Growth of Elements of Various Sizes Found in Cultures of a Stable *Proteus* L Form

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SUMMARY

Samples of a liquid culture of *Proteus* L9 were transferred to agar blocks containing the same medium as the liquid culture. Photomicrographs were made of the slide cultures thus obtained, and the diameters of a large number of individual L elements determined. The measurements indicated that the elements of the L cultures studied could be assigned to two classes (I and II) with regard to their size. The elements of class I had an average diameter of 0.33μ (standard deviation 0.071 ; standard error of the mean 0.006μ); the corresponding figures for class II were 0.94 , 0.21 and 0.02μ , respectively. The growth of individual L elements in the slide cultures was followed during incubation at 30° . Only elements having a diameter > 0.6 to 0.7μ , i.e. elements belonging to class II, enlarged measurably or formed microcolonies. Calculations based on measurements made on L elements growing on streak plates confirmed these observations.

INTRODUCTION

Elements of various sizes are found in all cultures of bacterial L forms. Many investigators have noted in such cultures the occurrence of granular particles of a size approaching the resolving limit of the light microscope, in addition to elements of a size comparable to that of a normal bacterium. Whether the granular particles play a role in the life cycle of the L forms has been debated for several years (Kellenberger, Liebermeister & Bonifas, 1956; Tulasne, Minck, Kirn & Krembel, 1960; Klieneberger-Nobel, 1960; Weibull & Beckman, 1961). To elucidate this point some experiments with slide cultures observed by the phase contrast microscope (Knöll, 1944) have been performed by the present authors. A stable L form derived from *Proteus mirabilis* (Klieneberger-Nobel, 1956; Weibull & Beckman, 1961) was studied.

METHODS

Organism. The *Proteus* L form studied has been designated as strain L9 (Klieneberger-Nobel, 1956). It was derived from a strain of *Proteus mirabilis* (Weibull & Beckman, 1961).

Growth conditions. Samples of the L form were obtained for experimental purposes from stock cultures grown in 200 ml. Erlenmeyer flasks containing 50 ml. of the liquid serum-free medium described by Abrams (1955). No penicillin, however, was included in the medium used in the present work. The cultures were incubated at 30° for 24 hr. on a rotary shaker (100 rev./min.) and stored at room temperature.

Subcultures were made weekly. Bacterial material for slide cultures was obtained by growing *Proteus* L9 on Abrams's medium as described above for about 24 hr.

Preparation of slide cultures. Agar blocks approximately 1 mm. thick and containing 0.8 % (w/v) agar were used. The cultures were sealed with Vaspar (petroleum jelly). For precise location of individual L elements and colonies, Formvar films provided with lattice-like ruled areas were attached to the coverslips (Taubeneck, 1959).

Light microscopy. Photographs were taken using a $\times 90$ Leitz phase contrast oil-immersion objective and a $\times 10$ compensating eyepiece. Negatives obtained on Gevaert Graphic Ortho O5 plates were enlarged twice when printed to give a final magnification of $\times 2600$. The sizes of individual L elements were measured either on the photographic prints or under the microscope. In the latter case a $\times 25$ eyepiece equipped with a micrometer was used. The distance between the rulings of the micrometer scale corresponded to $0.7\ \mu$ in the microscopic preparation when the oil immersion objective was used.

RESULTS

Preparation of medium for the slide cultures

Factors influencing the growth of *Proteus* L9 in pour plates and streak plates were studied by Weibull & Lundin (1961). No growth was obtained when fresh unsupplemented Abrams's medium (1955) was used for preparing the plates. When, however, 10 % (v/v) inactivated horse serum was added to Abrams's medium, viable counts of about 5×10^8 /ml. were obtained in a 24 hr. *Proteus* L9 culture. An agar content of 0.7–0.8 % (w/v) was found to be optimal for growth.

A solid serum-free medium, giving only about 40 % lower viable counts in pour plates than the serum-containing medium described above, was prepared by centrifuging at 15,000 *g* an over-night culture of *Proteus* L9 grown in unsupplemented liquid Abrams's medium, killing the remaining viable L elements in the supernatant fluid by boiling or autoclaving this fluid, and adding 0.8 % (w/v) agar. This medium will subsequently be called 'old' Abrams's medium. It has now been found that the addition of 10 % (v/v) horse serum to 'old' Abrams's medium increases the viable counts three to four times. Consequently this serum-containing medium was chosen for the experiments described below. Some control experiments with fresh Abrams's medium supplemented with serum gave, however, essentially the same results.

The appearance of the slide cultures before incubation

Plate 1, fig. 1, shows the appearance of an uninoculated slide culture as viewed under the phase contrast microscope. The microscope was focused on the Formvar film which was attached to the lower surface of the coverslip and thus was in close contact with the agar surface. The dark lines represent replicas of the rulings in the glass matrix used for preparing the Formvar film (Taubeneck, 1959). The sides of the square are $25\ \mu$ apart.

Plate 1, fig. 2, and Pl. 2, fig. 4, show the appearance of a slide culture inoculated with *Proteus* L9 elements, previously grown in Abrams's liquid medium for about 24 hr. at 30°. It can be seen that most of the L elements were approximately spherical and had sizes varying from the resolving limit of the microscope to several μ .

Figure 1 shows the results of measurements performed on three slide cultures inoculated with different *Proteus* L9 cultures as described above. The measurements were made immediately after the inoculation. The diameters of all *L* elements (about 400) reproduced on three photographic prints were measured. It can be seen that the *L* elements can be assigned to two classes (I and II) according to their size. The average diameter of the particles belonging to class I is about 0.3μ and that of the particles of class II is about 0.9μ . More than 97 % of the latter particles have a diameter $> 0.6\mu$ and more than 99 % of the former ones have a diameter $< 0.5\mu$. About 90 % of the particles of class I have a diameter $< 0.4\mu$.

Fig. 1. Frequency of elements of various sizes in slide cultures of *Proteus* L9 before incubation. The *L* elements had previously been grown in Abrams's (1955) liquid medium.

The appearance of the slide cultures after incubation

Plate 1, fig. 3, and Pl. 2, fig. 5, show the appearance of two different slide cultures of *Proteus* L9 after incubation at 30° for 17 and 9 hr., respectively. Identical fields of view of these cultures before incubation are shown in Pl. 1, fig. 2, and Pl. 2, fig. 4. It can be seen that most of the larger *L* elements (diameter $> 0.8\mu$) grew considerably during incubation. Some of these elements, however, lysed or remained unaltered in size. According to the photographs several of the small *L* elements (diameter $< 0.6\mu$) were more or less displaced during the incubation of the slide

cultures. However, none of these elements with certainty increased their size as judged from the study of these photographs. Altogether, photomicrographs of slide cultures of eight different batches of *Proteus* L9 before and after incubation at 30° for 7–18 hr. were studied. The size of all particles exhibiting growth as judged by the unaided eye was determined. According to these measurements only particles of a diameter $> 0.6\text{--}0.7\mu$ enlarged definitely during incubation. This value corresponds with the lower size limit of the L elements belonging to the particle class II in Fig. 1.

The average diameter of 102 L elements from four different batches of *Proteus* L9 and having a diameter $> 0.6\mu$ was found to be $0.94 \pm 0.02\mu$ (the $\pm$ sign indicates standard error of the mean) before incubation and $1.43 \pm 0.05\mu$ after incubation for 7–17 hr. at 30° in slide cultures. The difference is statistically significant. Observations on about 200 L elements of a diameter $> 0.6\mu$ showed that 76 % of these particles grew during incubation. The rest showed no increase in size or lysed.

The appearance of about 1000 L elements from eight batches of *Proteus* L9 and belonging to the class I of Fig. 1 (diameter $< 0.6\mu$) was studied before and after incubation at 30° for 7–18 hr. It never was observed with certainty that such an element grew to such an extent that after incubation it should be assigned to the class II of Fig. 1. Furthermore, the diameters of 150 sharply focused small L elements were measured on photographic prints representing unincubated slide cultures. The same measurements were made on about the same number of incubated elements. In this way, the average diameter of L elements belonging to the class I of Fig. 1 was found to be $0.33 \pm 0.006\mu$ before incubation and $0.32 \pm 0.008\mu$ after incubation. Thus the measurements performed did not indicate any growth of L elements of a diameter $< 0.6\mu$ during incubation in the slide cultures.

Experiments with L cultures fractionated by means of differential centrifugation

The results given above agree with the findings of Weibull & Beckman (1961), obtained by means of chemical and radiochemical methods. According to these workers there is very little or no biosynthetic activity of small L elements (most of them having a diameter $< 0.3\mu$, as measured electron microscopically). To compare more closely the data obtained by means of the chemical methods with the results of the slide culture studies described in the present paper, L cultures were fractionated by means of differential centrifugation essentially as described by Weibull & Beckman (1961). A suspension of small L elements obtained by this procedure was suitably diluted in 'old' Abrams's medium and slide cultures of the suspension were prepared. As can be seen from Pl. 2, fig. 6, almost all of the microscopically observable elements are bodies near the resolving limit of the light microscope. Only occasionally larger elements were found (as indicated by the arrow in Pl. 2, fig. 6). The average diameter of about 100 sharply focused small bodies was found to be $0.35 \pm 0.008\mu$. After 18 hr. incubation the corresponding figures were $0.34 \pm 0.009\mu$, i.e. no measurable growth of these bodies could be observed. At the same time, however, contaminating L elements of larger sizes enlarged considerably and often formed microcolonies (Pl. 2, fig. 7). It should be pointed out that the size of the small elements in the fractionated L cultures (average value 0.35μ) was practically the same as that of the small L elements found in unfractionated cultures (average value 0.33μ).

Experiments with streak plates

In all the slide culture experiments described above, the *L* elements were incubated in close contact with ruled Formvar films which in turn were attached to the coverslips. This arrangement made it easy to find again after incubation any individual *L* element studied or photographed before incubation, even when the slide culture was removed from the microscope stage in the meantime. On the other hand, it could be argued that the growth of the small *L* elements might be inhibited more than that of the larger ones under the prevailing semi-anaerobic conditions. To test this possibility cultures of *Proteus* L9 were suitably diluted with 'old' Abrams's medium and spread as uniformly as possible on plates containing the same medium and 0.8 % (w/v) agar. The plates were incubated at 30° for about 24 hr. Agar blocks were removed from the plates before and after incubation and studied under the phase contrast microscope. Before incubation the number of *L* elements having a diameter of ≤ 0.4 , $0.4-0.7$ and $\geq 0.7 \mu$ was determined in several fields of view using the ocular micrometer. After the incubation, the number of microcolonies formed (average diameter about 20μ) was determined in the same way. Table 1 gives the results of these experiments. Some of the *L* cultures were fractionated by means of differential centrifugation. The fractionation was essentially carried out as described by Weibull & Beckman (1961).

Table 1. *Number of L elements of various sizes present in L cultures grown in Abrams's liquid medium and number of microcolonies formed after incubation of these cultures for about 24 hr. on streak plates containing 'old' Abrams's medium plus 0.8 % agar-agar and 10 % horse serum*

The *L* cultures used in two experiments (3 and 4) were fractionated by means of differential centrifugation according to Weibull & Beckman (1961). The diameter of the *L* elements was measured by means of an ocular micrometer. The $\pm$ sign indicates standard error of the average values given.

Expt.	No. of <i>L</i> elements per microscopic field of view and having a diameter of			No. of microcolonies after incubation
	$\leq 0.4 \mu$	$0.4-0.7 \mu$	$\geq 0.7 \mu$	
1	57.7 ± 8.8	1.56 ± 0.25	7.39 ± 0.67	4.61 ± 0.20
2	87.0 ± 8.6	4.90 ± 0.41	8.20 ± 0.53	5.50 ± 0.29
3	c. 350	0.79 ± 0.17	0.23 ± 0.06	0.19 ± 0.03
4	c. 500	0.37 ± 0.07	0.24 ± 0.08	0.40 ± 0.04

When unfractionated *L* cultures were studied (Table 1, Expts. 1 and 2) the number of microcolonies formed on the streak plates was about 30 % lower than the number of *L* elements of a diameter $\geq 0.7 \mu$ present on the plates before incubation. Experiments with slide cultures (see the preceding sections of this paper) indicated that about 75 % of similar *L* elements enlarged and that the rest remained unchanged or lysed during incubation at 30°. Thus the results of these two methods of investigation agree well. In any case the results of the streak plate experiments gave no evidence indicating that *L* elements having a diameter $< 0.7 \mu$ were able to grow.

The experiments with fractionated *L* cultures (Table 1, Expts. 3 and 4) gave essentially the same results as the experiments with whole *L* cultures, even if in

the former case the number of microcolonies was almost as high as (Expt. 2), or slightly higher than (Expt. 4) the number of L elements of a diameter $\geq 0.7\mu$ present in these cultures before incubation. The total number of L elements of a diameter $> 0.4\mu$, however, was still higher and that of the L elements having a diameter of $\leq 0.4\mu$ was far higher.

DISCUSSION

Many attempts have been made to determine the size of the smallest viable elements present in cultures of bacterial L forms. Generally the filtration technique has been used. From results obtained by this technique, Kellenberger *et al.* (1956), Kliceneberger-Nobel (1956), Tulasne & Lavillaureix (1958) and Rada (1959) concluded that the smallest viable elements of the stable *Proteus* L forms investigated had a diameter of about 0.3μ . However, even when filtration experiments are carefully performed, the results obtained are not always easily interpretable. For example, soft, spherical particles may be forced through the filter pores in the form of threadlike elements. Such elements would have a considerably smaller diameter than the original spheres. A too-low value might thus be obtained for the smallest filterable elements of the L culture. Another source of error in a filtration experiment is due to the gradual plugging up of the filter pores. The number of elements of a certain size recovered in the filtrate may thus be considerably lower than that of the same elements in the original culture.

The technique of observing the L elements in slide cultures as described in the present paper eliminates the above-mentioned drawbacks of the filtration method. On the other hand the slide culture method implies that the L elements can be observed in the light microscope. Moreover, elements of a certain kind occurring at a very low frequency as compared to the total number of elements ($< 1:10^3$) may not be observed when the microscopic method is used.

The results obtained during the present investigation indicate, among other things, that individual elements of *Proteus* L9 of a diameter $< 0.6\mu$ at the most very seldom grow. However, this finding does not necessarily contradict the results of the filtration experiments quoted above, since only between 1000 and 2000 L elements were studied in the present investigation and the result of the filtration experiments can be explained if only a few of about 10^5 particles having a diameter of 0.3μ are viable.

On the other hand, the slide culture experiments clearly indicate that the great majority of the small L elements (diameter $< 0.6\mu$) do not grow under conditions that are very favourable for growth of the L culture as a whole. Therefore these elements probably do not play a major role in the ordinary life cycle of *Proteus* L9. The fact that the L elements with regard to their size can be assigned to two different classes, one of which contains only or almost only non-viable elements and the other mainly viable elements, is also noteworthy in this connexion.

Our results agree with the data recently published by Roux (1960). This worker studied an unstable L form of *Vibrio cholerae*, and stable and unstable L forms of *Proteus* P18. By using the micromanipulation technique Roux found that granular elements released from large L bodies never gave rise to colonies. Under the same cultural conditions up to 50 % of the large L bodies themselves were viable.

Weibull & Beckman (1961) showed that small L elements, most of them having

a diameter $< 0.3\mu$ as measured electron microscopically, exhibited a very low biosynthetic activity, if any. These elements were obtained by fractionation of whole *L* cultures by means of differential centrifugation. Essentially the same fractionation procedure was used in some of the present experiments. In agreement with the findings of Weibull & Beckman the isolated, small *L* elements did not grow in slide cultures. From measurements on photographs of the slide cultures the average diameter of these *L* elements was found to be 0.34 to 0.35μ . Since, however, relatively large systematic errors may be involved, especially in the light microscopic size determinations, essentially the same kind of fractionated *L* elements were very probably studied by the present authors and Weibull & Beckman (1961).

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EXPLANATION OF PLATES

The magnification in all figs. is $\times 2600$.

PLATE 1

Fig. 1. Slide culture of *Proteus L9* before inoculation as viewed under the phase contrast microscope. The microscope was focused on the Formvar film which was attached to the lower surface of the coverslip and thus was in close contact with the agar surface. The dark lines represent replicas of the rulings in the glass matrix used for preparing the Formvar film.

Fig. 2. Slide culture of *L* elements immediately after inoculation with a liquid culture of *Proteus L9* previously grown in Abrams's liquid medium.

Fig. 3. Same culture as in Fig. 2 after 17 hr. incubation at 30° . Identical fields of view in Figs. 2 and 3.

PLATE 2

Fig. 4. A slide culture of L elements other than that shown in Pl. 1 immediately after inoculation with a liquid culture of *Proteus* L9 previously grown in Abrams's liquid medium.

Fig. 5. Same culture as in Fig. 4 after 9 hr. incubation at 30°. Identical fields of view in Figs. 4 and 5.

Fig. 6. Slide culture of L elements immediately after inoculation with a liquid culture of *Proteus* L9 previously grown in Abrams's liquid medium. The culture had been fractionated according to Weibull & Beckman (1961).

Fig. 7. Same culture as in Fig. 6 after 18 hr. incubation at 30°. Identical fields of view in Figs. 6 and 7.