FACTORS INFLUENCING THE GROWTH OF A STABLE L FORM OF *PROTEUS MIRABILIS*

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For several years after the discovery of the bacterial L forms (Klieneberger, 1935), these forms were grown on media solidified with agar. But Tulasne (1950) showed that L forms of *Proteus vulgaris* could be grown on the surface of liquid media. Dienes (1953) confirmed these observations.

These workers used media enriched with serum for their experiments with the L forms. In 1954, however, a serum-free, chemically defined medium was devised for the growth of L forms of *P. vulgaris* and *Proteus mirabilis* (Medill and O'Kane, 1954). A similar medium was described by Abrams (1955). Tulasne, Terranova and Lavillaureix (1955) reported that vitamins of the B group can be substituted for serum in media designated for the growth of L forms.

The influence of various factors on the growth of a stable Proteus L strain, such as concentration of serum and agar in the media used, has been investigated during the present study. The relationship between the number of observable, microscopic elements in an L culture (total count) and the number of colony-forming elements (viable count) has also been studied.

MATERIALS AND METHODS

Organism. The Proteus L form used was obtained from E. Klieneberger-Nobel, Lister Institute of Preventive Medicine, London. It has not shown any signs of reversion during its history and has been designated as strain L9 (Klieneberger-Nobel, 1956). Tests performed in this laboratory have shown that the normal Proteus strain from which Proteus L9 was derived does not produce acid and gas from maltose and does not produce indole. This strain thus exhibits the properties of *P. mirabilis* (Taubeneck, 1956).

Growth conditions. Samples of the L form for experimental purposes were obtained from stock cultures grown in 250-ml Erlenmeyer flasks containing 50 ml of the serum-free, liquid medium described by Abrams (1955). No penicillin was, however, included in the medium. The cultures were incubated for 24 hr at 30 C on a rotary shaker (100 rev/min) and stored at room temperature. Subcultures were made weekly.

Counting procedures. Total counts of the elements in cultures of *P. mirabilis* 9 and L9 were performed by the method described by Weibull (1960). Viable counts were made by the pour plate and the streak plate methods. Dilutions of bacterial cultures were made using 50-ml volumetric flasks and 1-ml graduated pipettes. In each determination 150 to 1,000 colonies were counted.

Light microscopy. Photographs were taken using a 90× Leitz phase contrast immersion objective and a 10× compensating eyepiece. Negatives obtained on Gevaert Graphic Ortho plates were enlarged twice when printed to give a final magnification of 3,000×.

RESULTS

Experiments with liquid media. Proteus L9 grows well on Abrams' serum-free medium and does not revert to the bacillary form when penicillin is omitted (Weibull, 1958; Weibull and Beckman, 1960). Abundant growth is also obtained in other media, e.g., in brain heart infusion broth (Difco) and in the solution of salts and tryptose-digested peptone used by Kandler and Kandler (1956). In serum-free media, the L growth consists of single spherical elements or microscopic aggregates; in the presence of serum, macroscopic aggregates of L elements are often predominant.

It has been noted that large inocula are needed to obtain growth in serum-free media. To study this quantitatively, a number of 250-ml Erlenmeyer flasks each containing 50 ml of medium, were inoculated by serial dilutions with decreasing volumes of an overnight culture of Proteus L9. Incubation took place at 30 C on the rotary shaker. Table 1 gives the results of these experiments.

TABLE 1

Influence of amount of inoculum on growth of Proteus 9 and L9 in liquid cultures (50 ml medium in 250-ml flasks)

Organism	Medium	Minimal Volume of Inoculum*
Proteus 9	Abrams'	$<10^{-4}$
Proteus L9	Abrams'	6×10^{-1}
Proteus L9	Abrams' + 20 μg vitamin B_2/ml	6×10^{-1}
Proteus L9	Abrams' + 10% serum added *after* inoculation	8×10^{-2}
Proteus L9	Abrams' + 10% serum added *before* inoculation	$<10^{-4}$

* Volume of inoculum is expressed as per cent of fresh medium for obtaining growth of the organism within 10 days. The figures represent average values obtained from four separate experiments.

As could be expected, normal Proteus cells grow rapidly in Abrams' medium, even when a small inoculum is applied (minimal volume of inoculum less than 10^{-4}% of the fresh medium; Table 1, line 1). On the other hand, an inoculum, representing about 1% of the fresh, serum-free medium, had to be used to obtain growth of Proteus L9. The addition of vitamin B_2, as recommended by Tulasne et al. (1955) did not change the state of matters (Table 1, lines 2 and 3). When serum is added to Abrams' medium before inoculation, growth can be obtained after some days even with small inocula (Table 1, line 5). When serum is added after inoculation, however, relatively large inocula are again needed to obtain growth of the L form (line 4).

Experiments with pour plates. Tables 2 and 3 illustrate the precision of the pour plate method for the determination of viable counts when applied to Proteus L9 cultures. Abrams' medium supplemented with 10% horse serum was used in these experiments.

Table 2 shows the results of viable counts performed on two separate cultures diluted 10^7 times. From the figures in this table it can be concluded that the standard deviations of the counts are slightly greater than could be expected from a random distribution of the L bodies in the diluted cultures.

Generally two final dilutions (in most cases 1:10^6 and 1:10^7) were prepared of an L culture when the viable count of this culture had to be determined. As is shown in Table 3, the same counts for the undiluted cultures were obtained (within the experimental errors) irrespective of the final bacterial dilutions used when pouring the plates.

The influence of the agar concentration on the growth of Proteus L9 in pour plates was investigated within a concentration range of 0.6 to 1.3%. The results of four separate experiments are summarized in Fig. 1. As can be seen, an agar concentration between 0.7 and 0.8% seems to be optimal for the formation of Proteus L9 colonies in the medium studied.

The serum concentration did not markedly influence the growth of Proteus L9 in pour plates within the concentration range of 10 to 20%. A comparison between pour plates prepared using serum before and after inactivation at 56 C for 30 min showed that, on an average, the inactivation process increased the number of colonies by about 20%.

To study whether serum is indispensable for the growth of Proteus L9 in pour plates, such

TABLE 2

Precision of viable count determinations performed using the pour plate method

Expt	No. of Plates Poured	Avg No. of Colonies per Plate	Standard Deviation	Standard Error
I	13	69.9	9.1	2.5
II	11	67.2	9.0	2.7

Abrams' medium, supplemented with 10% horse serum, was used for preparing the plates and diluting bacterial samples.

TABLE 3

Determinations of the number of viable L elements in Proteus L9 cultures

Dilutions Used for Pouring Plates	No. of Expts Performed	Estimated No. of Viable Elements in Undiluted Cultures, Relative Values
1:10^6	17 + 5	(100)
1:10^7	17	107 ± 5
1:10^8	5	112 ± 9

The determinations were carried out using the pour plate method. Average values of data obtained from a number of experiments are given.

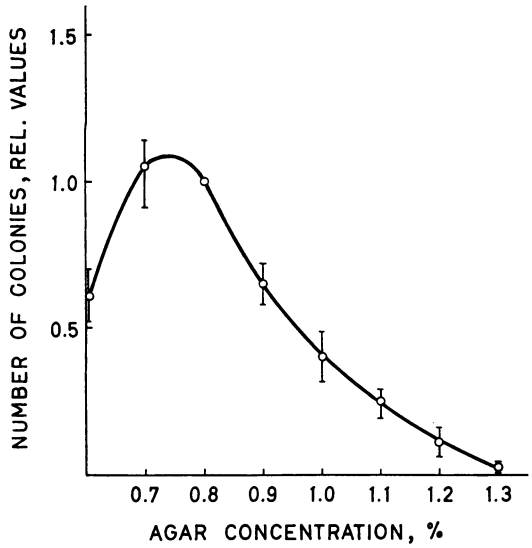

Fig. 1. Influence of the agar concentration on growth of Proteus L9 in pour plates. Abrams' medium (1955) supplemented with 10% horse serum was used as growth medium. The figures represent average values of data from four independent experiments. In each experiment the plates were inoculated with identical samples of a diluted Proteus L9 culture. The number of colonies found per plate in a medium containing 0.8% agar is given as being equal to unity.

plates containing fresh Abrams' medium supplemented with or devoid of serum were prepared. In some of the serum-free plates, vitamin B_2 was included at a final concentration of 10 μg/ml. Another type of growth medium was prepared by centrifuging overnight cultures of Proteus L9 at 15,000 $\times$ *g* and killing remaining viable L elements in the supernatant by heating this fluid at 56 C for 1 hr. All plates were inoculated with the same amount of a diluted Proteus L9 culture (the medium used for preparing the plates also served as dilution liquid). Table 4 gives the results of these experiments. To facilitate a comparison between the growth-promoting properties of the media tested, the figures of this table express the following ratio: number of colonies obtained per plate containing a certain medium:number of colonies obtained per plate using Abrams' medium supplemented with 10% serum.

It can be seen from the figures in Table 4 that a batch of Abrams' serum-free medium previously used for growing Proteus L9, but then centrifuged and sterilized, supported the growth of Proteus L9 almost as effectively as fresh Abrams' medium supplemented with serum. No growth whatsoever was obtained in the other serum-free media tested.

Experiments with streak plates. Dried plates were inoculated with 0.1 ml of a known dilution of a Proteus L9 culture. Carlsberg micropipettes (Levy, 1936) were used and the inoculum was spread with a sterile, bent glass rod.

Fig. 2 shows the influence of the agar concentration on the growth of Proteus L9 on streak plates. Within the concentration range of 0.8 to 1.2% the most abundant growth was obtained using the lowest agar concentration. This is in accordance with the results obtained from experiments with pour plates (Fig. 1).

As is shown in Table 5 streak plates gave slightly lower viable counts than pour plates, when identical samples of Proteus L9 cultures were counted.

Comparison between total and viable counts of cultures of Proteus 9 and Proteus L9. The cultures studied were grown in Abrams' liquid medium. Samples were taken from 4 to 48 hr after the inoculation of the cultures. The viable counts were performed by means of the pour plate method; plates containing Abrams' liquid medium sup-

TABLE 4

Comparison between the number of L9 colonies obtained in pour plates using various media

Medium	No. of Colonies, Relative Values
Abrams' medium + 10% serum	(1.00)
Abrams' medium, no serum	0.00
Abrams' medium + 10 μg vitamin B_2/ml, no serum	0.00
Abrams' medium, used for growth of Proteus L9, centrifuged and sterilized, no serum	0.62 $\pm$ 0.13

All plates were inoculated with identical amounts of a diluted Proteus L9 culture (the same medium was used in the plates and as dilution liquid). The figures above give the ratio: (number of colonies per plate using a certain medium): (number of colonies obtained using Abrams' medium supplemented with 10% serum). The figures represent average values of data obtained from four independent experiments.

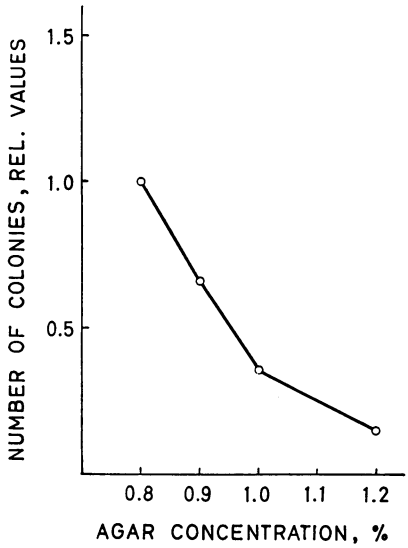

Fig. 2. Influence of the agar concentration on growth of Proteus L9 on streak plates. Abrams' medium supplemented with 10% horse serum was used as growth medium. Average values of data from two independent experiments are given. In each experiment the plates were inoculated with identical samples of a diluted Proteus L9 culture. The number of colonies found per plate on a medium containing 0.8% agar is given as being equal to unity.

plemented with 0.8% agar and 10% horse serum were used (the serum was omitted in the experiments with Proteus 9). The total counts were performed according to Weibull (1960). Table 6 gives the results of five independent experiments.

According to the total counts, at the most about 5×10^9 individual L bodies, or aggregates of these bodies, were found in the Proteus L9 cultures studied (Table 6, experiments I to III). The viable counts were considerably lower, ranging between 0.3 and 52% of the corresponding total counts. The discrepancy between viable and total counts was most marked in the 48-hr cultures.

Between 50 and 90% of the L elements in the cultures studied had a diameter greater thao 0.5 μ.

A rather close agreement between the total and viable counts of the normal Proteus cultures (Proteus 9) investigated was obtained (Table 6, experiments IV and V).

Morphological studies on L bodies suspended in serum-free and serum-containing media. Cultures grown in Abrams' medium were used for these studies. About 20 ml of the cultures were centrifuged and resuspended at 45 C in about 0.5 ml of fresh Abrams' medium supplemented with 0.7% agar and, in some of the experiments, 10% horse serum. Small drops of the suspensions obtained were placed on a slide and a cover slip was placed on the drop, which was allowed to spread. The slide preparations were sealed with vaspar. Photomicrographs were taken of the preparations immediately after the agar had gelled, and after 3 days. Fig. 3 shows the results of the morphological studies. One of the photographed preparations (Fig. 3, *c* and *d*) contained serum, the other (Fig. 3, *a* and *b*) did not. It can be seen that the L bodies disintegrated completely within 3 days in the serum-free medium, whereas they remained almost unaltered in the presence of serum.

TABLE 5

Comparison between the growth of Proteus L9 in pour plates and on streak plates

Expt	Viable Counts	
	Streak plates	Pour plates
I	$(2.36 \pm 0.17) \times 10^8$	$(2.89 \pm 0.12) \times 10^8$
II	$(5.14 \pm 0.36) \times 10^8$	$(7.11 \pm 0.26) \times 10^8$

The figures give the viable counts of the undiluted bacterial cultures studied. The plates were prepared using Abrams' liquid medium supplemented with 0.8% agar and 10% horse serum.

TABLE 6

Total and viable counts of Proteus L9 and Proteus 9 cultures grown in Abrams' liquid medium

Organism	Expt	Age of Culture (hr)	Total Count ($\times 10^8$)	Viable Count ($\times 10^8$)
Proteus L9	I	4	5.49 ± 0.50	3.14 ± 0.13
Proteus L9	II	24	35.3 ± 2.9	5.34 ± 0.25
		48	41.6 ± 2.3	0.69 ± 0.10
Proteus L9	III	4	4.00 ± 0.33	0.19 ± 0.02
		24	53.2 ± 1.8	7.17 ± 0.32
		48	45.9 ± 1.1	0.15 ± 0.07
Proteus 9	IV	24	52.8 ± 4.1	60.2 ± 3.1
Proteus 9	V	24	59.8 ± 3.6	65.0 ± 4.8
		48	123 ± 8.0	119 ± 5.2

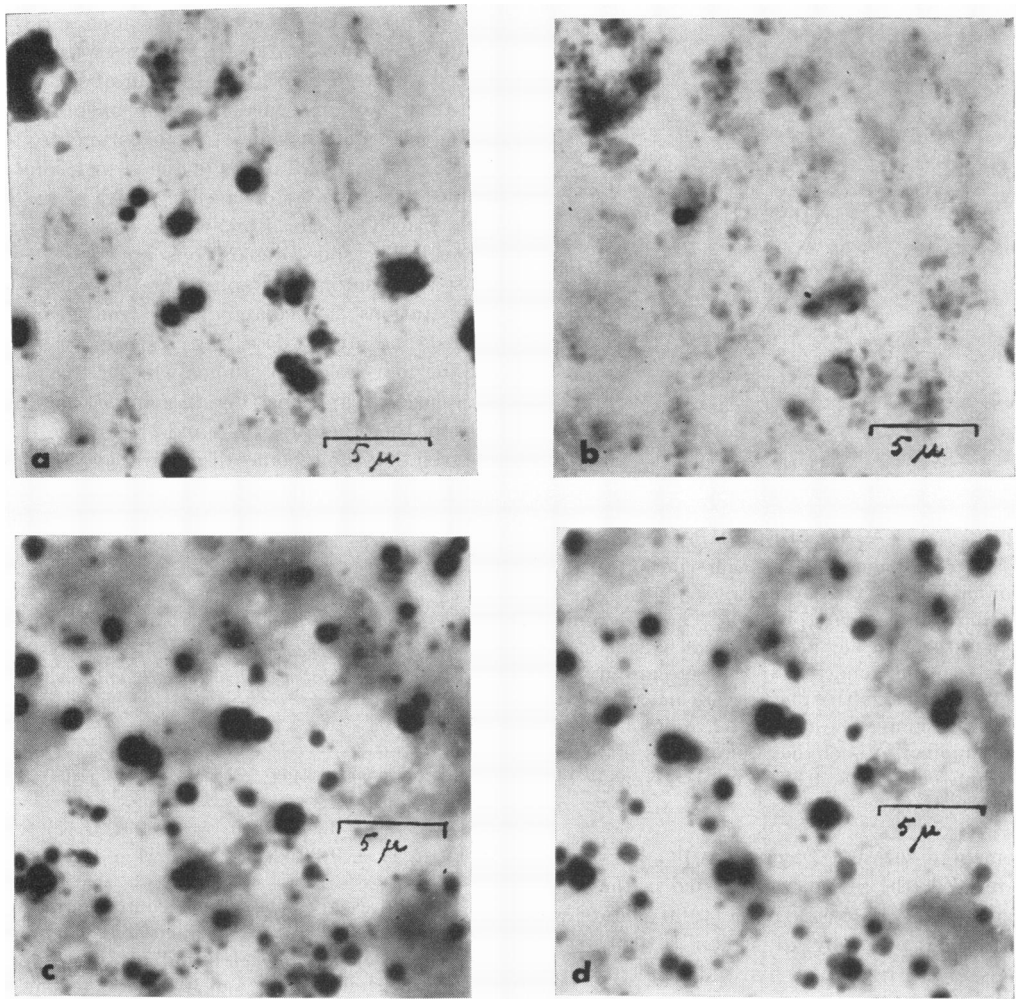

Fig. 3. Proteus L9 bodies suspended in a serum-free medium (*a* and *b*) and in a medium containing 10% horse serum (*c* and *d*). The photomicrographs were taken immediately after the preparation of the suspensions (*a* and *c*), and 3 days later (*b* and *d*).

DISCUSSION

Various factors promoting or inhibiting the growth of *P. mirabilis* strain L9 have been investigated in the present study. Determinations of the number of viable elements in L cultures by means of the pour plate technique have previously been reported by Kellenberger, Liebermeister, and Bonifas (1956) and by Landman, Altenbern, and Ginoza (1958). It appears from our investigations that the pour plate and the streak plate methods give consistent results in the case of Proteus L9. Thus, heating the L bodies for a short time to 40 to 45 C does not seem to reduce their viability.

The precision attained in our counting experiments with Proteus L9 is comparable to that reported by Snyder (1947) using normal bacteria. No evidence was obtained indicating destruction or aggregation of L bodies during the preparation of the bacterial dilutions. Similarly, no dispersion of the aggregates of L bodies which are normally present in cultures of Proteus L9 seemed to occur.

The amount of agar included in the solid media used markedly influenced the results of the plate counting experiments. Similar findings have been reported by Kandler and Kandler (1956) and by Lorkiewicz, Marciniak, and Żelazna (1956). The

agar concentration which is optimal for the growth of L forms seems, however, to vary from one strain to another.

It has been shown (Kandler and Kandler, 1956; Lorkiewicz, 1958; Weibull and Beckman, 1960) that at least some stable L forms, among them Proteus L9, can be grown in the absence of serum. However, data presented in the present paper show that large inocula must be used to obtain growth of Proteus L9 in liquid, serum-free media. Moreover, growth in pour plates is not obtained when fresh, serum-free Abrams' medium is used for preparing the plates. Either 10% serum has to be added to obtain growth or a batch of medium must be used that has previously been used for growing Proteus L9. These findings seem to indicate rather that serum protects the L bodies from poisonous substances present in fresh media than that it contains substances directly promoting the growth of Proteus L9. This is in accordance with the findings of Medill and O'Kane (1954) and Lorkiewicz (1958). Lorkiewicz found that L forms of *P. mirabilis* could be grown in a serum-free medium to which active carbon had been added. According to Medill and O'Kane, substances inhibiting the growth of L forms are present in yeast extract and in hydrolyzed casein containing vitamins, but not in hydrolyzed, vitamin-free casein.

The detoxicating role of serum in cultures of L forms is confirmed by the photomicrographs presented by us. The structure of the L bodies is entirely destroyed within a few days in a fresh, solid medium containing no serum, whereas the appearance of the L bodies remains practically unaltered for several days in the presence of serum.

According to our experiments, vitamin B_2 cannot be substituted for horse serum in media designed for the growth of Proteus L9 (Tulasne et al., 1955).

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SUMMARY

Various factors influencing the growth of a stable L form of *Proteus mirabilis* (Proteus L9) in solid and liquid media have been investigated by means of quantitative methods. The optimal agar concentration for the growth of this L form is about 0.75%. Serum does not seem to be a growth factor for Proteus L9 in the usual sense of this word. Instead it probably inhibits poisonous substances in the medium, which in the absence of serum causes the cell membrane to disintegrate.

The number of viable elements in cultures of Proteus L9, as estimated by the pour plate and streak plate methods, is considerably lower than the corresponding number of microscopically observable bodies.

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