Cytological and Chemical Studies of the Growth of Protoplasts of *Bacillus megaterium*

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PLATES 139 TO 143

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ABSTRACT

Aeration of protoplasts of *Bacillus megaterium* in a succinate buffered nutrient broth led to marked growth similar to that already described by McQuillen, and the degree of chromatin synthesis in these growing forms prompted a combined cytological and chemical study. Growth was followed by phase contrast and by Feulgen stains, as well as by lipide phosphorus, nucleic acid, and protein analyses. In slide cultures, growth and compression led to monstrous flattened forms with readily visible, but coalescent nuclear structures. In fluid cultures, the protoplasts grew as phase dense spheres. Orderly reproduction of apparently discrete nuclear bodies was observed during the initial hours of spherical growth, but in older cultures, the chromatin arrangement tended to be more haphazard and was influenced by the concentration of Mg ions. In the same medium, protoplasts free of lysis showed a linear rise in optical density, while vegetative cells exhibited an exponential increase. However, protoplasts were able to synthesize DNA at the same rate as vegetative cells, but their increase of RNA was always less. Thus, as they grew, the ratio RNA/DNA fell. The lipide P increased in proportion to the expanding surface. With growth and lysis, large amounts of water-insoluble slime accumulated. Analyses indicate it to be a phospholipoprotein material containing some RNA.

INTRODUCTION

Bacterial protoplasts, particularly those of *B. megaterium*, have been objects of considerable interest and study since their controlled formation by lysozyme digestion was first described (15). As McQuillen has shown, protoplasts aerated in suitable media are capable of considerable growth and assume shapes suggestive of cell division (8, 9). During the course of a combined chemical and cytological study of protoplasts, it was observed that the extensive increase in size of growing protoplasts was accompanied by a disproportionate enlargement of their chromatin material. This preliminary observation prompted the further chemical and cytological studies which are the subject of this paper.

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Methods

The culture used was *Bacillus megaterium* strain KM. Protoplasts were, for the most part, formed from rejuvenated vegetative cells obtained by the methods of Landman and Spiegelman (7). Once formed, the protoplasts were collected by centrifugation and after one wash, resuspended again in a small volume of fresh stabilizing medium containing a sucrose-phosphate-$MgSO_4$ (S-P) system (0.3 M sucrose, 0.1 M phosphate buffer at pH 6.1, 0.016 M $MgSO_4$). The medium used to grow protoplasts was, on occasion, also used as a stabilizing medium for protoplast formation. This growth medium was a succinate buffer system recommended by Spiegelman (12); 0.5 M disodium succinate, 0.1 M KCl, 1.0 per cent enzymatic casein hydrolysate (Nutritional Biochemicals), 10^{-3} M $MgSO_4$, 10^{-4} M $MnSO_4$. The pH was adjusted to 6.5. The magnesium salt of hexose diphosphate (HDP, Schwartz and Co.) was added at one of two concentrations: 6 and 0.6 mg./ml. Solid medium for protoplast growth was made

by incorporating agar (1.5 per cent Difco noble agar) into the above fluid medium.

Direct observation in dark phase contrast of growth of protoplasts was achieved with slide cultures. A small drop of protoplasts, suspended in either the S-P medium or the succinate broth on a coverslip, was mounted onto a thin strip of the agar medium. Many of the protoplasts adhered to the coverslip and were compressed by the agar. Those at the sides of the agar strip received variable amounts of nutrients and oxygen, so providing suitable material for phase contrast observation.

For studies of growth in fluid medium, an aliquot (1 to 3 ml.) of a thick protoplast suspension in S-P medium was pipetted into 100 to 200 ml. of the succinate medium in a ½ or 1 litre flask, to give an optical density at 650 mμ (OD 650) between 0.300 and 0.500. The flasks were aerated on a to and fro platform of 2 inch excursion at 80 strokes/minute in an incubator at 32.5°C.

Samples for chemical analysis (10 to 20 ml.) were chilled and centrifuged in round bottomed, thick walled tubes, at 10,000 *g* (Serval SS-1), and the residues analyzed for lipide-phosphorus (P) and nucleic acids by a fractionation procedure similar to that of Schneider (10), following modifications already described (2). Total P on the lipide extract was estimated by a modification of King's method (2).

The residues remaining after nucleic acid extraction were ashed by a micro adaptation of Beet's (1) permanganate method, and the nitrogen content measured by titration of distilled ammonia. The nitrogen content (× 6.25) was used as an expression of total protoplast protein.

Protoplast and cell counts were readily obtained with the Petroff-Hauser counting chamber under the high dry objective (43×) of the phase contract microscope (Bausch and Lomb).

Cytological Procedures:

Unless otherwise stated, the fixative used was the acetic acid–ethanol (1:3) mixture found previously to preserve the nuclear structure of protoplasts, both in the cell and after liberation by lipase (11). Protoplasts growing against glass, were fixed by running the fixing fluid under the coverslip. Protoplasts in fluid culture were smeared on coverslips and then dropped into a Columbia staining pot filled with acid-alcohol. After 10 minutes, the slips were rinsed in 70 per cent alcohol, then stored (up to 24 hours) in 70 per cent alcohol, until studied.

For nuclear staining, the preparations were hydrolyzed at 60°C. in N HCl or N TCA for 7 to 8 minutes. Both Schiff's reagent and azure A, reduced with SO_2 after Huebschman (6), were used to stain DNA.

Fat stains were made, using Sudan black B dissolved in a solvent of ethylene glycol, alcohol, and water (40:40:20). Formaldehyde-fixed smears mounted in the solvent were observed in dark phase contrast, as the stain was run under the coverslip. Broth-grown protoplasts, fixed in bulk with buffered formaldehyde, were suspended in the stain, centrifuged, and washed with solvent or 70 per cent alcohol.

EXPERIMENTAL RESULTS

Growth of protoplasts was studied in both slide cultures and in aerated fluid medium. With the former, only the morphological methods were used: first, phase contrast microscope on the living mounts, then light field microscopy on Feulgen-stained preparations. The results of studies of growth in fluid culture are presented separately.

Growth of Protoplasts in Slide Cultures

Dark phase contrast.—Protoplasts mounted on succinate agar show various stages of growth (Figs. 1 to 4) after 10 hours incubation at 35–37°C. Some protoplasts beyond the edge of the nutrient block grew only slightly, but possessed well differentiated and contracted chromatin bodies. Such condensed nuclear structures were invariably found in protoplasts in these slide cultures. The compression in a strongly cationic medium is presumed to contribute to this effect (3).

Although compression causes an apparent increase in size, the larger protoplasts in Fig. 1 have probably grown to some extent. The size of the chromatin regions (transparent areas) suggests that synthesis of this material has also occurred. Near the edge of the agar strip, the growth was more marked (Fig. 2). In addition to aeration and nutrition, the degrees of development appeared to depend partly on room available for growth. That is, protoplasts in isolated islands grew to monstrous flattened forms (Fig. 3), while in more heavily populated regions, enlargement was more limited. The crowded cells and their enclosed nuclear bodies showed signs of degeneration and fusion. Division of the chromatin of these structures was not seen in these growths on coverslips, although some showed configurations suggestive of abortive attempts in this direction (Figs. 2 and 4). In such nuclear bodies the phase dense central regions ("cores") appeared to have grown and divided as the chromatin increased.

Feulgen stains of protoplasts grown against glass were, in contrast to those usually obtained with bacteria, exceptionally intense. Stained

areas were examined mounted in water (Fig. 5) and in acetocarmine (Fig. 6) (4). In water mounts the cell membrane and cytoplasm were almost invisible, while in acetocarmine these structures were sufficiently tinted to be seen and photographed.

Acetocarmine also differentiated scattered inclusions from the rest of the cytoplasm. These inclusions appeared as clear areas in the stained preparation and could also be readily seen in phase contrast pictures (Figs. 2 and 4) as dark granules. These bodies are dense, approximately 0.20 μ in diameter, and apparently attached to the protoplast membrane. They can be collected as a yellow pellet after sonic disruptions of protoplast ghosts (16) or from lipase-digested protoplasts (11). Similar granules have been described in another organism and are assumed to be the mitochondrial equivalents of the bacterial cell (5). Regardless of the degree of growth of the protoplasts, the size of these cytoplasmic inclusions remained within the range of those seen in "resting" protoplasts. Their number did increase, however, especially in protoplasts well nourished and aerated (Figs. 6, 8, and 9). It is interesting to note that these phase dark granules absorbed the stain Sudan black B. However, subsequent work with broth-grown protoplasts has indicated that they were not simply neutral fat inclusions.

The thinness of the slide-cultured protoplasts, and especially of their chromatin bodies, could be seen in part of a smear, in which the sheet of compressed growth had become detached and buckled off the coverslip (Fig. 7).

The well isolated islands of protoplasts possessed nuclear bodies with large, and in some instances, double central cores (Fig. 8). And on occasion, also, large protoplasts were found with an open network of Feulgen-positive material (Fig. 9), which suggests a filamentous nature for the chromatin masses. Such structures were seen in cells that were degenerating, at the edge of a group, or in other well isolated regions. The chromatin of protoplasts undergoing degeneration in crowded areas, however, did not unravel into filaments but, instead, coalesced. Such nuclear material often formed huge aggregates (Fig. 10).

Protoplasts growing in slide cultures were watched for periods up to 10 hours in the hope of seeing division. Increase of both cytoplasmic and nuclear material was readily seen (Fig. 11), but in numerous periods of observations, division of the protoplast was never observed in these slide cultures. On the contrary, the opposite, coalescence of adjacent protoplasts, was often seen (Fig. 12). A similar fusion of the protoplasts of *Bacillus anthracis* was observed by Stähelin (13).

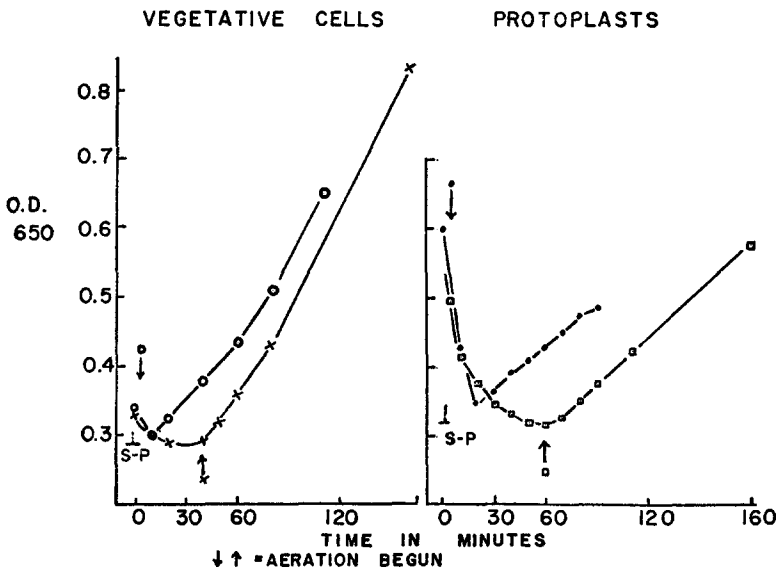
Growth of Protoplasts in Fluid Culture

Comparison of the cytology with changes in the chemical composition of growing protoplasts necessitated the use of fluid cultures. Preliminary studies of the optical density changes during adjustment, aeration, and lysis warrant some mention.

Protoplasts stabilized in sucrose phosphate underwent a marked contraction when they were transferred to the 0.5 M succinate growth medium, and a marked rise in optical density accompanied this transfer. The contraction was transient, however, and as the protoplasts adjusted to the strongly cationic medium, they reexpanded and the optical density of the suspension fell (Text-fig. 1).

Freshly prepared protoplasts were able to adjust to this transfer without lysis. Nucleic acid analyses revealed that, in keeping with other studies of the effects of cationic stabilizing media (3), the adjusted protoplasts suffered a loss of from 10 to 15 per cent of their RNA, but showed no loss of DNA. Following the adjustment, the chromatin existed as a condensed ring or beaded structure (Fig. 16), having aggregated from a more diffuse form in the S-P stabilized protoplasts (3). Stale or aged protoplasts prepared in S-P medium, adjusted poorly to the succinate medium transfer. Many lysed immediately or when aeration was begun. Protoplasts formed from cells suspended in the growth medium were slower in forming, but were similar both in appearance and growth capabilities to those formed first in S-P and then transferred to the growth broth. For most of the experiments, however, protoplasts were formed in the S-P medium, since the time for complete formation was so short (10 minutes).

Aeration of suspensions of protoplasts adjusted to the growth medium was invariably accompanied by a linear rise in optical density with time (Text-fig. 1). Moreover, controls that had been incubated for 3 hours without aeration showed an immediate and linear increase in optical density only when aeration was begun. This finding is in



TEXT-FIG. 1. Optical density changes which accompany the transfer of vegetative cells and protoplasts from S-P medium to succinate broth, and the subsequent effect of aeration begun immediately after transfer (↓), and after adjustment (↑) to the growth medium. Transfer was achieved by adding 1 ml. of a thick suspension of cells or protoplasts in S-P medium to 50 ml. of succinate broth. Point *S-P* is the O.D. level attained by a control dilution in the S-P medium.

agreement with the linear rise in dry weight found by McQuillen (8). No lysis could be seen by phase microscopy during the period of linear increase of optical density. When lysis did occur, it could be detected both in the phase contrast microscope and in the colorimeter. With lysis, the linear rise of the optical density fell off and small flocks of slime appeared in the cultures.

The time of onset of lysis varied with different lots of protoplasts and appeared to bear some relation to the rate of growth. The more rapid the initial rise in density, the sooner lysis would occur, but in most studies, lysis began after 2 to 3 hours of aeration. Occasionally a culture would show one or two cycles of growth and lysis and these displayed a jagged optical density curve, *i.e.* linear growth periods broken by periods of lysis. After each period of lysis the slope of the growth curve was less than that of the previous one. That is, within certain limits, the slope of the linear rise in optical density was related to the concentration of growing protoplasts.

Morphology of Protoplasts Growing in Fluid Culture

Phase Contrast Microscopy:

The marked increase in size attained by protoplasts grown in aerated fluid cultures was readily followed by phase contrast examination of wet mounts (Figs. 13 to 15). When such mounts were made without compression, the protoplasts were usually too dense to reveal internal structures by phase microscopy. In fluid cultures, then, the protoplasts grew with remarkable uniformity and in all dimensions, and this increase in size was obviously the result of *de novo* growth, and not of coalescence of adhering forms. As the forms grew larger and presumably more fragile, lysis occurred. With lysis, the protoplasts became more sticky, and the formerly well isolated forms now tended to adhere to one another, giving the impression of dividing forms. However, examination of fixed and stained adherent protoplasts indicated the separate nature of the forms (Figs. 21 and 22). In none of the experiments followed did any of the protoplasts divide. Some very large protoplasts did show bleb-like protrusions, but these were not seen to separate. In these studies, the end result of a protoplast's growth was a large sphere which invariably went on to lysis—not to division.

Nuclear stainings of protoplasts grown in succinate broth are shown in Figs. 16 to 20 from 0 time to 4 hours. For the first 2 hours of growth in succinate medium containing 6 mg. HDP/ml.,

the nuclear material appeared to increase as a somewhat orderly array of evenly sized granules. The striking increases in chromatin material seen by 3 and 4 hours (Figs. 19 and 20) are in keeping with the 8.5- and 11-fold increases in DNA encountered at these respective time periods. It will be noticed, however, that the chromatin arrangement now appeared more haphazard than in the younger forms. If, however, the HDP level of the medium was reduced (0.6 mg./ml.), the more orderly nuclear pattern characteristic of the earlier stages of growth persisted longer (Fig. 21), but did show some disarray later (Fig. 22). At the lower level of HDP it was also possible to obtain larger protoplasts. That is, lysis tended to occur earlier at the higher of the two levels of Mg-HDP used, and, as the growing protoplasts approached the period of lysis, disorganization of the chromatin bodies occurred. The effect of the 6 mg. level of the Mg salt of HDP on chromatin arrangement could be obtained by adding an equivalent amount of Mg ions (*ca.* 10^{-2} M), but not by adding barium HDP or the free acid.

The above observations of the effect of Mg ions on protoplast growth led to some studies of the morphology of vegetative cells of *Bacillus megaterium* growing in the same succinate broth. The appearance after 3 hours growth was as follows: with 6 mg./ml. of Mg HDP (or the equivalent of $MgSO_4$), the cells grew as relatively thin rods possessing condensed or aggregated compact chromatin bodies; in succinate broth without added Mg (*i.e.* at 10^{-3} M Mg), the cells were larger and possessed bulbous ends with chromatin bodies which were likewise larger and unequally dispersed; at the 0.6 mg. level of Mg HDP (or equivalent of $MgSO_4$), the cells grew in normal proportions and possessed typical patterns of chromatin.

In spite of these differences in morphology, growth of cells was excellent in all of the media. Nevertheless, from the point of view of cytology, growth appeared best for both cells and protoplasts when the succinate-fluid medium was supplemented with additional Mg ions (final concentration 5×10^{-3} M).

Change in Protoplast Volume with Growth and Fixation:

Measurements of the diameters of 10 to 30 protoplasts at each different period of growth were taken from photomicrographs and used to calculate protoplast volume. The average diameter of fixed protoplasts was smaller than that of living ones. Thus a marked, but relatively constant decrease in calculated volume was found (Table I). As might be deduced from the above photomicrographs, measurements in any one aerated sample were remarkably close to the mean value.

TABLE I

Effect of Ethanol Acetic Acid Fixation on the Volume of Protoplasts Calculated from Diameter Measurements

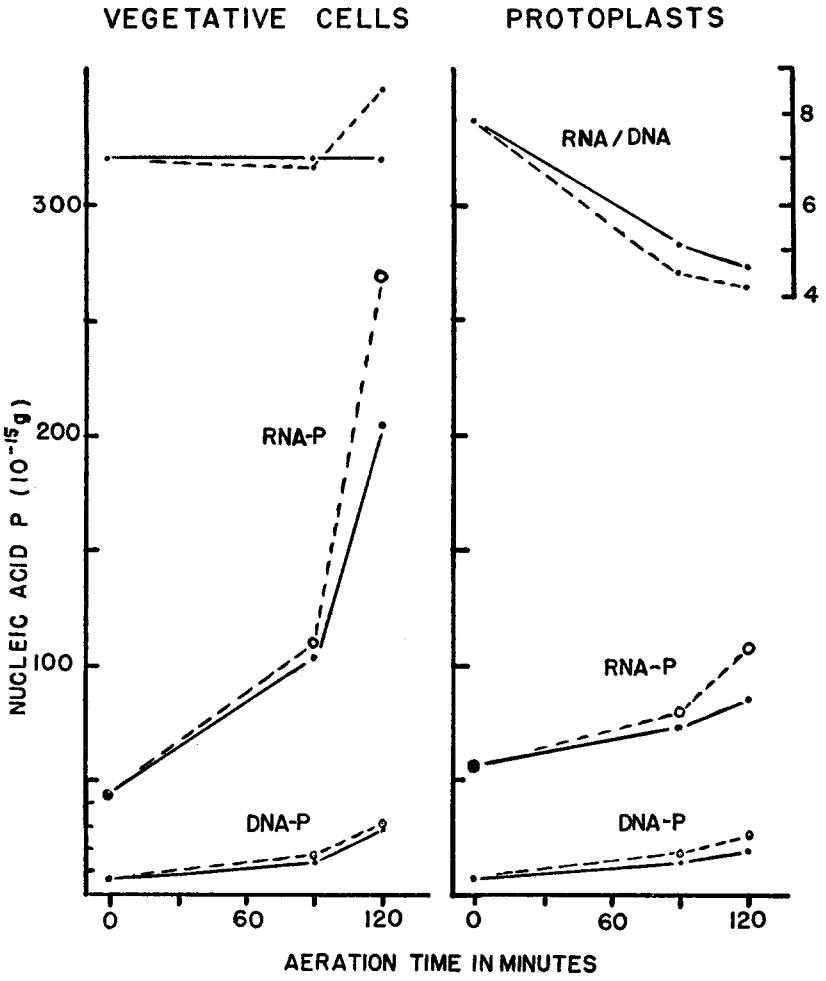
Hrs. of aeration	Volume in phase contrast	Volume in fixed smears	Decrease in volume
	μ^3	μ^3	*per cent*
0	5.9	3.45	40
$2\frac{3}{4}$	50.9	33.5	34
4	105.5	65.4	38

Chemical Analyses of Growing Protoplasts

Initial nucleic acid analyses of centrifuged samples of the culture after 2 to 3 hours of aeration revealed marked increases in DNA-P, but only moderate increases in RNA-P. It was found that such samples were composed of both intact protoplasts and slimy debris, and Feulgen stains indicated that much of the chromatin material of lysed protoplasts was present in the culture and recovered in the pellet. It was then discovered that the true nucleic acid content of the growing protoplasts could be determined even in the presence of lysis, if the cultures were first poured through a pad of 8 layers of surgical gauze, for such filtration removed all visible flocks and slime and produced even suspensions of protoplasts, which after centrifugation yielded a non-slimy pellet that could be readily resuspended. Feulgen stains of such filtered cultures revealed that now all the chromatin was within the protoplasts. Counts of the filtered culture were used as a basis on which to express the results.

A Comparison of the Nucleic Acid Content of Growing Protoplasts and Vegetative Cells:

The optical density and nucleic acid changes of a typical comparison of growth of protoplasts and cells in succinate-citrate broth are shown in Text-figs. 1 and 2. The contraction and adjustment discussed above which occurred when cells and protoplasts suspended in S-P medium were transferred into the cationic growth broth are also shown in Text-fig. 1 as optical density changes. Vegetative cells showed a less marked contraction (rise in optical density) on inoculation to the growth medium than did the protoplasts



TEXT-FIG. 2. The nucleic acid content and the RNA/DNA ratios of vegetative cells and protoplasts growing in aerated succinate broth containing 0.6 mg. (⊙ - - - ⊙) and 6.0 mg. (● —— ●) HDP (Mg. salt)/ml. For this comparison, nucleic acid P is expressed as the amount per cell-unit inoculated and per protoplast analyzed.

and, as indicated by the earlier fall in optical density to a constant level, vegetative cells also adjusted to the medium sooner. On aeration the optical density of the vegetative cell culture rose exponentially. On the other hand, as mentioned above, the optical density of the protoplast culture rose linearly.

In order to compare the nucleic acid changes as shown in Text-fig. 2, the number of vegetative cell units inoculated was used as a basis of the cell calculations, and the number of protoplasts analyzed for the protoplast calculations. In this way, the nucleic acid content of growing and dividing vegetative cells could be compared with that of growing protoplasts. The number of cell units inoculated was derived either from a direct count or by quantitatively converting a sample of cells into protoplasts and counting these. Both methods gave the same count. When the nucleic acid content of growing protoplasts was compared to that of growing and dividing vegetative cells, both forms appeared to be able to synthesize similar quantities of DNA, but the protoplasts contained much less RNA. Thus the ratio RNA/DNA of vegetative cells remained constant or rose slightly, while that of protoplasts fell markedly (Text-fig. 2).

This chemical information agrees well with the cytological appearance. In vegetative cells, where division occurs, the ratio of chromatin mass to cytoplasmic space is maintained; whereas, in protoplasts with no division, the cytoplasmic space

TABLE II

*Lipide Phosphorus, Nucleic Acid, and Protein Content of Growing Protoplasts as Functions of Counts and Protoplast Volume**

	Hrs. of aeration	Fractions as a function of counts ($g \times 10^{-15}$/protoplast)				Fractions as a function of volume ($g \times 10^{-15}/\mu^3$)			Ratio RNA: DNA: Protein
		Lipide P	RNA	DNA	Protein	RNA	DNA	Protein	
Exp. I	0	2.0	540	44	650	93	7.8	110	12.1:1:14.6
	2	3.6	735	117	1448	47	7.6	93	6.2:1:12.3
	10	3.35	1810	371	4900	35	7.1	93	4.9:1:13.2
Exp. II	0	—	570	64	606	97	10.8	103	8.8:1: 9.4
	1.5	—	1205	161	1310	76	10.1	83	7.5:1: 8.1
	3	—	3460	549	—	68	10.0	—	6.3:1: —
	4	—	4160	685	6300	65	10.6	98	6.1:1: 9.2

* Volumes were calculated from mean diameters of 10 to 30 protoplasts in phase contrast photomicrographs of unfixed smears.

becomes crowded with chromatin (Figs. 16 to 22). When protoplast and vegetative cell growth were compared at different levels of Mg HDP (or Mg ions) as outlined above, in spite of marked differences in the appearance of the nuclear material, no consistent difference was found in the optical density curves. However, by 2 hours, a greater amount of RNA-P was found in both vegetative cells and in protoplasts grown at the lower level of Mg HDP (Text-fig. 2).

Lipide-P and protein of growing protoplasts also increased as well as the nucleic acids (Table II). If it is assumed that the lipide-P is an indication of the amount of protoplast membrane, then it would appear that membrane material was formed, rather than simply stretched as the protoplast grew. Moreover, during the first 4 hours of aeration, the amount of lipide-P of the protoplasts expressed as a function of cell surface, was a reasonably constant value. Protein or DNA expressed as a function of volume, showed no striking change. RNA concentration, however, steadily decreased as the protoplasts grew larger. These relationships are reflected in the ratios expressed as a function of DNA (Table II).

Ancillary Studies of Grown Protoplasts

Effect of Compression:

The marked difference in phase contrast appearance between broth-grown protoplasts and those grown in slide cultures, indicated that the compression previously found useful for visualizing the internal structures of resting protoplasts (3) might be used to advantage on these giant forms (Figs. 14 and 15). As compression was applied, the phase-light nuclear structures became visible as discrete regions (Fig. 24). With further compression the gel-like nuclear material coalesced to a single mass of indefinite form, while excess compression led to rupture with loss and degradation of the contents (Fig. 25). Once the nuclear bodies had coalesced by compression, they remained in this form when the pressure was released and the flattened protoplasts were allowed to become more spherical.

The effect of fixation (formaldehyde, osmium, or ethanol–acetic acid) on compressed protoplasts, was followed in the phase microscope. The delicate phase dense regions or cores of the nuclear structure became more prominent—in agreement with earlier work (11)—and were Feulgen-negative, while the phase light region of these compressed nuclear bodies was found to be the site of the DNA.

Acetocarmine "Squashes" of Protoplasts:

Compression of grown protoplasts in the combined fixative and stain acetocarmine resulted in both aggregation and staining of the nuclear bodies. Like Feulgen stains on similar material (Figs. 5 to 8), the deeply stained chromatin was wound about unstained cores (Fig. 23).

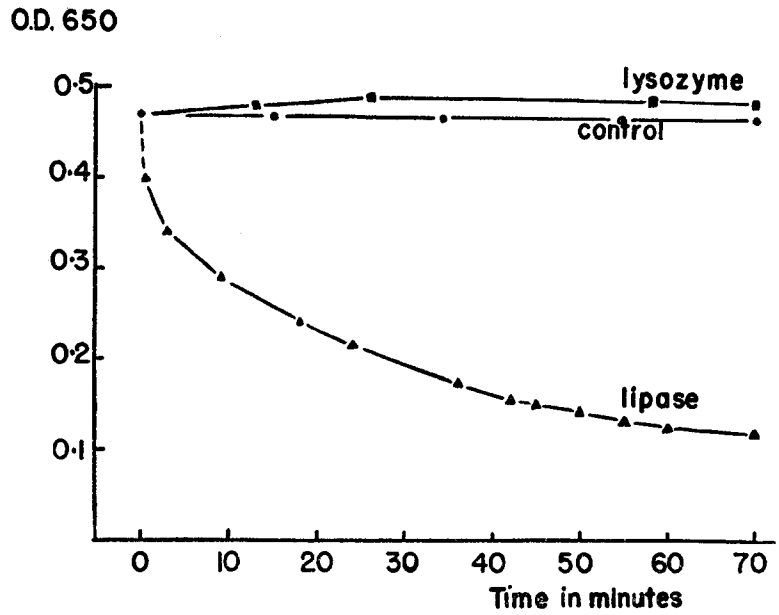
The Effect of Lipide Solvent Extraction on the Fat Staining of Grown Protoplasts:

As with slide-grown protoplasts, the phase dense beads of those grown in fluid culture readily absorbed Sudan black B. Similar protoplasts fixed (formaldehyde) and stained in bulk, likewise showed fat staining of these cytoplasmic inclusions. After hot lipide-solvent extraction, these particles were still present, but now had a low affinity for the stain. It was interesting to note that, before lipide extraction, the limiting membrane of the protoplast was slightly stained, while after extraction it was not. The central cores of the nuclear regions of grown protoplasts failed to show lipide staining.

Studies of a Slimy Byproduct of Protoplast Growth and Lysis:

With prolonged aeration (6 hours) of growing protoplasts, considerable quantities of white strings of slimy material accumulated in the cultures. A quantity of this slime was collected and, unlike the nuclear slime (3), was found to be water insoluble. With repeated water washings entrapped protoplasts and debris were removed, and a white tangle of material remained.

In the phase contrast microscope, this product appeared to be a tangled membranous structure (Fig. 26). It stained faintly with basic dyes before, but not



TEXT-FIG. 3. Effect of lipase and lysozyme on the membranous by-product of growing and lysing protoplasts.

after, treatment with N HCl (60°C. 5 to 10 minutes). It was Feulgen-negative.

Analysis of a water-washed sample indicated that it contained less than 0.5 per cent of its dry weight as DNA, some 3 per cent RNA, 2.5 to 3 per cent phospholipides, and 7.5 per cent nitrogen. Lipase, but not lysozyme, readily clarified a suspension of the material (Text-fig. 3), and after 70 minutes of digestion (75 per cent fall in OD 650), 76 per cent of the lipide-P and 90 per cent of the RNA was dissolved. Alkali titrations of a sample revealed maximum buffering (pKa) at pH 10.8, while back titration with acid led to maximum precipitation (isoelectric point) at pH 5.2. The above analyses indicate this membranous material is a phospholipoprotein containing some 3 per cent of its dry weight as RNA. Its similarity to the membranes isolated from freshly formed protoplasts (14), suggests a similar origin. From 150 ml. of culture which originally received 12 to 15 $\times$ 10^9 protoplasts (*ca.* 15 mg.), 13 mg. of this coalescent membrane material were recovered after 6 hours' growth and lysis.

DISCUSSION

These chemical and cytological studies of protoplasts grown 10 to 20 times their original volume, have helped confirm the findings from the studies of structure and composition of smaller, freshly formed protoplasts (3, 11). The aggregated nuclear body of the protoplast is capable of division into a series of units (Figs. 21 and 22) similar in arrangement to those of vegetative cells or "resting" protoplasts. The nuclear structure of these giant protoplasts, like that of the small ones, appears in both fresh and fixed forms to possess two types of material—a chromatin gel or net, and an associated core. Growth and DNA synthesis are invariably accompanied by an increase in the size and in the number of cores. Protoplasts growing in compressed smears possess chromatin completely aggregated around a variable number of cores. Protoplasts growing in aerated cultures containing an excess of Mg^{++}(10^{-2} M) possess chromatin unequally dispersed about irregular achromatic cores. In medium containing less Mg^{++}(5×10^{-3} M), growing protoplasts possess chromatin arranged more uniformly around smaller centres of more equal size.

Division was not observed in these studies. This is not in keeping with the findings of McQuillen (8, 9), who, observing dumbbell-shaped forms after prolonged aeration (8 to 10 hours) in a somewhat different medium, concluded that division was occurring. It is equally likely that such forms could be adherent protoplasts stuck by the slime of the culture. In the present studies, such forms were seen in both slide cultures and in wet mounts of fluid cultures.

They were not seen to separate, but they would often coalesce. The linear rise of optical density of protoplasts growing in the absence of lysis, certainly is characteristic of a growing and non-dividing system.

In spite of the absence of its cell wall, the protoplast of *B. megaterium* is as capable of DNA synthesis as is the complete cell. The growing protoplast can also form protein and lipide-P in amounts sufficient to maintain a constant concentration. It is suggested that the failure to produce a heavier membrane to counteract the increased tension which accompanies the larger diameter, leads to the greater fragility of the larger forms.

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Explanation of Plates

Plate 139

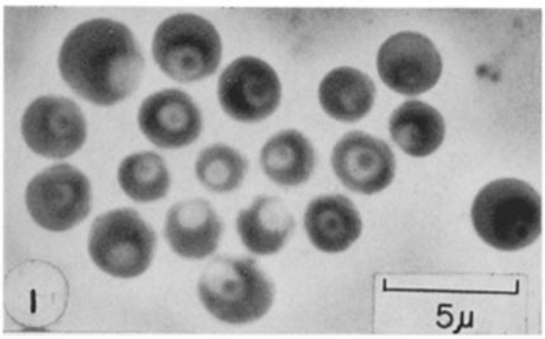
Figs. 1 to 4. Dark phase contrast photographs of protoplasts in various stages of compressed growth in a 10 hour slide culture on succinate-casein agar. Magnification as indicated by the marker.

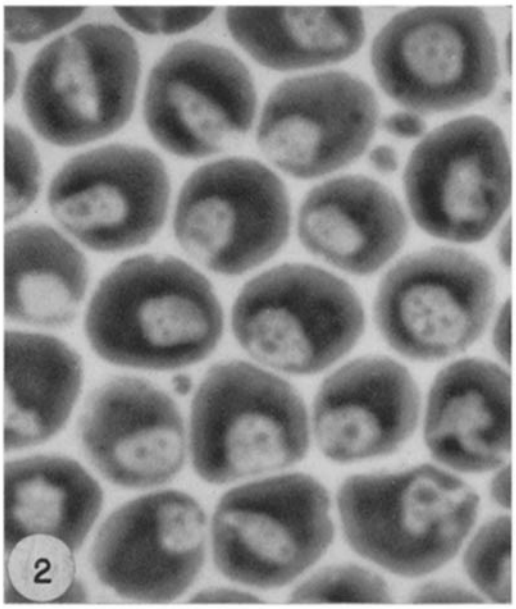
Fig. 1. Protoplasts poorly nourished or aerated which showed only slight growth.

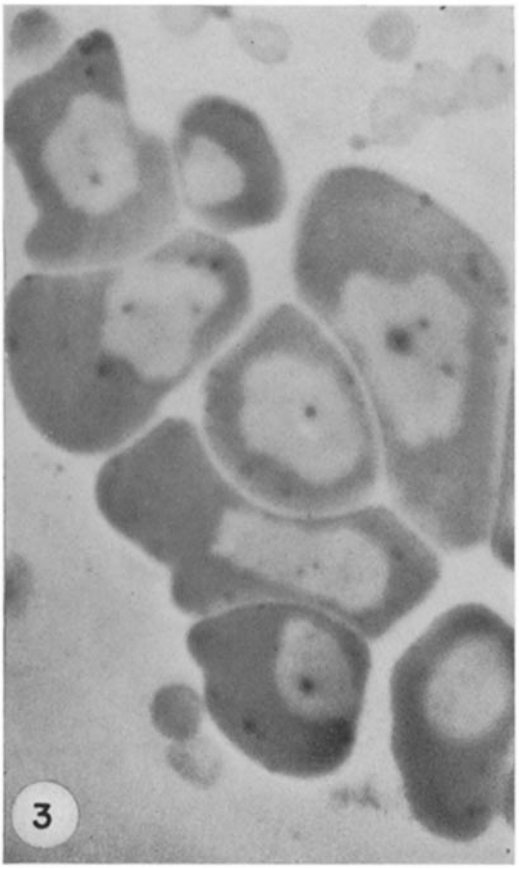
Fig. 2. Protoplasts growing in close quarters near the agar block.

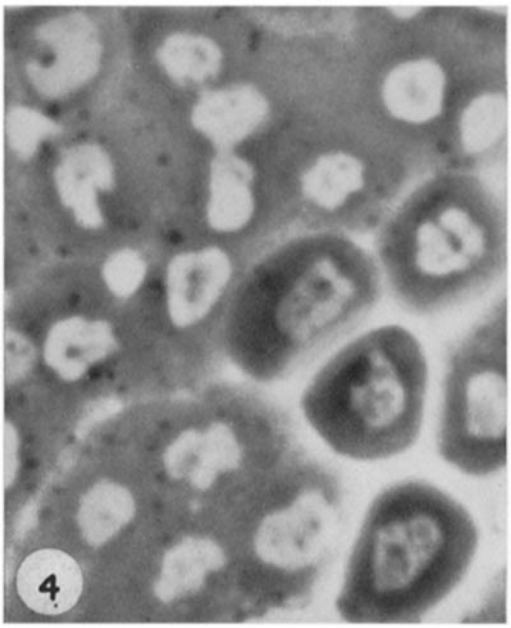
Fig. 3. Giant forms which developed in well isolated structures near the agar. The small round objects are "ghosts" of lysed protoplasts.

Fig. 4. A sheet of coalescent protoplasts probably lysing following growth in a crowded area of the smear.









(Fitz-James: Growth of protoplasts)

PLATE 140

FIGS. 5 to 10. Photomicrographs of protoplasts growing in slide cultures. Acid-alcohol–fixed, Feulgen-stained. Magnification as indicated.

FIG. 5. Area showing varying degrees of growth and size of nuclear bodies. Water-mounted.

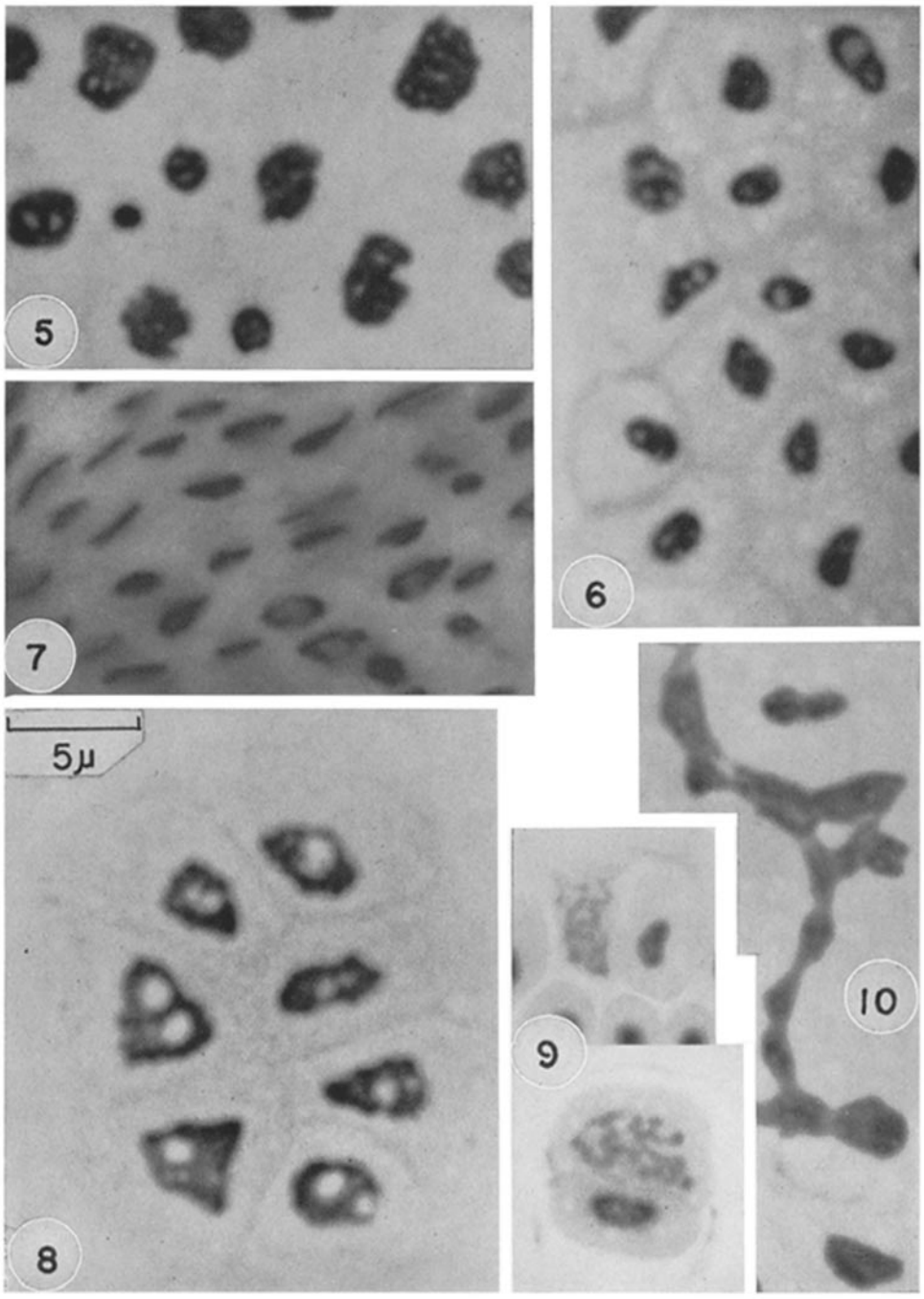
FIG. 6. Zone of moderate uniform growth. Mounted in acetocarmine.

FIG. 7. Part of a sheet of Feulgen-stained protoplasts, with flat forms lying at right angles to the plane of the coverslip. Showing compression of the protoplasts and their contained nuclear bodies.

FIG. 8. An island of seven protoplasts, showing marked growth of both cells and nuclear bodies. The nuclei possess separated cores, but nuclear division has not occurred.

FIG. 9. Two areas from the same culture, showing filamentous forms of Feulgen-positive material. The upper protoplast is breaking apart.

FIG. 10. Lysis of protoplasts in a region of crowded growth, showing coalescent nuclear bodies (see also Fig. 4).



(Fitz-James: Growth of protoplasts)

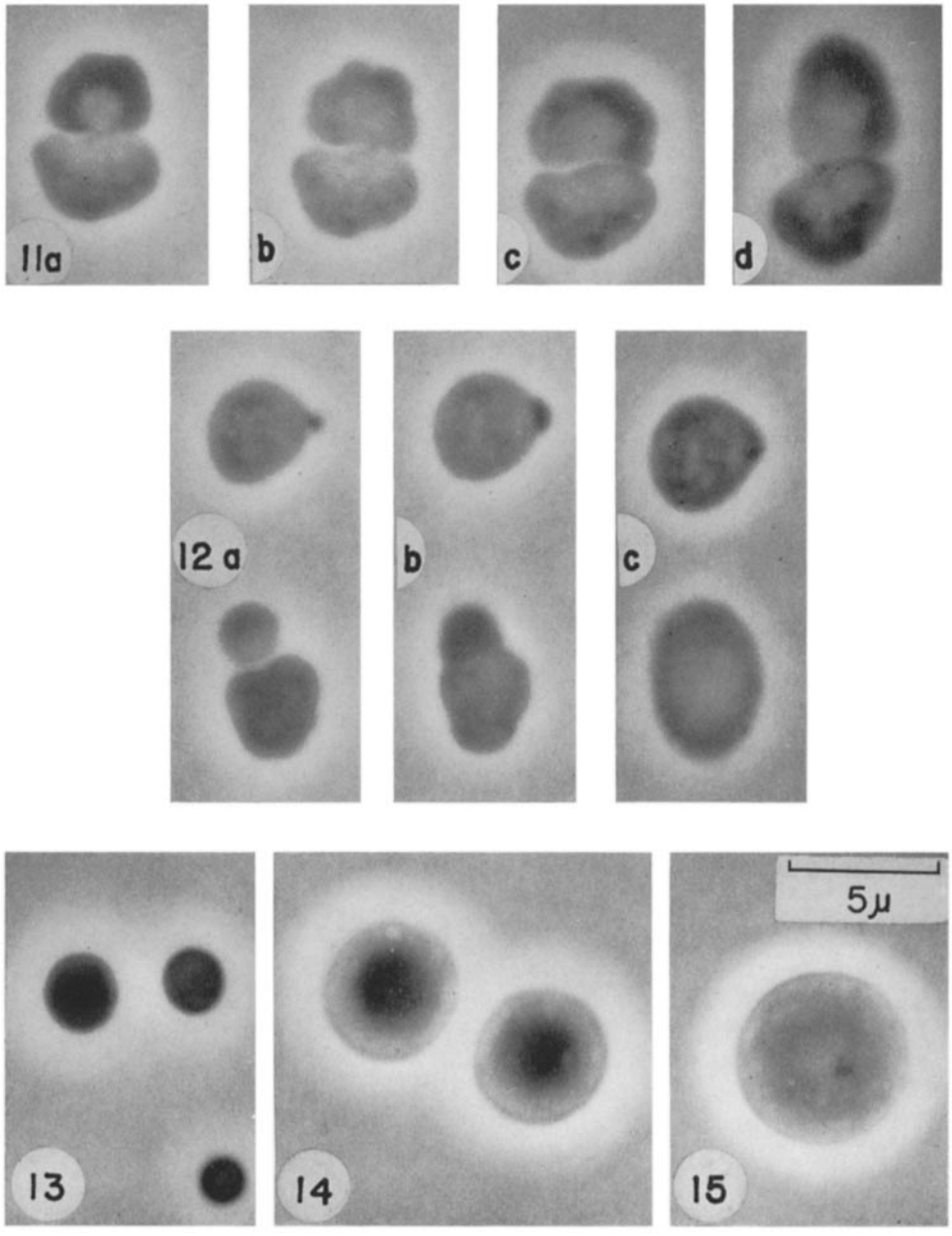
PLATE 141

FIG. 11. Phase contrast photographs of two closely adhering protoplasts growing slowly in a slide culture. (*a*) 5 minutes after mounting; (*b*) at 30 minutes; (*c*) at 2 hours; (*d*) at 7 hours.

FIG. 12. A field adjacent to that of Fig. 11 at (*a*) 5 minutes, (*b*) 2 hours and (*c*) 7 hours, showing the coalescence of two adjacent protoplasts and the growth of another.

FIGS. 13 to 15. Phase contrast appearance of protoplasts growing in succinate-casein broth containing HDP at 0.6 mg./ml. Fig. 13 at 0 minutes, Fig. 14 after 3 hours' and Fig. 15 after 4 hours' aeration.

Magnification as indicated by marker.



(Fitz-James: Growth of protoplasts)

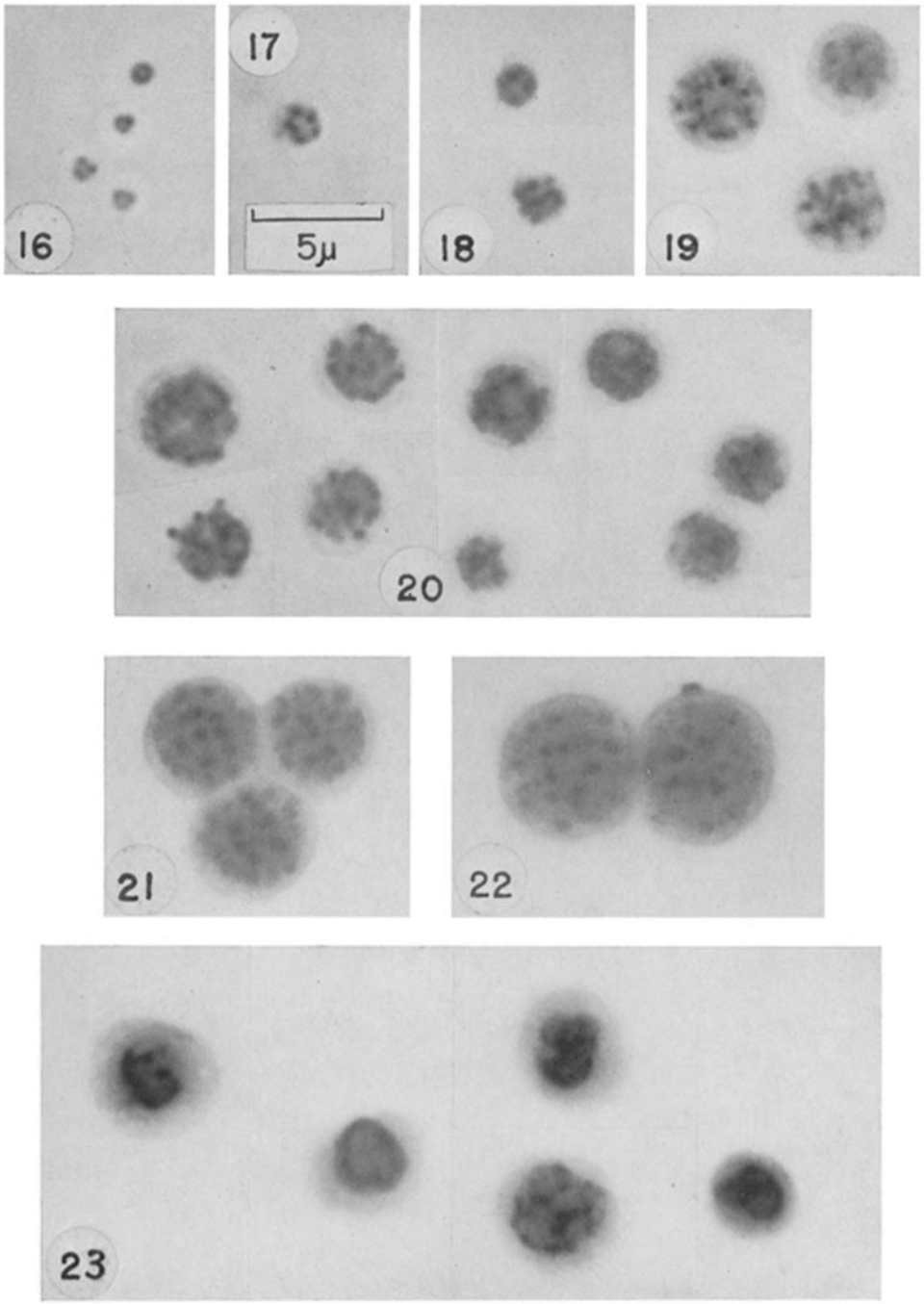
PLATE 142

FIGS. 16 to 22. Nuclear stains of protoplasts growing in succinate-casein broth. Acid-alcohol-fixed. Magnification as indicated.

FIGS. 16 to 20. Growth in broth containing 6 mg. HDP/ml., showing the size and nuclear arrangement at 0 time, Fig. 16; 1 hour, Fig. 17; 2 hours, Fig. 18 (the focal plane of the top protoplast is above the centre); 3 hours, Fig. 19; and 4 hours, Fig. 20. Stained with SO_2-azure A.

FIGS. 21 and 22. Growth in medium containing 0.6 mg. HDP/ml., showing the larger size of protoplasts and the more orderly arrangement of nuclear bodies attained after 3 (Fig. 21) and 4 hours' (Fig. 22) aeration. Feulgen-stained, mounted in acetocarmine.

FIG. 23. Protoplasts mounted with compression in acetocarmine after 5 hours' growth; chromatin has stained and lies around unstained cores. Cytoplasm is poorly preserved. Magnification as indicated in Fig. 17.



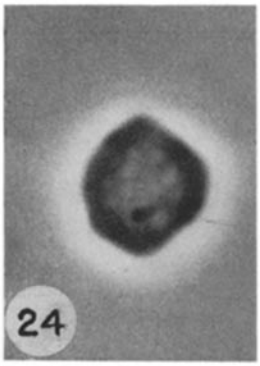
(Fitz-James: Growth of protoplasts)

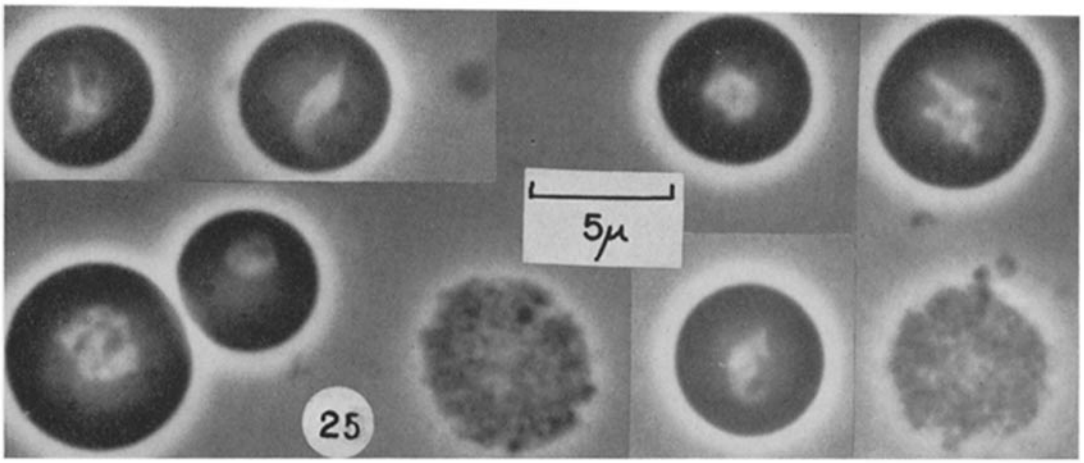
PLATE 143

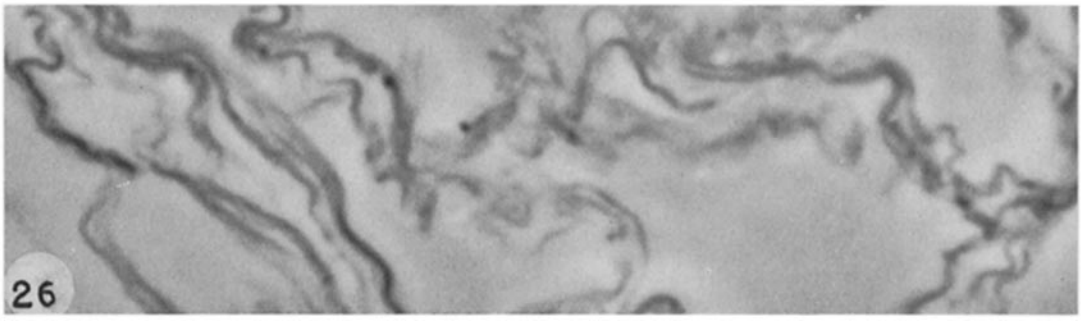
FIGS. 24 and 25. Effect of compression in the phase contrast appearance of protoplasts aerated for 3 hours in fluid culture. Control appearance is shown in Fig. 14 and magnification in Fig. 25.

FIG. 25. Further compression leads to aggregation of the nuclear material. Excess compression causes rupture and loss of phase density.

FIG. 26. Phase contrast appearance of water-washed membranous material collected from growing and lysing protoplasts. Magnification as indicated in Fig. 25.







(Fitz-James: Growth of protoplasts)