GROWTH OF BACTERIAL L FORMS AND BACTERIAL PROTOPLASTS

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Numerous morphological, physiological, and biochemical studies have been carried out on bacterial forms exhibiting a pronounced osmotic fragility (for review see Weibull, 1958*b*). Naked bacterial protoplasts, obtained by means of a complete removal of the cell wall by lysozyme in a sucrose medium (Weibull, 1953) offer an example of such an osmotically fragile structure. Osmotically sensitive forms may also result when the cell wall is only partly degraded. Such structures have been named spheroplasts (Hurwitz *et al.*, 1958; Michael and Braun, 1959). The so-called bacterial L forms, which are highly pleomorphic and which may arise when normal bacteria are subjected to an unfavorable environment, are also markedly sensitive to changes in the osmotic pressure of the surrounding medium (for reviews see Dienes and Weinberger, 1951; Klieneberger-Nobel, 1951; Tulasne, 1953; Schellenberg, 1954).

The present paper describes some investigations concerning the growth of a stable L form, of bacterial protoplasts, and of the normal bacteria from which these forms were derived. Liquid media were used for the experiments. Samples of the bacterial material were taken at various times during growth and were analyzed chemically. The data obtained show that the growth characteristics of the bacterial forms studied agree in many respects. Differences, however, also exist.

MATERIALS AND METHODS

Organisms. The L form used was obtained from Dr. E. Klieneberger-Nobel, Lister Institute of Preventive Medicine, London. It was originally produced by penicillin treatment of *Proteus vulgaris* strain 9, and has not shown any signs of reversion during its history. It has been designated as strain L9 (Klieneberger-Nobel, 1956).

The strain M (Baumann-Grace and Tomcsik, 1957) of *Bacillus megaterium* was used for the preparation of protoplasts.

Growth conditions. The strains 9 and L9 of *P. vulgaris* were grown in 3-L Erlenmeyer flasks containing 500 ml of the liquid, serum-free medium described by Abrams (1955) for the cultivation of L forms. No penicillin was, however, included in the medium. The cultures were incubated at 30 C on a rotary shaker at 100 rpm. The flasks were inoculated with overnight cultures of the normal bacteria or the L form, grown as described above in Abrams' medium. To obtain satisfactory growth of the L form, large inocula, about 30 ml, had to be used.

For the preparation of protoplasts, *B. megaterium* was grown overnight on the shaker mentioned above and in the medium described by Gladstone and Fildes (1940). A thick suspension of cells (4 mg bacterial dry weight/ml) in 0.5 M sucrose buffered with phosphate (pH 7.0, final concentration 0.02 M) was prepared and treated with lysozyme (final concentration 100 μg/ml). When the cells had been converted into protoplasts the suspension was poured in 500-ml Erlenmeyer flasks containing 150 ml of the medium used by Fitz-James (1958) in his growth experiments with protoplasts. The flasks were then incubated at 30 C in a Warburg thermostat and agitated at 60 to 80 strokes/min (length of strokes, 8 cm).

Chemical analysis. Samples of the bacterial cultures were taken at suitable intervals and immediately centrifuged at 3000 rpm at 5 C. The sediments were subjected to the analytical procedures outlined below.

For the determination of lipid phosphorus, nucleic acids, and protein nitrogen, samples were subjected to the fractionation procedure described by Schneider (1945). As a control, a few samples were also fractionated according to Schmidt and Thannhauser (1945). To remove completely the inorganic phosphate of the media from the samples before the extraction with alcohol-ether (Schneider, 1945), the material, precipitated with cold trichloroacetic acid, was washed four times with the same reagent.

Furthermore, to effect a complete extraction of the nucleic acids, the bacterial material was treated two or three times with hot trichloroacetic acid (Fitz-James, 1955).

In the alcohol-ether extracts obtained from bacteria treated with cold trichloroacetic acid, total phosphorus was determined according to Allen (1940). In extracts prepared with hot trichloroacetic acid or with potassium hydroxide ribonucleic acid (RNA) phosphorus was determined according to Schneider (1945). The deoxyribonucleic acid (DNA) phosphorus present in the trichloroacetic acid extracts was determined according to Burton (1956). The total content of nucleic acid phosphorus in trichloroacetic acid extracts was determined by measuring the ultraviolet absorption at 260 mμ, using the extinction values for pure nucleic acids (extinction per mole of phosphorus) given by Ogur and Rosen (1950). The nitrogen content of trichloroacetic acid-insoluble material was determined using the Kjeldahl method.

Samples of yeast nucleic acid (Hopkin and Williams Ltd.) and of salmon DNA (Mann Research Laboratories Inc.) were used as standards in the colorimetric determinations of RNA and DNA phosphorus. The phosphorus content of these standards was determined by Allen's method (see above).

Biuret positive material in L forms and protoplasts was determined according to Gornall *et al.* (1949). The bacterial sediments were treated with 0.1 N NaOH at room temperature. Complete dissolution occurred within 15 min. No biuret determinations were carried out on normal bacteria, since these were not completely dissolved in 0.1 N NaOH.

For the determination of diaminopimelic acid, samples of the cultures to be investigated were centrifuged and the precipitates were dried *in vacuo* after washing with acetone. The diaminopimelic acid content of the dried samples was estimated by the method of Rhuland *et al.* (1955), as described by Weibull and Bergström (1958). It was found unnecessary, however, to run the hydrolyzed bacterial samples through an anion-exchange column to remove cysteic acid, as the blue color given by this acid on the chromatograms faded away after about 24 hr. After this time, the presence of diaminopimelic acid was revealed by a yellow spot located approximately at the same place as the cysteic acid (Rhuland *et al.*, 1955). No such yellow spot appeared on chromatograms run on oxidized and hydrolyzed samples of bovine albumin. The simplified procedure just described obviously prevents any losses of diaminopimelic acid after the hydrolysis of the material to be analyzed. Known amounts of diaminopimelic acid were run in parallel with the samples. The accuracy of the estimation was judged to be ±30 per cent.

Dry weight determinations. Samples of L forms for dry weight determinations could not be prepared by washing cells in distilled water, because of the osmotic fragility of these forms. Instead, samples of the cultures were centrifuged in graduated tubes, the supernatant liquid was carefully sucked off, the walls of the centrifuge tubes were wiped with filter paper and the volume of the sediment was read. The sediment was then suspended in distilled water, transferred to a weighing glass, dried at 100 C, and weighed. The dry weight (x) of the bacteria was calculated according to the formula

$$x = A \cdot \frac{1 - B \cdot V/A}{1 - 4.7B} \qquad (1)$$

Here, A is the dry weight in grams of the centrifugal sediment, V its volume (ml). B is the dry weight content of the medium (g/ml). It is assumed that the bacteria contain 80 per cent (w/w) of water (Porter, 1946) and that the partial specific volume of the nonaqueos material of the bacteria is 0.7.

It is evident from formula (1) that the method for dry weight determinations given above becomes less precise with increasing dry weight content of the medium. In accordance with this, it was found that the method gave irreproducible results when applied to bacteria suspended in the medium of a very high dry weight content such as used by Fitz-James (1958) for growing protoplasts. Thus the dry weight of protoplasts could not be determined according to formula (1). Instead, as an approximate estimation of the dry weight of protoplasts, the sum of their content of biuret positive material, nucleic acids, and lipids was taken. The following formulas were used for the estimation of nucleic acids and lipids: RNA = 10.5 × RNA phosphorus; DNA = 10.1 × DNA phosphorus; lipids = 31.8 × lipid phosphorus (Schneider, 1945; Weibull and Bergström, 1958).

TABLE 1

Dry weight determinations carried out on normal cells of Proteus and on the corresponding L form. Direct determinations and values calculated according to formula (1)

	Normal Cells, Washed	Normal Cells, Unwashed	L Form, Unwashed
Volume of centrifugal sediment (ml)	—[a]	0.27	0.20
Dry weight of sediment (g)	—	0.0397	0.0256
Dry weight (g) of bacteria calculated	—	0.0370	0.0230
Dry weight (g) of bacteria directly determined	0.0356	—	—

The bacteria were grown in Abrams' medium. The dry weight of this medium was 0.0282 g/ml. The values given in columns 1 and 2 refer to two identical samples of normal cells of *Proteus vulgaris*.

[a] Value not determined.

Turbidity measurements. The turbidity values given in the following refer to measurements in the Beckman spectrophotometer at 700 mμ using 1-cm cuvettes.

Light microscopy of L forms. Ten to thirty ml of the cultures to be studied were centrifuged and the sediment was resuspended at 45 C in 0.2 to 0.5 ml of Abrams' medium to which 0.5 per cent agar had been added. A small drop of this suspension was placed on a slide and a cover slip was carefully placed on the drop, which was allowed to spread. After a few minutes of cooling, the agar gelled. In this way a microscopic preparation of a few microns in thickness was obtained in which the Brownian movement of the L bodies was completely inhibited. 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 3000×.

RESULTS

Dry weight determinations. The relationship between the turbidity of the bacterial cultures investigated and their content of cell material. The method described previously (see Materials and Methods) for estimating the dry weight of bacterial material without washing, was checked using normal cells of Proteus grown in Abrams' medium. The bacterial culture was divided into two equal parts, which were centrifuged at 1000 × G. One sediment was washed once with distilled water by centrifugation at 20,000 × G, and its dry weight was determined after drying at 100 C. The dry weight of the bacteria present in the other sediment was calculated according to formula (1). The results are shown in table 1. It is seen that the calculated dry weight of the unwashed cells is about 4 per cent higher than that of the washed cells. It is known, however, that material leaks out of bacterial cells when the organisms are washed with saline or distilled water (Palmstierna, 1956).

Figure 1 illustrates the relationship turbidity/bacterial dry weight in cultures of *P. vulgaris* strains 9 and L9. Samples of the cultures were taken at suitable time intervals during incubation and the dry weight of the bacterial material in the samples was determined using formula (1). It should be pointed out, however, that no reproducible relationship could be established between the content of bacterial material in cultures of Proteus strain L9 and their turbidity, when the cultures had been incubated for more than 30 hr. Thus, separate dry weight determinations had to be performed on such cultures when deemed of interest.

Curves similar to that shown in figure 1*a* were obtained when studying cultures of *B. megaterium*, grown in the media described by Fitz-James (1958) and Gladstone and Fildes (1940). In these cases, however, the dry weight of the cells was determined after washing them twice with distilled water, since the method used for Proteus gave irreproducible results when applied to bacteria grown in media of a very high dry weight content (see Materials and Methods).

The turbidity/dry weight curves obtained as described above were used for estimations of the amount of bacterial material present in cultures designated for chemical analyses (see below).

Comparison between different methods for the determinations of nucleic acids. Samples of about 50 mg (dry weight) of bacterial cells from cultures incubated for 16 to 20 hr were fractionated according to the procedures described

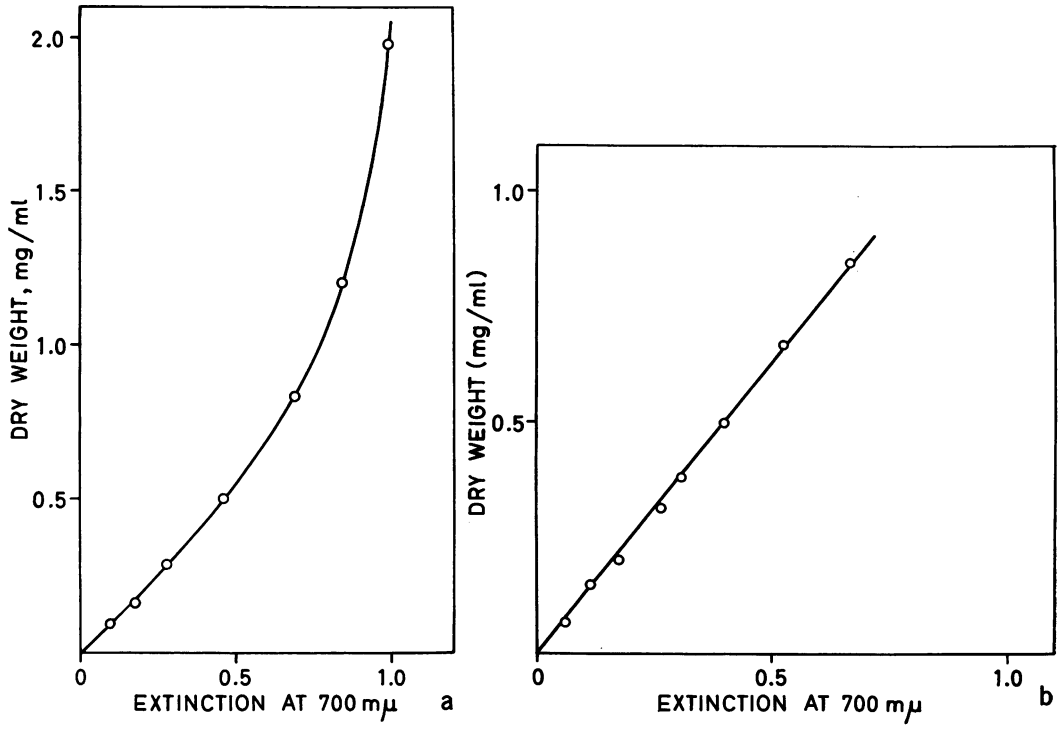

Figure 1. The relationship turbidity/bacterial dry weight in cultures of *Proteus vulgaris*. Samples of the cultures were taken at suitable time intervals during incubation and the dry weight of the bacterial material in the samples was determined using formula (1). *a*, Normal bacteria; *b*, the L form.

by Schneider (1945) and by Schmidt and Thannhauser (1945). The nucleic acid content of the KOH and trichloroacetic acid extracts obtained was determined by the orcinol and diphenylamine reactions, by total phosphorus determinations, and by measurements of ultraviolet absorption. The results are given in table 2. In this table the total amount of nucleic acid phosphorus found in each kind of bacterial cell, as determined by the orcinol and diphenylamine reactions after fractionation according to Schneider, is given as being equal to unity.

It can be seen that the various analytical methods applied agree reasonably well. One exception exists, however. The total nucleic acid content of *B. megaterium*, as determined by phosphorus analyses, is about twice that obtained by the colorimetric methods and by ultraviolet measurements. In all probability, other phosphorus-containing substances were present apart from the nucleic acids in the trichloroacetic acid extracts in question. The presence of such substances in trichloroacetic acid extracts from various gram-positive bacteria has been noted by Mitchell and Moyle (1954).

The determinations of nucleic acids referred to in the following were carried out on material fractionated according to Schneider (1945).

Growth experiments with P. vulgaris: a comparison between the growth characteristics of normal bacteria and the L form. Several cultures of strain 9 and L9 of *P. vulgaris* were investigated throughout the growth cycle. It was found that the growth characteristics of both strains varied from one experiment to another in many respects. However, some traits always recurred. Thus, as is shown in figure 2, no major difference could be noted between the growth rate of the normal bacteria and the L form. The RNA phosphorus content of both normal bacteria and the L form increased sharply during the first few hours of growth and then decreased markedly. On the other hand, the content of DNA phosphorus and lipid phosphorus remained fairly constant or fluctuated irregularly. The protein

TABLE 2

Nucleic acid analyses on bacterial material fractionated according to Schneider (1945) or according to Schmidt and Thannhauser (1945)

Compound Analysis	Fractionation	*Proteus vulgaris* Normal Cells	*Proteus vulgaris* L Form	*Bacillus megaterium* Normal Cells	
				I[a]	II[b]
Total nucleic acid phosphorus, orcinol + diphenylamine	S[c]	1.00	1.00	1.00	1.00
	STS[d]	1.01	0.94	0.94	1.04
Total nucleic acid, ultraviolet absorption	S	0.88	0.87	0.88	0.89
	STS	0.97	0.93	1.09	1.02
Total nucleic acid, phosphorus determination	S	0.87	0.91	2.54	1.60
Ribonucleic acid phosphorus, orcinol	S	0.68	0.72	0.85	0.87
	STS	0.69	0.67	0.80	0.93
Ribonucleic acid phosphorus, ultraviolet absorption	STS	0.65	0.64	0.82	0.90
Deoxyribonucleic acid phosphorus, diphenylamine	S	0.32	0.28	0.15	0.13
	STS	0.31	0.27	0.14	0.11
Deoxyribonucleic acid phosphorus, ultraviolet absorption	STS	0.32	0.29	0.26	0.12

The total nucleic acid content of each kind of bacterial material, as determined by the colorimetric reactions for ribonucleic acid and deoxyribonucleic acid on material fractionated according to Schneider (1945), is given as being equal to unity.

[a] Cells grown in medium described by Gladstone and Fildes (1940).

[b] Cells grown in medium described by Fitz-James (1958).

[c] Fractionation according to Schneider (1945).

[d] Fractionation according to Schmidt and Thannhauser (1945). Ribonucleic acid phosphorus was determined in the KOH extracts, deoxyribonucleic acid phosphorus in the trichloroacetic acid extracts (Schneider, 1946).

content (6.25 × amount of nitrogen in trichloroacetic acid-insoluble residue) was found either to increase during growth or to remain approximately constant. The L form was found to contain 10 to 20 per cent more biuret positive material than protein.

Thus the data referred to above do not reveal any major differences between the growth characteristics of the normal Proteus investigated and the corresponding L form. The same is true for the chemical composition of the two bacterial forms.

Diaminopimelic acid content of Proteus strain L9. One of us (Weibull, 1958*a*) previously analyzed this strain for diaminopimelic acid. The L bodies were precipitated in the culture medium with cold trichloroacetic acid. In three experiments, performed with separate cultures, the precipitate was found to contain about 0.2 per cent diaminopimelic acid (w/w). In a fourth experiment, however, no diaminopimelic acid could be detected in the bacterial material.

To establish whether the diaminopimelic acid content of Proteus strain L9 varies markedly during growth, samples from a culture were taken at approximately the times indicated in figure 1*b* and analyzed for diaminopimelic acid. In all samples the presence of diaminopimelic acid could be established. However, the diaminopimelic acid content increased from about 0.1 per cent during the first hours of growth to about 0.5 per cent during the phase of decline. It should be pointed out in this connection that the growth conditions and the experimental procedure employed were not exactly the same as those used earlier (see Materials and Methods and the publication by Weibull, 1958*a*).

Similar experiments were performed with normal Proteus strain 9. In young cells, about 0.4 per cent diaminopimelic acid was found; in cells from the stationary growth phase, 0.7 per cent. These figures indicate that the normal Proteus definitely contain more diaminopimelic

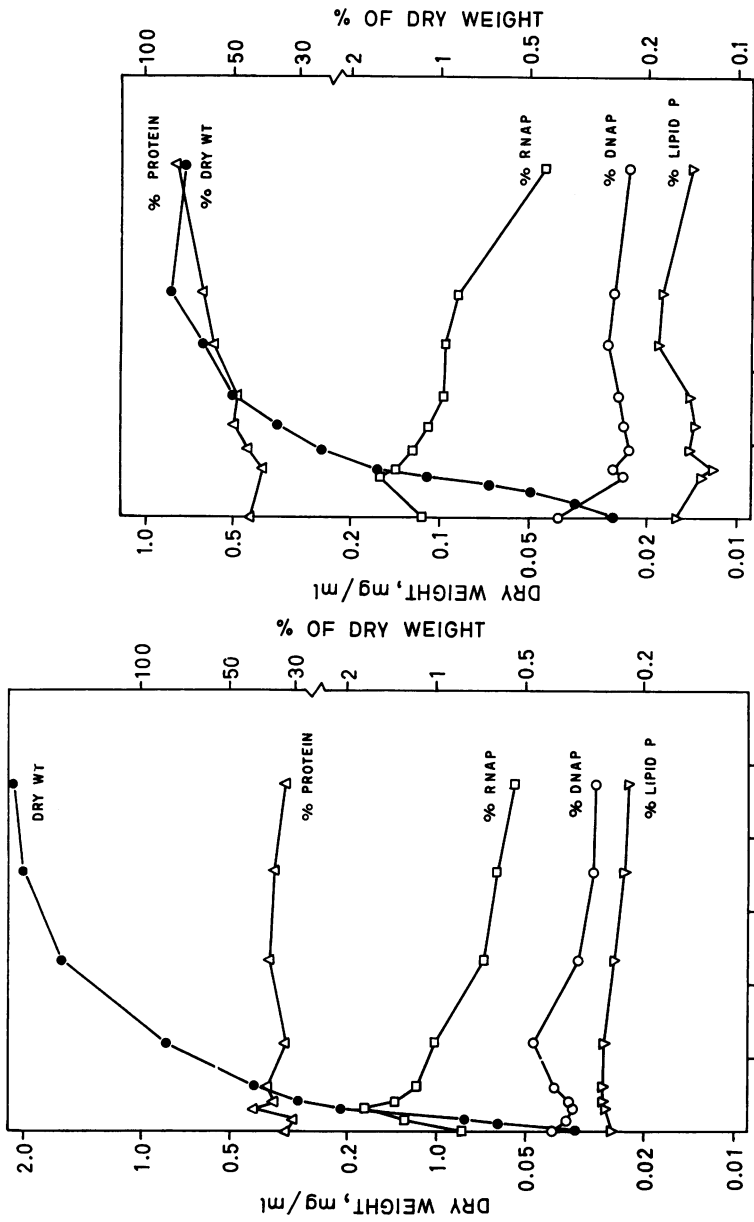

Figure 2. Growth characteristics of *Proteus vulgaris*, normal cells (*a*) and the L form (*b*). The values on the ordinate are plotted on a logarithmic scale. The content of protein, ribonucleic acid phosphorus (*RNAP*), deoxyribonucleic acid phosphorus (*DNAP*), and lipid phosphorus (*P*) are expressed as percentage of the dry weight of the bacteria.

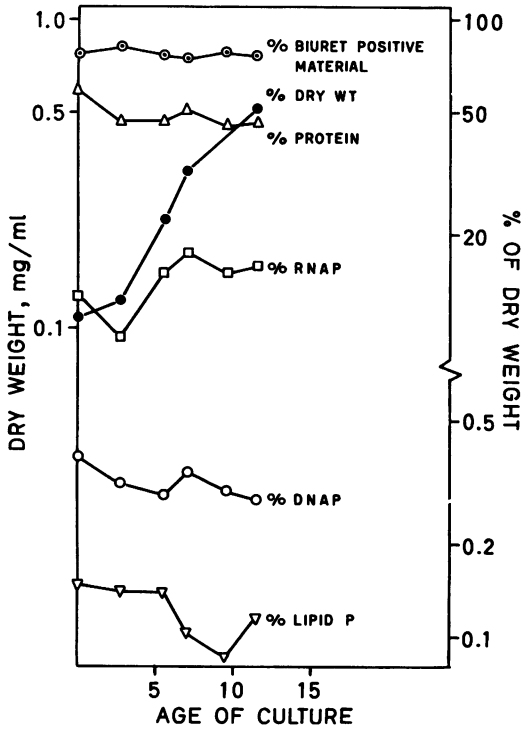

Figure 3. Growth characteristics of protoplasts of *Bacillus megaterium* strain M. Dry weight comprises the sum of biuret positive material, RNA, DNA, and lipids. The values are plotted as in figure 2.

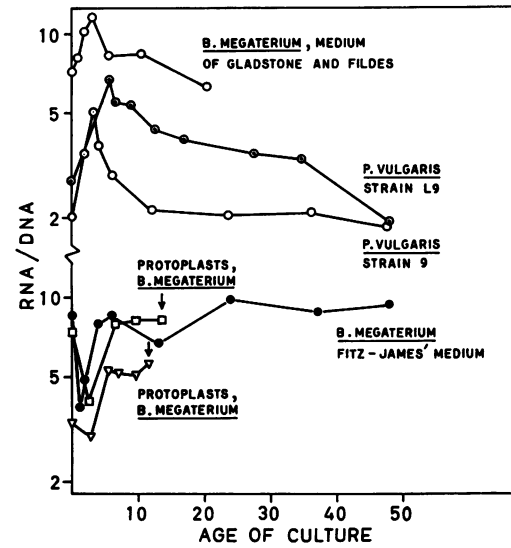

Figure 4. The ratio RNA/DNA of normal bacterial cells, L bodies, and protoplasts during growth. The values are plotted as in figure 2. The arrows indicate onset of lysis of protoplasts.

acid than the corresponding L form (strain L9) during the earlier part of the growth cycle, but that the difference is not very pronounced toward the end of growth.

Morphology of Proteus strain L9 during growth. The bulk of the matter observed microscopically in the L cultures consisted of spherical bodies of varying sizes. The diameter of these bodies ranged between 0.2 μ (the resolving power of the microscope used) and 10 μ (figure 5). Bodies having a diameter of 0.2 to 0.3 μ were found in greater numbers in old cultures than in young ones (figures 5*a* and 5*b*). This may, however, be due to disintegration processes occurring during the preparation of the slides.

Apart from the spherical bodies mentioned above, aggregates of morphologically ill-defined material were found in the L cultures. Aggregates of the spherical bodies were also seen, but most of these bodies appeared single.

Growth experiments with cells and protoplasts of B. megaterium. Experiments similar to those described in the preceding section were performed with intact cells of *B. megaterium* strain M, grown in the media described by Gladstone and Fildes (1940) and by Fitz-James (1958). In most respects the results were similar to those obtained from experiments carried out with *P. vulgaris*. The variations of the RNA content of bacterial cells that have been described in the preceding paragraphs were, however, noted only to a minor degree in *B. megaterium* cells grown in Fitz-James' medium.

Figure 3 illustrates a growth experiment with protoplasts of *B. megaterium* strain M. For several reasons the data obtained cannot be directly compared with those obtained with normal bacterial cells or with the Proteus L form. First, as has already been pointed out (see Materials and Methods), the dry weight of the protoplasts could only be approximated. In figure 3 "dry weight" thus means the sum of biuret positive material, RNA, DNA, and lipids. Second, growth ceased after about 12 hr, when the protoplasts began to lyse. Third, the growth observed was restricted to about a 5-fold increase in dry weight. However, in

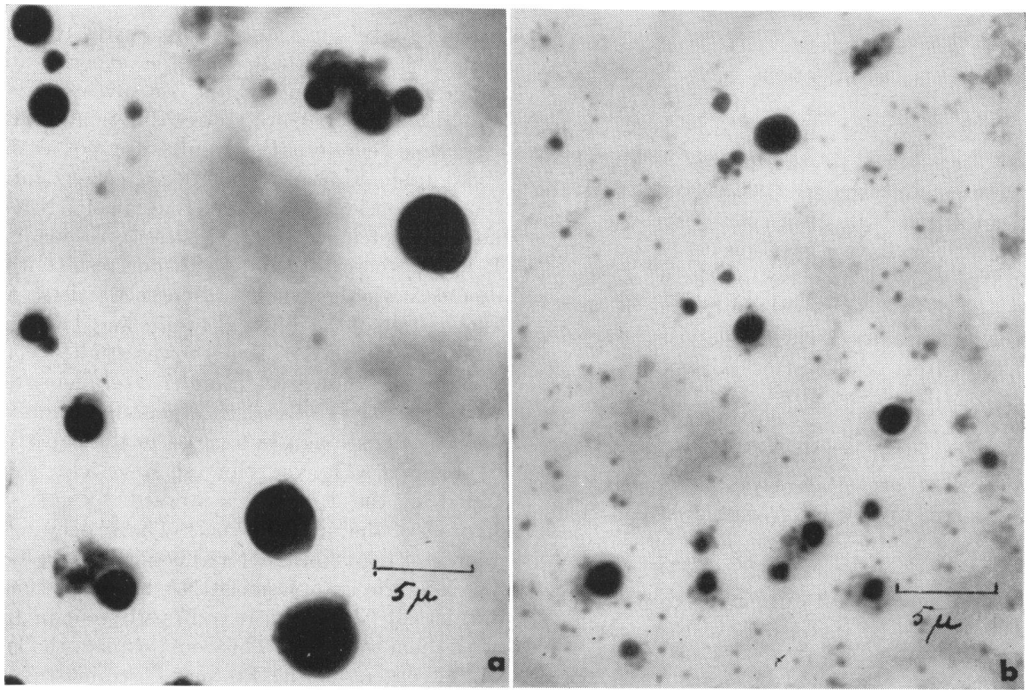

Figure 5. Proteus vulgaris strain L9: *a*, 2 hr after inoculation into fresh medium; *b*, 18 hr after inoculation.

several experiments of the kind illustrated in figure 3, it was found that the RNA content of the protoplasts decreased before growth started, but then increased considerably and attained a rather constant value during the latter part of growth. The content of DNA, protein, and biuret positive material remained approximately constant throughout the experiments. The content of lipid phosphorus decreased somewhat during growth or fluctuated irregularly. The protoplasts were found to contain about 50 per cent more biuret positive material than protein.

In his investigation on the growth of protoplasts of *B. megaterium* strain KM, Fitz-James (1958) found, among other things, that the ratio RNA/DNA of these protoplasts falls during growth, whereas the same ratio of intact cells remains constant or increases slightly. In his experiments, Fitz-James used as inoculation material rejuvenated cells or protoplasts obtained from such cells. The rejuvenation was accomplished by diluting a culture of stationary cells with about five volumes of fresh medium and incubating until the turbidity of the culture had approximately doubled (Landman and Spiegelman, 1955). A growth medium of very high ionic strength was used (Fitz-James, 1958). Figure 4 shows the RNA/DNA ratio of intact cells and protoplasts of *B. megaterium* strain M during growth in the same medium (see the lower part of the figure). It can be seen that the RNA/DNA ratio of both protoplasts and cells decreases immediately after incubation. The protoplasts do not grow appreciably during this period (figure 3). After the initial decrease, the RNA/DNA ratio increases sharply and then attains a more or less constant value.

It should be pointed out, however, that in the experiments illustrated in figures 3 and 4, the cells or protoplasts used for inoculation were not rejuvenated. When protoplasts of rejuvenated cells were studied, falling RNA/DNA ratios during growth were noted, which is in accordance with Fitz-James' findings.

The changes in the RNA/DNA ratio of *B. megaterium*, grown in the medium described by Gladstone and Fildes (1940), and of *P. vulgaris* (normal cells and L form) are included in figure 4. It is evident that the RNA/DNA ratio first rises in all these kinds of bacterial material but then decreases more or less continuously.

DISCUSSION

Numerous investigations have been carried out concerning changes in the chemical composition of bacteria during growth, especially those pertaining to the nucleic acids. Thus Malmgren and Hedén (1947) found that the concentration of ultraviolet-absorbing substances in cells of gram-positive and gram-negative bacteria increases on the whole during the first hours of growth but then decreases. Using chemical methods for analysis, Levy *et al.* (1949), Morse and Carter (1949), Fish *et al.* (1950), Mitchell and Moyle (1951), Price (1952), Sherratt and Thomas (1953), Wellerson and Tetrault (1955), Cavanna *et al.* (1955), and Kandler *et al.* (1956) established that the fluctuations observed by Malmgren and Hedén were mainly due to variations in the RNA content of the bacterial cell, the DNA content being rather constant during the growth cycle.

Our studies on intact cells of *P. vulgaris* and *B. megaterium*, grown in conventional media, have in the main confirmed the results of the above-mentioned workers concerning the variations in the RNA and DNA content of bacterial cells. In addition, it was found that fluctuations also occur in the protein and lipid phosphorus content of the bacteria studied, but these fluctuations were less pronounced and less reproducible than the variations in the RNA content.

Our investigations show that under the prevailing conditions the growth characteristics of the Proteus L form investigated agree in many respects with those of the normal bacteria from which it was originally derived. It has furthermore been shown that the chemical composition of both bacterial forms vary within rather wide limits during growth. This makes it difficult to establish to what extent the normal Proteus and the L form differ chemically from each other. It has been demonstrated, however, that the diaminopimelic acid content of normal Proteus strain 9 is higher than in Proteus strain L9, at least when not too old cultures are compared. In this connection it can be mentioned that earlier investigations by one of us (Weibull, 1958*a*) concerning the presence of diaminopimelic acid in Proteus strain L9 have been confirmed. Thus stable L forms may contain this acid, even if very often this is not the case (Kandler and Zehender, 1957).

Our study links up closely with that of Kandler *et al.* (1956), but the experimental conditions prevailing in the two investigations and the analytical procedures differ in several respects. Therefore the results are not easily comparable. However, Kandler *et al.* evidently found a considerably higher content of RNA in normal Proteus cells than in the corresponding L form, whereas according to our results it is doubtful whether such a difference exists. An explanation of the different results may be found in the appearance of the L forms studied. The L material studied by Kandler *et al.* consisted to a large degree of vesicles, apparently more or less empty (see figures 6 and 7 in the paper by Kandler *et al.*). Very few such vesicles were found in the L cultures studied by us (see figure 5 of the present paper). The vesicles may represent nonviable bacterial bodies, from which much of the cytoplasmic RNA has leaked out, but in which the nuclear bodies still remain and with them the DNA. This could explain the low RNA content of the Proteus L strain studied by Kandler *et al.* In a similar way the analytical figures obtained by Vendrely and Tulasne (1953) could be explained.

It is known that the average size of the cells in a bacterial culture diminishes during the growth cycle (Porter, 1946). It is of interest in this connection to note that the average size of the morphological elements found in cultures of Proteus strain L9 also diminishes during growth. If it is assumed that diaminopimelic acid is present only in the surface layers of Proteus strain L9 (Weibull, 1958*a*), this could explain why the diaminopimelic acid content of this L form increases during growth. Furthermore, the fact that the L bodies do not increase in size during the major part of the growth cycle strongly suggests that these bodies are as capable of dividing as normal bacteria. Our experiments on protoplasts of *B. megaterium*, on the other hand, like those of Fitz-James (1958), strongly suggest that these bodies do not divide during growth, at least not in the succinate medium used by Fitz-James and by us. Whether or not division occurs in the phosphate medium described by McQuillen (1955) can only be ascertained by further experiments.

According to our findings, the growth characteristics of the protoplasts of *B. megaterium* strain M seem to be rather similar to those of

intact cells of the same organism, provided that the same medium is used for growing the two bacterial forms. The most striking difference established so far seems to be that the protoplasts do not divide. The production of extracellular slime by the protoplasts is also worth mentioning.

Fitz-James (1958) found that the RNA/DNA ratio of protoplasts of *B. megaterium* strain KM decreases during growth, whereas the same ratio of intact cells of this bacterium remains constant or increases slightly. Most probably, however, this difference is characteristic mainly for rejuvenated cells and protoplasts. Our findings indicate that, if the rejuvenation procedure is omitted, the RNA/DNA ratio of protoplasts during growth follows much the same course as that of intact cells.

The content of biuret positive material in the protoplasts was found to be much higher than that of the proteinaceous material insoluble in hot trichloroacetic acid. To a smaller degree, the same is true for the Proteus L form. This suggests that some of the proteins of the bacterial material studied are more or less soluble in trichloroacetic acid or that considerable amounts of peptides are present in this material.

Bacterial protoplasts, prepared by lysozyme treatment of intact cells, and bacterial L forms no doubt resemble each other in several respects. It is not difficult, however, to point out certain differences. Thus the L bodies divide freely during growth, whereas it is doubtful whether protoplasts can divide at all. The growth of protoplastt is much more limited than that of the L forms. Media supporting the growth of L forms seem to be unsuitable for growing protoplasts and vice versa. The complex structures found in cultures of L forms should also be mentioned in this connection, as well as the fact that typical cell wall components such as diaminopimelic acid may be present in L forms but not, by definition, in true protoplasts.

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SUMMARY

Studies have been performed concerning the changes during growth in the chemical composition of normal cells of Proteus, a stable Proteus L form, intact cells of *Bacillus megaterium*, and protoplasts of the same bacterium. When growth occurred in conventional, liquid media of moderate ionic strength, the ribonucleic acid (RNA) content of normal cells of *Proteus vulgaris* and *B. megaterium* and of the Proteus L form increased during the first hours of growth, but then decreased. The same was found to be true for the ratio RNA/DNA (deoxyribonucleic acid).

Protoplasts of *B. megaterium* were grown in a liquid medium of high ionic strength. The RNA/DNA ratio of the protoplasts, as well as cells of intact *B. megaterium* grown in the same medium, decreased before growth started, then increased and remained approximately constant during the latter part of the growth cycle.

The content of DNA, lipid phosphorus, and protein in the bacterial forms mentioned above remained approximately constant during growth or fluctuated irregularly.

The content of diaminopimelic acid in the Proteus L form was found to be lower than in normal Proteus cells, especially during the early part of the growth cycle.

The main morphological elements found in cultures of the Proteus L form studied were spherical bodies of varying sizes. The average size of these bodies increased during the first hours of growth, but then decreased.

The size of the protoplasts of *B. megaterium* increased continuously during growth, and no division phenomena were observed. The protoplasts started to lyse when their dry weight had increased about five times.

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