of difficulties and errors arise which are absent when working with a fluid culture. Whereas a culture in fluid medium can be investigated repeatedly with no limitations of the number of counts and the intervals between them, a colony on a solid medium, when once used for a count, no longer exists. Further, the number of organisms inoculated on to a plate must be limited to allow for the growth of separate colonies, whilst the exact number of colonies on a plate is largely a matter of chance, however great the care in preparing the plates. The same element of chance operates for the distribution of colonies on the available surface, so that, of the already limited number of colonies, only a few are sufficiently comparable to be suitable for counts.

The same strain of *B. coli* was used throughout the experiments. The principle of the method was to prepare plates of the organism so as to obtain isolated colonies. Two different dilutions of a suspension from a young culture were prepared, and one loopful of each was spread over the surface of a large plate containing 20 c.c. of Lemco agar. The plates were incubated at 37° C. The colony whose count was to be determined was cut out with the underlying and surrounding medium and was broken up in 1 c.c. of broth by rubbing the surface of the medium with a wire loop and vigorous shaking for several minutes so as to obtain a suspension of the organisms. 9 c.c. of broth were added. The number of organisms contained in this suspension was then estimated by the usual plating methods. From the suspension of the colony in 10 c.c. broth at least two, or even up to seven, independent series of dilutions were made in distilled water. As the final dilution of each series, one was chosen that was estimated to give between 50 and 300 colonies on a plate. This last dilution was then made in clear agar at 52° C. and was poured into a plate. As never more than 10 min. elapsed from the time of preparation of the first dilution in water to the time of pouring the plate, the use of distilled water as a diluent was thought to be innocuous. In a few experiments in which a series of dilutions was made in broth for comparison, the difference between the number of colonies in the two series was not greater than in two series prepared in distilled water. The dilutions were made with straight graduated 0·3 c.c. pipettes by transferring 0·1 c.c. into 10 c.c. of diluent, using a fresh pipette for every dilution as stressed by Wilson, Twigg, Wright, Hendry, Cowell & Maier (1935). The counts were made after 24 hr., since preliminary experiments showed that the number of colonies did not increase after that time.

Under ideal conditions of preparation of suspensions and of dilutions the individual counts for any one colony would be distributed as a Poisson series. Under the actual experimental conditions such a distribution was not, and perhaps could not, be realized. Certain errors are inherent in the technique and make the variation between plate counts of the same colony exceed slightly those that would be expected if chance alone was operative. The error of this method has been analysed by Jennison & Wadsworth (1940); and the error in our observations seems to be of the same order as obtained by them.

After a few preliminary experiments it became obvious that the population of a colony was dependent on its position relative to neighbouring colonies, so that it was found necessary to measure the degree of crowding to which the colonies were exposed and to classify them according to this characteristic. This classification was carried out in the following way. Two disks were cut from cardboard, one with a radius of 1 cm., the other of 1·5 cm., the centres of the disks being marked. Each disk was placed in turn on the glass plate under the medium, with the centre mark exactly under the centre of the colony to be investigated, and the colonies which were on the areas bounded by the circles were counted. Colonies which were lying partly in the circles were counted as full, half or naught, according to how much of them was inside the circle. The two figures found for the number of colonies inside the two circles were added up, and in this way an index number obtained for the colony under investigation. For instance, a colony may have one neighbouring colony nearer than 1 cm. and 2 colonies farther than 1 cm. but nearer than 1·5 cm. Over the smaller circle 1 colony will be counted, over the larger one, 3. By adding them up we obtain the index figure 4. As the colony nearer than 1 cm. is counted over both circles it is contained twice in the index number, the colonies at a distance of 1·0–1·5 cm. only once. The same index number 4 would be obtained in the case of two colonies nearer than 1 cm., and no colony between 1 and 1·5 cm.; or in the case of no colony nearer than 1 cm. and 4 colonies 1–1·5 cm. distant. This index number takes account of the fact that decreased distance of colonies means increased influence, but it is a very rough measure and would be much improved, for instance, if the colonies farther distant could also be taken into account. However, for the present investigation, which deals with approximate figures, it proved to be adequate. Other more complicated schemes of giving an index number to a colony (e.g. no. of colonies/distance^2) yielded slightly varying figures, but the order and significance of the results obtained were the same. The use of the total number of colonies on the plate is not a sufficiently accurate measure of their effect as near colonies have more influence than distant ones. The following grouping was finally adopted:

Group	Colonies with index numbers
I	0
II	1– 4
III	5–10
IV	11–16
V	17–24
VI	25–33

Of each group 1–12 colonies were subjected to counts of their viable populations at each stage of development from the 2nd up to the 8th day of incubation. Altogether more than 80 counts were performed after the method had been tried out and its error determined in a large number of preliminary counts. The figures given for the viable count of any colony in Table 1 are the averages of the counts of at least two series of dilutions put up from that colony.

The error in the differences of viable counts between colonies of the same group and of the same age is difficult to assess. The error is made up of several components of which the error caused by technique as, for instance, differences in the preparation of medium, is probably the least important. Biological variation probably plays a considerable role. The greatest contribution to the error is doubtless made by the unavoidable arbitrariness of any grouping which may separate colonies that biologically are similar, and group together others that live under different biological conditions with regard to their neighbours.

RESULTS

The average of the logarithms of all the counts made on the colonies in a particular group examined after the same number of days' incubation are the points plotted on Fig. 1. At 2 days' incubation groups I–IV show no significant difference in the number of viable organisms present in the colonies, though already the excessive crowding of colonies in groups V and VI results in significantly lower viable counts. With the passage of time the differences between the groups increase until at 6 days groups I, II, III and VI are all significantly different. (Groups IV and V were not examined as long as this.)

The results obtained for group I showed very considerable differences between colonies of the same age. This irregularity only appears so because the total number of colonies on the plate, though all at distances greater than 1·5 cm., was not taken into account. On plates with a total of more than 10 colonies the maximum of the population was reached on the 4th or 5th day and a decline was shown on the 6th day. On plates with less than 10 colonies there was still an increase in population from the 5th to the 6th day. The highest living population was obtained with a colony standing singly on a plate on the 6th day, namely, 9980 millions.

In group II also the colonies differed considerably in the time required to reach the maximum and to commence to decline. On an average the maximum was reached on the 4th day and numbered 2000–3000 millions. The highest number obtained with a colony in this group was 3050 millions on the 4th day.

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In group III the maximum was reached on the 2nd to the 4th day and was 1500–2300 millions. A good example of the influence of the total number of colonies on the populations in this group was given in the following experiment. Two plates were poured and inoculated at the same time; the total number of colonies coming up on one was 43 and on the other 233. From each plate one colony of the same index number, 8, and apparently of the same size, was counted on the 3rd day. The colony from the plate with 43 colonies had a population of 1270 millions, that from the plate with 223 colonies, a population of 850 millions.

In group IV the maximum was 1300–1700 millions, reached on the 2nd or 3rd day. In groups V and VI the maxima were reached on the 2nd day and were about 600 and 300 millions respectively.

If the cause of the reduced viable count as a result of nearby colonies is either the exhaustion of essential foodstuffs or the accumulation of inhibitory products, we should expect an increased effect with greater crowding as shown in Fig. 1. The adverse effect of crowding is also greater the longer it has time to act; for example, the greatest difference between the viable population of group I and group VI is developed on the 8th day, whereas the

Table 1. *Details of viable counts*

The figures in the first column are the viable populations of every colony counted, which was grown under the same conditions and counted by the same method. Each figure is an average of 2–7 independent counts of the same colony. The index number is derived by adding the number of colonies (x) within 1 cm. radius of the colony under observation to those ($x+y$) within 1·5 cm. radius. It is therefore expressed by $2x+y$. The fractions denote that a colony was lying just on the circle. The brackets denote that on the day of incubation shown in it the colony or half colony preceding the bracket was cut out and used for a viable count.

Viable count in millions	Days of growth	Total cols. on plate	Cols. within 1 cm. radius (x)	Cols. within 1·5 cm. radius ($x+y$)	Index no. ($2x+y$)
			Group I		
2440	3	15	0	0	0
4550	4	18	0	0	0
4220	4	18	0	0	0
6050	4	6	0	0	0
6220	4	6	0	0	0
3870	4	9	0	0	0
1140	4	18	0	0	0
3050	5	6	0	0	0
8050	5	9	0	0	0
4370	5	11	0	0	0
2800	5	15	0	0	0
8600	6	6	0	0	0
5160	6	11	0	0	0
9980	6	1	0	0	0
2810	6	18	0	0	0
2700	8	15	0	½ (3rd day)	0
			Group II		
1835	2	44	2	2	4
2915	2	19	0	1½	1½
1770	3	9	½	2	2½
1120	3	18	½	1½	2
1160	3	138	0	4	4
1290	3	138	0	4½	4½
2830	4	11	0	½	½
1650	4	44	1	2½	3½
3050	4	19	0	1	1
2500	5	18	½	1+½ (4th day)	2
1250	5	—	½	1½+1 (3rd day)	3
920	5	44	0	3	3
2100	5	19	½	1	1½
1480	6	18	½	1½+1 (4th day)	3
2035	6	19	0	2½	2½

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Viable count in millions	Days of growth	Total cols. on plate	Cols. within 1 cm. radius (x)	Cols. within 1·5 cm. radius ($x+y$)	Index no. ($2x+y$)
			Group III		
1540	2	64	1½	7	8½
1280	2	43	1	7	8
2280	2	71	1½	5½	7
1760	2	35	2½	4½	7
730	3	—	1	6	7
710	3	—	3	7	10
2175	3	44	2	4	6
1270	3	43	3½	5	8½
850	3	223	1	7	8
1010	3	138	1	5½	6½
1370	3	138	2	5½	7½
1320	3	138	2	5	7
1160	3	138	2	5	7
1530	3	71	½	4	4½
2275	3	19	1	4	5
1300	3	35	3½	5	8½
1010	4	18	1½	4	5½
1000	4	18	1	3	4
690	4	—	1½	5	6½
1000	4	64	2	4	6
580	4	71	½	4+1 (3rd day)	5½
470	4	35	2	6	8
540	5	—	3	4	7
830	5	64	1½	3½	5
880	5	71	1½	3½	5
520	6	—	1	4	5
340	6	—	½	5	5½
620	6	64	1	3+1 (5th day)	5
425	6	44	½	3½+1 (5th day)	5
700	6	18	½	4+1 (4th day)	5½
400	6	71	2	5+1 (5th day)	8
480	7	18	1½	3+½ (6th day)	5
225	8	—	2½	5½	8
			Group IV		
1060	2	44	4	7	11
1690	2	72	3	8	11
1370	3	44	3	7	10
1100	3	72	3	8½	11½
825	4	131	6	10	16
750	4	44	3	8	11
720	4	72	3	9	12
1530	4	43	3	9	12
			Group V		
425	2	131	4	14	18
705	2	107	4	14	18
405	3	131	7	16+1 (2nd day)	24
200	4	131	8	15	23
			Group VI		
336	2	107	11	16	27
169	3	—	6	23	29
140	4	—	7	18½	25½
140	5	—	6	24	30
58	7	—	9½	17	26½
14	8	—	9	20	29

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smallest difference is on the 2nd day. This greater number of viable organisms in isolated colonies suggested that solid media might illustrate the principle of equivalent maximal growth on a certain area of plate analogously to Bail's maximum density of population in liquid media. Fig. 2 was constructed to show that in these experiments this law had been fulfilled within the limits of experimental error. The lower number of viable organisms in crowded colonies is just about compensated for by the increased number of colonies in unit area, so far as the actual number is concerned. The attainment of the maximal population, however, is reached much more quickly with large numbers of colonies in a given area than it is with a few isolated colonies. The total number of colonies on a plate that are capable of giving visible growth is well known to be diminished below expectations by overcrowding. In fact, Wilson (1922) introduced a correcting factor into plate counts for those that failed to develop as a result. This lack of development was, however, regarded mainly as due to almost coincident colonies being counted as single colonies rather than to any direct inhibition of growth.

The influence of certain variations was investigated to some extent. The strain used was one which very readily dissociated and, though in most experiments colonies of the S-type were used, some of the experiments concerning groups I and II colonies were carried out with colonies showing R-sectors, entirely smooth ones not being available.

In a number of experiments pure cultures of R-variants were used in comparison with smooth colonies of the original strain. Throughout, the R-colonies showed a maximal living population about 50% higher than for the S-colonies; they reached the maximum earlier and on the whole declined more rapidly.

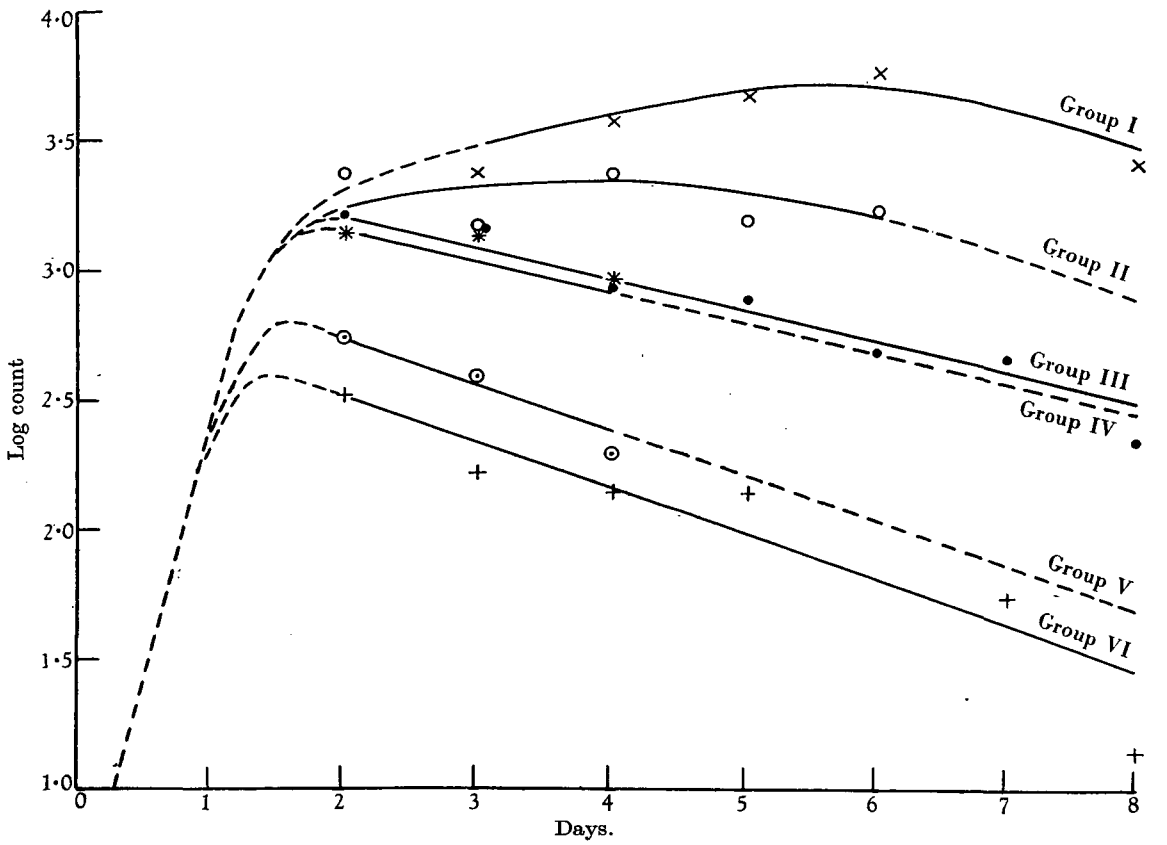

Fig. 1. Showing the development of viable populations in six groups of colonies which differed as to the degree of crowding to which they were exposed. The dotted lines represent interpolated results. The results found by experiment are represented by signs, × for group I; ○ for group II; ● for group III; ✱ for group IV; ⊙ for group V and + for group VI. Each sign represents the average of 1–12 colonies counted.

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A few experiments were carried out under different cultural conditions. Colonies grown on 10% unheated blood agar seemed to have a maximal population only slightly higher than corresponding colonies on plain Lemco agar but to decline more rapidly. One experiment was performed at room temperature and followed up at irregular intervals for nearly 4 weeks. The maximum reached for colonies of group III was 2970 millions on the 6th day, the corresponding colonies at 37° C. showing a decline to a number of 690 millions on the 4th day when counts were begun. After 27 days the population at room temperature was still 134 millions. These figures, which show that at room temperature the maximum is higher and reached later and that the decline is very gradual, are in good agreement with the figures given by Gotschlich for the populations on agar slopes incubated at 37 and 22° C. respectively.

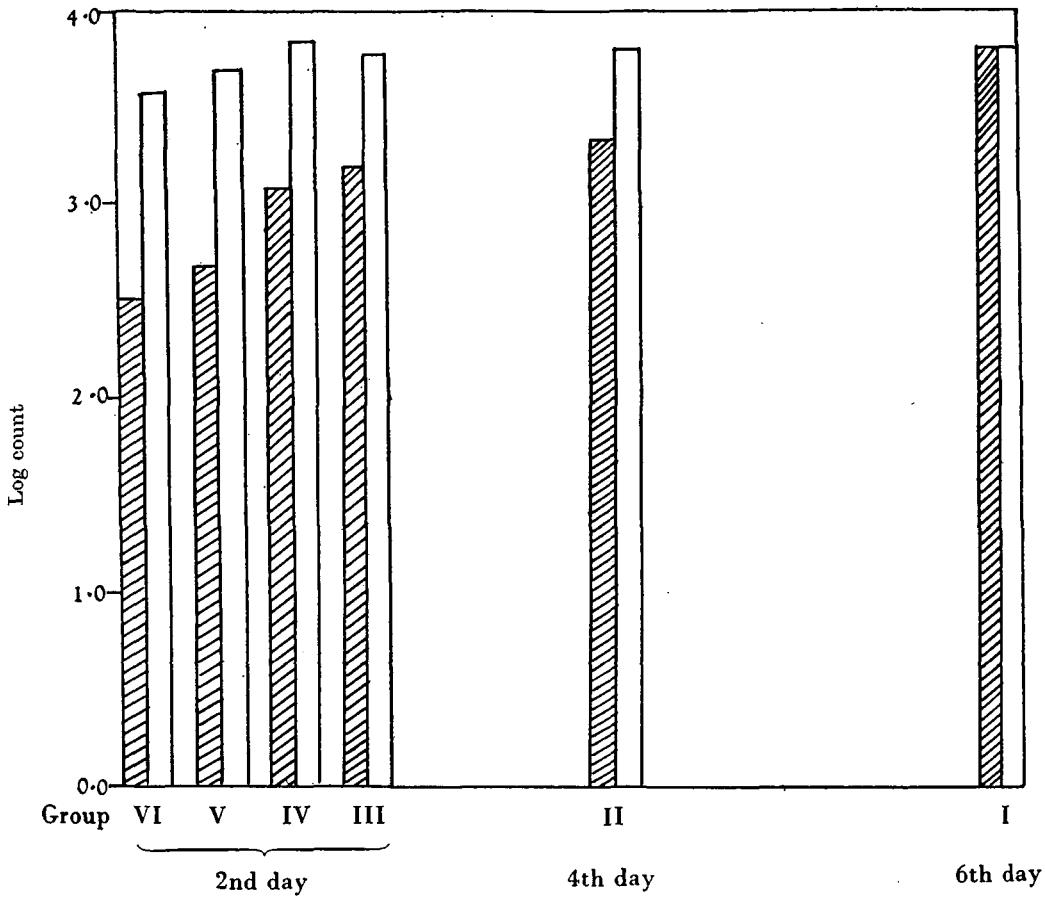

Fig. 2. The shaded columns represent the maximum viable populations of colonies of each group. All the counts on the day of development when colonies of a particular group reach their highest population were averaged. Thus the column for group I is the average of all counts of colonies in this group on the 6th day of incubation. Similarly, the figure for group V is the average of all group V colony counts on the 2nd day of incubation. The unshaded columns represent the maximal number of viable organisms which could theoretically be present if the growth of colonies of the different groups were imagined as covering equal areas (circles of 1·5 cm. radius) on the days of maximal growth for that particular group. The tops of these columns are at approximately the same level, indicating that the sums of the maximal colony populations are equal per unit area.

DISCUSSION

In considering these results and their significance one conclusion becomes obvious, namely, that the maximal population reached by a colony during its life is, all other circumstances being constant, strictly dependent on the number and distance of

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neighbouring colonies. The more colonies there are on the plate and the nearer they are to the colony investigated, the smaller will be the maximum number of living individuals which can be maintained in the colony.

Underlying these facts are, obviously, phenomena of diffusion, i.e. diffusion of nutrient substances to the colony and of products of metabolism away from it. An isolated colony can for a long time keep up the flow of these substances; neighbouring colonies will tend to diminish the concentration gradient of nutrient substances surrounding the colony under consideration and diminish its available nutrient material; they will also increase the concentration of metabolic products around this colony and thus oppose the diffusion of these substances away from it. As diffusion in agar takes time it is quite clear that the shorter the distance between the colonies, the earlier will they feel this interference with the diffusion processes. This has been borne out by experiments which show that crowded colonies start their decline on the 2nd day, whereas the more isolated ones can go on to increase their viable population for days.

The eventual effect of all these diffusion processes around colonies is to make the sums of the maximal viable populations of all the colonies on a certain area equal to that of any area of the same size whether the colonies on such an area are sparse or crowded. This fact of a maximum viable population per area bears some resemblance to the phenomenon of 'maximal populations' as described by Bail for fluid cultures. In the case of a solid medium, however, this is a purely theoretical concept which cannot be experimentally confirmed. In any area, which can be experimentally subjected to counting the viable populations on it, the colonies will be at very different stages of their development; some may have reached the maximal population, others may not have reached it, or passed it. Only the theoretically calculated sum of their maximal viable populations will give the constant which is the maximal viable population of that area. This figure is capable of being found experimentally in one case only, namely, when all the colonies on that area are equidistant, that is, belonging to the same group, and the count carried out on the day when they have all reached their maximal viable populations. In all other cases the figures obtained experimentally will be below that of the theoretical maximal population of that area, but approaching it as the distance between colonies comes nearer to being equal.

Bail claimed that a maximal population represented the mature state of a culture in the same way as mature individuals of any species of higher animals or plants reach a certain size which is an inherent property of the species. It is impossible at the moment to define a relation between this concept and the results of the investigation here presented.

CONCLUSIONS

1. A method to measure roughly the crowding of colonies on the surface of an agar culture is described.

2. The maximal viable count of a colony of *B. coli* on nutrient agar is shown to be quantitatively dependent on the number and distance of other colonies on the plate.

3. It is higher the less colonies there are in the culture and the greater the distance between them.

4. The time when the maximal viable count is reached depends on the same factors as the number. The more isolated a colony stands the longer its population will go on increasing, and its maximal viable population will be reached days later than that of colonies under crowded conditions.

5. The sum of the theoretically calculated maximal viable counts of all colonies is equal per area of culture surface.

6. The possible relationship of these results to those obtained in liquid media, and the factors influencing growth (such as availability of food supplies) are discussed.

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