ISOLATION AND CHARACTERIZATION OF NUCLEAR BODIES FROM PROTOPLASTS OF BACILLUS MEGATERIUM

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In the course of experiments involving the enzymatic treatment of protoplasts from Bacillus megaterium it was discovered (Landman and Spiegelman, 1955) that exposure to a lipase preparation resulted in their physical dissolution accompanied by a marked loss in optical density. Centrifugation of such lipase lysates at 10,000 G for 5 min yielded pellets containing a large percentage of the deoxyribonucleic acid (DNA) concentrated in a gelatinous layer (Spiegelman, 1956). Similar enrichment for DNA was noted by centrifugation of osmotically lysed at the same speeds (Spiegelman, 1957). These observations implied that the DNA of protoplasts was associated with a structure large enough to be sedimented at relatively low speeds.

Preliminary phase contrast studies suggested that the lipase had dissolved the protoplast membrane permitting the solution of the mass of cytoplasmic material and the release of pre-existent cellular structures. Such digests contained many small (μ) vesicular objects, visible in the phase contrast microscope, which were concentrated during centrifugation in the fraction containing the bulk of the DNA.

The correlation between the chemical and phase contrast observations suggested that these lipase resistant structures were the nuclear bodies of the bacterial cells. An investigation was, therefore, undertaken to explore this possibility further. The present paper details the evidence obtained for the discreteness and pre-existence of these structures and describes their chemical and cytological properties.

The experiments summarized indicate that digestion of protoplast membranes in hypertonic buffer systems provides a method of liberating nuclear bodies from bacteria in a state which permits their ultimate isolation from other components of the cell.

MATERIALS AND METHODS

Media for conversion to protoplasts. Strain KM of Bacillus megaterium was employed in the present investigation. The methods of protoplast formation were essentially those described by Landman and Spiegelman (1955). In addition to the stabilizing media described by them phosphate buffered sucrose solution (Weibull, 1953) and 0.5 M sodium phosphate were used as suspending media in the conversion of vegetative cells to protoplasts. Other media were also examined in an attempt to obtain constant chemical composition. Of those tested 0.5 M sodium citrate, 0.5 M succinate, and a mixture of equal parts of 0.90 M succinate and 0.90 M sodium citrate were the most promising. Unless noted otherwise, all buffer systems employed were adjusted to pH 7.6.

Lipase purification. A crude lipase preparation (steapsin) from Nutritional Biochemical Corporation was the starting material employed. A two step purification (Glick and King, 1933) was carried out to remove contaminating nucleolytic and proteolytic enzymes. Two grams of the crude lipase were suspended in 100 ml of 10 per cent sodium chloride, stirred at 0 C for 30 to 40 min and centrifuged at 2000 rpm for 30 min. The supernatant, which contained the bulk of the enzyme, was saturated with MgSO₄ at 0 C and then spun at top speed in a Servall SS-1 for 15 min. The pellet was dissolved in 10 per cent NaCl and insoluble material removed by centrifugation. The resulting solution was dialyzed against distilled H₂O for 48 hr in the cold, a procedure found necessary to remove substances which lead to the breakdown of DNA in treated preparations.
Subsequent to the present investigation, it was discovered that the lipase was undergoing degradation during the dialysis against the distilled water. In more recent preparations this dialysis is carried out at pH 5. During the dialysis the lipase precipitates in the dialysis bag. The precipitate is then collected by centrifugation and dissolved in 10 per cent sodium chloride. Any insoluble material is removed by centrifugation.

Lipase digestion of protoplasts. In all studies reported here protoplasts were suspended in buffer to an optical density of approximately 0.3 at 650 mμ. Lipase was added to give a final concentration of 40 to 50 μg/ml of protein, and CaCl₂ to a final level of 1 X 10⁻³ M. Suspensions were incubated at 30 C. Shaking was used for most of the chemical studies but was generally omitted in cytological studies in order to avoid physical distortion of the nuclear bodies. Clearing time varied with the pH and with the buffer used. On completion of the digestion, the lysate was centrifuged at 10,000 G for 5 min on a Servall SS-1 to recover fractions of interest. The buffer system used for digestion was also employed for washing of the pellets.

Analytical procedures. The methods of Ogur and Rosen (1950) and Schneider (1948) were used for nucieic acid analyses. Both methods gave similar results.

A test for protein deficient in aromatic amino acids was devised after the quantitative amino acid procedure of Moore and Stein (1948). The ninhydrin was recrystallized from water or ethyl alcohol. Protamine sulfate was used as a standard in amounts up to 50 μg. The protein solutions in 0.2 M NaOH were adjusted to pH 5.9 to 6.3, and 0.5 ml aliquots were taken so as to contain no more than 50 μg of protein. To these were added 1.0 ml of the ninhydrin solution and the tubes heated on a steam bath for 20 min. Five ml of 1:1 n-propanol-water were added and the solution allowed to stand for 20 min at room temperature. All tubes were then read on a Klett colorimeter using a no. 54 filter. Protein was also measured by the Folin procedure as modified by Lowry et al. (1951).

Density gradient centrifugation. Sucrose gradients for the separation of protoplast fractions were prepared in 30 ml centrifuge tubes by layering an equal volume of 1 M sucrose on 2 M sucrose. A one-half volume of water was then layered on top. A stirring rod was plunged into the mixture once or twice, and the preparation then left for 24 hr in the cold in order to establish a final gradient. Suspensions for study were layered on the gradient and spun in the Spincot swinging bucket head (SW 25.1) at 20,000 rpm for 20 to 25 min. The layers were drawn off with a syringe, spun down, and resuspended in 0.01 M phosphate buffer at pH values appropriate for subsequent enzymatic tests.

Immunological analysis. Complement fixation tests were performed as described by Venn and Gerhardt (1956).

Dark phase-contrast microscopy. All coverslips and slides were treated at 400 C for 20 min to insure freedom from grease. Fresh material was examined as wet mounts under coverslips sealed with paraffin.

Fixed preparations mounted in buffer were also examined in phase, as were water mounts of Feulgen-stained preparations. Phase contrast photographs were made using the phase equipment of Bausch and Lomb in conjunction with either an Xl-No. 11 or a B-No. 58 filter onto Kodak Royal Pan sheet film at a magnification of 1950X, and were enlarged twice for presentation in this paper.

Fixation methods. A number of fixatives were used in this study and were applied in both smear and bulk procedures. Wet smears of material were exposed to osmium vapor followed by 70 per cent alcohol rinsing and storage. Bulk osmium fixation of suspensions of protoplasts and components derived from them was achieved by adding to the buffer suspension an equal amount of 2 per cent osmium tetroxide in the same buffer. After 1 to 2 hr at room temperature, or several hours (up to 8) in the cold, the samples were centrifuged and washed with buffer.

For acid alcohol fixation wet smears partly dried on coverslips were immersed in a mixture of 1 part acetic acid and 3 parts of ethanol for 10 min and rinsed in 70 per cent alcohol.

Newcomer's fixative (1953) was used in the same way as acid-alcohol. Smears could be left in this fixative in the cold for 2 to 4 days without apparent change. A 70 per cent alcohol rinse preceded staining or hydrolysis.

Formalin fixation of protoplasts and protoplast fractions was achieved by two procedures. At first, 37 per cent formaldehyde was added directly to the preparation to give a final concen-
tation of 5 to 10 per cent. The drop in osmotic pressure which occurs in this method caused some swelling of whole protoplasts before fixation was accomplished. In the second procedure, fixation was accomplished without swelling by the addition of an equal volume of the stabilizing buffer containing 10 to 15 per cent formaldehyde. After a 20 min treatment, preparations were spun and washed twice. Such bulk-fixed material was applied to coverslips, dried, and rinsed in 70 per cent alcohol before cytological study.

Staining. Nuclear stains were made after hydrolysis in either \( n \) HCl or \( n \) trichloracetic acid (TCA) at 60°C for 7 to 8 min. Hydrolyzed preparations, rinsed in water, were stained for 3 hr in Schiff's reagent or for 1 hr in 2 per cent Azure A freshly reduced with \( n \) HCl and 10 per cent potassium metabisulfitite (0.6 ml of each to 10 ml of Azure A). Feulgen-stained preparations were washed for 20 min in a stream of tap water, the Azure A in four changes of distilled water, and both were examined as wet mounts and photographed within 3 hr.

Optical equipment has been previously described (Fitz-James, 1953). Photographs on Panatomic X film were enlarged twice to give a final magnification of 3600 x.

Electron microscopy. The proper application of protoplasts and protoplast fractions to electron microscopy grids was hampered by the presence of large quantities of nonvolatile salts. Most of the electron micrographs presented here were made with material applied to grids by a method similar to that described by Kellenberger (1953). A small square of 2 per cent agar in succinate-citrate buffer was mounted on a slide. Formvar (0.3 to 0.5 per cent in ethylene dichloride) or collodion (1 to 2 per cent in amylacetate) was run across the surface of the block to form an even film. To the dried film the material was added as smears or tiny drops and the block fixed (if the material was not already bulk fixed) in the vapor of 2 per cent osmium for 4 to 5 min. The film was then floated off into distilled water. “Atheine” grids, 100-mesh (Smelstoth Highlight, Ltd., Bolton, Lancs., England), were applied and the grids and film lifted out carefully on a strip of dry, rough paper toweling and then dried and stored in a desiccator over fresh CaCl\(_2\). The specimens were shadowed with uranium at an angle of 18° and examined in the Philips E.M. 100A microscope. Magnifications as shown by the micron markers are comparable, but approximate.

EXPERIMENTAL RESULTS

I. Chemical studies. (1) Centrifugal fractionation of lipase lysates:—The course of a lipase digestion of protoplasts is described in figure 1. The protoplasts were prepared in sucrose-phosphate and transferred to succinate-citrate medium. As will be noted, the transfer results in an immediate rise in optical density. This change in optical properties is also observable as an increase in refractility in phase contrast. The addition of lipase to the protoplast suspension held at pH 7.4 initiates a drop in optical density which becomes rapid and severe in about 20 min. The lysis is virtually complete in 1 hr. It will be noted from figure 1 that the lipase acts much more slowly at pH 6.2.

When lipase lysates are centrifuged for 5 min at 10,000 G, the following three fractions become evident; a yellow bottom layer, a loosely packed gelatinous mass immediately above it, and finally a clear supernatant. The nucleic acid contents of these three fractions collected at two points in the history of a lipase digestion are summarized in table 1. Several features of these data are worthy of note. DNA tends to concentrate in the gel fraction and the extent of the concentration increases with the degree of lysis as measured by the optical density. On the other hand, more and more of the ribonucleic acid (RNA) is found in the supernatant as the digestion proceeds. The
TABLE 1
Distribution and recovery of nucleic acids in centrifugal fractions obtained during lipase digestion of protoplasts

Protoplasts were suspended at an O.D. of 0.3 succinate-citrate buffer at pH 7.4 in the presence of 50 μg/ml of lipase and 1 × 10^{-2} M CaCl₂. Samples for analysis were taken when the optical density had fallen to 50 per cent and 5 per cent of the initial readings. They were centrifuged at 10,000 G for 5 min and separated into the three recognizable fractions described in the text. Zero time samples were taken to provide a basis for estimation of total recovery.

<table>
<thead>
<tr>
<th>Per Cent of Initial Optical Density</th>
<th>Fraction</th>
<th>RNA, Per Cent of Total</th>
<th>DNA, Per Cent of Total</th>
<th>RNA/DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>Supernatant</td>
<td>33</td>
<td>14</td>
<td>26.4</td>
</tr>
<tr>
<td></td>
<td>Gel</td>
<td>9.5</td>
<td>44</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>Yellow Pellet</td>
<td>41</td>
<td>41</td>
<td>11.2</td>
</tr>
<tr>
<td>Per cent recovery</td>
<td>83.3</td>
<td>99</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>Supernatant</td>
<td>51</td>
<td>27</td>
<td>19.9</td>
</tr>
<tr>
<td></td>
<td>Gel</td>
<td>8.7</td>
<td>75</td>
<td>1.27</td>
</tr>
<tr>
<td></td>
<td>Yellow Pellet</td>
<td>7.6</td>
<td>0.12</td>
<td>66.0</td>
</tr>
<tr>
<td>Per cent recovery</td>
<td>67</td>
<td>102</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

RNA to DNA ratio of the entire protoplast is between 7 and 8. The gel fraction consistently approaches a ratio near unity with completion of the lipolysis. Finally, it will be noted that virtually all of the original DNA is recovered as acid precipitable material, whereas there is a progressive loss of polymerized RNA with time during the lysis.

(2) Structural elements and the DNA content of the gel layer:—It has already been noted in the introduction that the small bodies released on lipase digestion of protoplasts were found concentrated in the gel layer. Their appearance and relative size is readily apparent in the phase contrast photomicrographs grouped in figure 13. A more detailed cytological analysis is given below. The present section is primarily concerned with the quantitative aspects of their association with DNA.

If these bodies are nuclear structures, and if there is one such per protoplast, it would be expected that the DNA per body should correspond to the DNA per protoplast. Counts of lysates and phase contrast observation of the lytic process indicated that the method of cell rejuvenation employed yielded protoplasts each of which released one phase visible body on digestion. It should be noted that 100 per cent recovery of these bodies was rarely obtained on dissolution of the protoplast, some of them disintegrating on being released. However, based on original protoplast counts, 70 per cent yields could be routinely obtained by most of the procedures employed.

Of interest here is the question whether a quantitative correspondence existed in a lysate between the number of countable bodies and the amount of DNA which is sedimentable by centrifugation at 10,000 G for 5 min. Table 2 summarizes the results of a representative experiment. The lysate contained a number of phase-contrast visible bodies corresponding to 67 per cent of the original protoplast input. In agreement, 70 per cent of the DNA was found to be sedimentable at speeds which are adequate to pellet the bodies. The DNA assignable to each body agrees within experimental error to the amount of DNA per protoplast. The quantitative relations are not drastically altered by one washing.

The data presented in the present section, are consistent with the conclusion that the DNA of the protoplast is attached to, or associated physically with, the phase contrast visible bodies present in lipase lysates.

(3) Differentiations of nuclear bodies from protoplast membranes:—Experiments were next undertaken to answer the following question: Are the phase contrast visible bodies found in lipase lysates entities distinct from resistant remnants of undigested protoplast membranes?

Protoplasts were ruptured by osmotic shocking (Weibull, 1953), or by treatment with 2 per cent ethyl acetate. The resulting preparations, which contain the protoplast membranes, were concentrated by centrifugation and then layered on the sucrose gradients described under Methods. These were then spun at 20,000 rpm for 20 min. Two well-defined layers were observed. The lowest and heaviest consisted primarily of membranous material, showing a tendency to coil into ropy masses. The chief microscopic characteristic of the upper layer was a large number of small refractible beads (figure 18). The membranous layer was completely dissolved on exposure to lipase under conditions which lead to the
Figures 2 and 3. Phase contrast photomicrographs of unfixed protoplasts.

Figure 2. Freshly formed protoplasts immediately after transfer to succinate-citrate buffer showing contraction and visualization of nuclear body as a darker ring in the refractile cytoplasm.

Figure 3. Protoplasts which have re-expanded in succinate-citrate buffer. Nuclear regions are now less dense and barely discernible in the densely refractile cytoplasm.

Figures 4 and 5. Dark phase contrast photomicrographs of protoplasts which were suspended, osmium-fixed (1 per cent) and washed in succinate-citrate buffer. The striking increase in phase differentiation reveals a variety of compact arrangements of the nuclear regions. 5a and 5b and accompanying line drawing show two focal planes through the same protoplast (a) at the level of the nuclear body, (b) showing the beaded structures outside of the nuclear region.
dissolution of the protoplast membrane. Drops in optical density of suspensions of this layer on treatment with either trypsin or chymotrypsin indicates that it contains, in addition to lipid, a protein component. The upper layer, consisting of the refractile beads, showed no visible reaction to any enzymes tried which, in addition to those already mentioned, included ficin, papain and wheat germ lipase.

That the membranous layer is characteristic only of ruptured protoplasts was shown by subjecting to gradient analysis a nuclear body preparation disrupted by a 1 to 2 min exposure to sonic oscillation. Here the membranous layer of highest mass density is completely missing. One does find the refractile beaded layer and above it a very light layer of material, resistant to lipase. Unfortunately, it proved difficult to collect enough of the latter fraction by these procedures for an adequate chemical analysis.

In any event, these data show that the membranous, lipase sensitive, fraction found on rupturing whole protoplasts is undetectable by these procedures in nuclear preparations. Further, the fact that the membranous fraction is completely dissolved by lipase, leaving no perceivable residue, argues against the hypothesis which would seek to explain the visible bodies seen in lysates of protoplasts in terms of lipase resistant remnants of protoplast membranes.

Another method of obtaining information pertinent to the same issue was suggested by Dyar's (1947) observation that the cell membrane can be stained by congo red after treatment with cetyl pyridium chloride. Protoplasts were fixed with Bouin's fluid and stained according to Dyar's procedure. The protoplasts were then disrupted by a 1 to 2 min exposure to sonic oscillation in a 10 kc Raytheon oscillator and the resulting preparation examined microscopically. The characteristic vesicular bodies, 1 μ in size, were observed and had the appearance usual for them in phase contrast subsequent to fixation. These bodies were completely unstained and could be easily distinguished from surrounding clumps of brightly stained membranes. Since none of these bodies showed evidence of the dye applied to the protoplast membrane it seems unlikely that they are residues of the latter which survived the treatment.

Finally, recourse was had to immunological procedures to settle the question of the existence of the nuclear structures distinct from adventitious material surrounded by residual membranes. Nuclear bodies were isolated in the usual manner described and were washed twice with 10 per cent sodium chloride to remove the lipase which was found to be anticomplementary. The resulting preparations were subjected to complement fixation studies kindly performed by Drs. Vennes and Gerhardt of the University.
Figure 9. Phase contrast of protoplasts partly digested with lipase at pH 7.4. Formalin fixed to increase contrast. The poor phase contrast visibility of the escaping chromatin is indicated by arrows. The larger empty vesicles are probably "ghosts" formed from partly digested protoplasmic membranes.

Figures 10 and 11. Trichloracetic acid (TCA)-Feulgen stains of partly digested protoplasts showing chromatin streaming from the protoplasts and the attachment therein to the faintly Feulgen-positive nuclear cores. Figures 9 and 10 are from the same smear fixed in dilute formalin. Figure 11 is a high contrast print of a different preparation more thoroughly fixed with buffered formalin.

Figure 12. A group of nuclear cores bearing thin coatings of chromatin and lying in a pool of partly digested cytoplasm. Buffered formalin fixed, trichloracetic acid (TCA)-Feulgen (control is figure 7). Line drawings refer to figure 10.

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of Michigan. The nuclear preparations were tested against antiprotoplast serum and the results are summarized in table 5. Adsorption of the antiserum with purified protoplast membranes did not decrease the titer against the nuclear fraction. Adsorption with nuclear bodies lowered the titer against their fraction but had no effect on the ability of the antiserum to react with membrane preparations. These cross adsorption tests demonstrated unequivocally that protoplast membranes and the lipase resistant structures are immunologically distinct entities. We will here presume the further supporting evidence yet to be presented and refer to the vesicular structures from here on as nuclear bodies.

(4) Chemical composition of nuclear fractions:—It was of interest to determine the chemical composition of the purified nuclear fraction. The natural criterion to apply is the attainment of constant composition on repeated purification. Attempts at successive washing of these fractions runs into difficulties. The agitation required to suspend the pellets results either in complete disruption of the visible bodies or in a physical separation of the DNA from them so that the two no longer sediment together. While these complications are difficult to eliminate completely, they can be minimized by the exercise of some care and the proper choice of washing medium. The probable reason for the ease with which this separation is effected will become apparent when the detailed structure of these bodies is considered in the section on electron microscopy.

As a possible auxiliary aid, analyses for
protein were carried out using both the Folin and the ninhydrin methods. If the megasterium structures are like other nuclei, they would be expected to contain proteins analogous to protamines or histones at higher levels relative to the mass of cytoplasmic proteins. This would be reflected in an increase in the ratio of ninhydrin to Folin values as the purification progressed. A constancy in this ratio could then be taken as an added indication of purity.

While a variety of washing media were tried, we here detail in table 3 the results obtained with two, i.e., 0.5 M succinate and the succinate-citrate mixture. In both instances, the media for the lipase lysis were also used in the subsequent washings of the nuclear fractions. The fraction prepared and washed in 0.5 M succinate illustrates a persistent loss of DNA at each successive washing. While there is some loss of RNA and protein in the course of the 5 resuspensions and centrifugations, these are far less severe and are, furthermore, not selective for either one. As a consequence, by the third washing, the RNA to protein ratio attains a constant value. It will also be noted that the ninhydrin to Folin ratio of the protein in the nuclear fraction is considerably higher than for the whole protoplast and that it reaches a constant value by the second washing.

It is evident from the data in the lower half of table 3 that the succinate-citrate is a superior medium for the purification of the nuclei. There is only a 10 per cent loss of DNA in 3 washings and this is matched by about an equivalent loss in the other 2 components being analyzed. By the end of the first wash ratios among the 3 components, as well as between ninhydrin and Folin values, are established which are maintained in the subsequent purification cycles. Table 4 compares the relative compositions with respect to DNA, RNA and protein of protoplasts, initial nuclear pellets, and nuclear fractions purified by several cycles of centrifugation.

II. Cytological studies. The chemical and preliminary microscopic observations presented in the previous sections suggest the existence of a nuclear structure to which the DNA is attached. The succeeding paragraphs provide direct visual evidence for the existence of such structures and offer further detail on their microscopic appearance and the mode of their association with the Feulgen-positive chromatinic material. The cytological studies to be described include examinations of intact protoplasts, the observable events occurring in the course of a lipase digestion, and finally the nature and appearance of purified nuclear bodies.

(1) Cytology of protoplasts.—A. Intact protoplasts. Because of the superiority of the succinate-citrate medium in maintaining constant nuclear composition, it was chosen for most of the studies to be described.

When protoplasts are formed in the sucrose phosphate medium and are washed over into the succinate-citrate buffer at either pH 7.6 or 6.1 the protoplasts become more refractile in phase contrast (figure 2). After some storage in the succinate-citrate medium, they revert to their original size and refractility (figure 3). When introduced into the succinate-citrate buffer, the chromatin takes on the appearance of a dark localized zone in the refractile cytoplasm. After the protoplasts expand to their initial volume in the succinate-citrate medium, the nuclear regions are still localized but become phase light regions which are difficult to record in photographs (figure 3). However, marked improvement in contrast can be achieved by osmium fixation (figures 4 and 5). Here, the nuclear bodies appear to be composed of a phase light zone surrounding a central dense structure. It will be noted that these regions correspond in size and general shape

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vesicular Bodies in Per Cent of Original Protoplast Input</th>
<th>Per Cent of Sedimentable DNA</th>
<th>DNA/Body in Picograms (10^-12 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protoplast</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipase lysate</td>
<td>67</td>
<td>70</td>
<td>.070 ± .007</td>
</tr>
<tr>
<td>1 × washed</td>
<td>61</td>
<td>56</td>
<td>.074 ± .007</td>
</tr>
</tbody>
</table>
to the nuclear bodies which have already been discussed.

Highly refractile beads located peripherally in the cytoplasm may also be seen but these bear no fixed relationship to the nuclear bodies (figures 5a and 5b). An electron micrograph of a protoplast membrane partly digested by lipase (figure 18) also shows these beads. Lipid stains with Sudan Black B revealed that the central core of the nuclear region was not a fat inclusion. It might be noted in passing that KM, unlike other strains of *B. megaterium* are remarkably free of large lipid inclusions.

Figure 6 and 7 show the location of chromatinic material exhibited by nuclear staining of fixed protoplasts formed from cells rejuvenated as described by Landman and Spiegelman (1955). Greater amounts of DNA and larger nuclear bodies can be obtained by extending the period of rejuvenation. The results of nuclear stains on such preparations are exhibited in figure 8.

The chromatin material of protoplasts fixed in the succinate-citrate invariably surrounds a central achromatic region. This sort of nuclear arrangement is similar to that observed in other bacterial forms (Robinow, 1957).

B. Partial lipase digests. Figure 9 shows in phase contrast an early stage of lipase lysis which was interrupted by the addition of unbuffered formalin. The phase-light nuclear areas become almost invisible when released by the partial digestion of the protoplast membrane. The arrows in figure 9 point to escaping nuclear structures. The large faint vesicles seen are osmotically active ghosts formed early by partially digested membranes. These are subsequently dissolved away by continued lipase action. Other smaller vesicular bodies, about half the diameter of the protoplasts, make their appearance in increasing numbers during the course of this digestion. These structures are not in good focus in figure 9 and are shown more clearly in figure 13. They resist digestion with lipase and have a tendency to shrink during fixation and hydrolysis. Figure 10 is a photomicrograph of a subsequent nuclear stain on an area adjacent to the field of figure 9. Escaping Feulgen-positive chromatin is seen streaming off the faintly staining bodies. In the whole protoplasts these achromatic bodies are the central cores on which the chromatin appears to be laid. After liberation with lipase, these cores are

*Figures 16 and 17.* Dark phase contrast (figure 16) and Feulgen stain (figure 17) of gelatinous residue sedimented at 10,000 G from protoplasts suspension digested with lipase at pH 6.1 in succinate-citrate buffer. Newcomer fixed, HCl SO₄-Azure A.
Figures. 18-25
TABLE 3

Nucleic acid and protein content of nuclear fractions during the course of repeated washings

Numbers represent μg per 10 ml. The protoplasts were suspended to an optical density of 0.3 at 650 mλ, and subjected to lipase digestion in the medium indicated which was also used for washing. All pellets were collected by centrifugation for 5 min at 10,000 G and referred to as 10 G5P. All volumes were made up to the original protoplast volume so that the numbers are comparable to the initial input. Absolute protoplast values are referable only to succinate medium. Protoplast ratios are applicable to both media.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>DNA</th>
<th>RNA</th>
<th>Protein (Folin)</th>
<th>RNA/DNA</th>
<th>DNA/Protein</th>
<th>RNA/Protein</th>
<th>Ninhydrin/Folin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protoplasts</td>
<td>300</td>
<td>2150</td>
<td>6200</td>
<td>7.15</td>
<td>.048</td>
<td>.347</td>
<td>.27</td>
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**Succinate medium**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>DNA</th>
<th>RNA</th>
<th>Protein (Folin)</th>
<th>RNA/DNA</th>
<th>DNA/Protein</th>
<th>RNA/Protein</th>
<th>Ninhydrin/Folin</th>
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</thead>
<tbody>
<tr>
<td>10 G5P</td>
<td>250</td>
<td>450</td>
<td>1200</td>
<td>1.80</td>
<td>.208</td>
<td>.375</td>
<td>.36</td>
</tr>
<tr>
<td>10 G5P washed:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1X</td>
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<td>293</td>
<td>1050</td>
<td>1.27</td>
<td>.220</td>
<td>.279</td>
<td>.41</td>
</tr>
<tr>
<td>2X</td>
<td>174</td>
<td>320</td>
<td>750</td>
<td>1.84</td>
<td>.232</td>
<td>.405</td>
<td>.45</td>
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<tr>
<td>3X</td>
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<td>5X</td>
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<td>285</td>
<td>600</td>
<td>7.12</td>
<td>.067</td>
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**Succinate-citrate medium**

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<thead>
<tr>
<th>Fraction</th>
<th>DNA</th>
<th>RNA</th>
<th>Protein (Folin)</th>
<th>RNA/DNA</th>
<th>DNA/Protein</th>
<th>RNA/Protein</th>
<th>Ninhydrin/Folin</th>
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<td>10 G5P</td>
<td>155</td>
<td>293</td>
<td>888</td>
<td>1.89</td>
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<td>10 G5P washed:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1X</td>
<td>151</td>
<td>162</td>
<td>490</td>
<td>1.07</td>
<td>.309</td>
<td>.331</td>
<td>.36</td>
</tr>
<tr>
<td>2X</td>
<td>142</td>
<td>145</td>
<td>450</td>
<td>1.02</td>
<td>.315</td>
<td>.321</td>
<td>.45</td>
</tr>
<tr>
<td>3X</td>
<td>140</td>
<td>149</td>
<td>460</td>
<td>1.06</td>
<td>.304</td>
<td>.324</td>
<td>—</td>
</tr>
</tbody>
</table>

found with various amounts of adherent chromatin. Line drawings of parts of figure 10 have been appended to the plate to augment the low contrast photomicrographs. Figure 11 is a similar residue from an analogous experiment with a pellet which was fixed with buffered formalin. Some free nuclear cores, slightly Feulgen-positive, are seen scattered in the background of figure 11. Figure 12 shows another group of such liberated bodies which, according to their more intense Feulgen reaction, possess a fuller content of DNA.

C. Feulgen stains on complete lipase digests and isolated nuclear fractions. Phase contrast photomicrographs of complete lipase digests of protoplasts are grouped in figure 13. Such prep-

*Figures 18-25.* Electron micrographs of uranium shadowed material from lipase digested protoplasts. Material was osmium fixed to grids by the procedure described in Methods. Magnification as indicated. Figures 23 and 24 are at the same magnification as figure 21.

*Figure 18.* Protoplast membrane with some adhering cytoplasm and dense beads showing an early effect of lipase digestion.

*Figures 19-21.* Chromatin gel at two different magnifications from the same gelatinous layer after lipase digestion at pH 7.4.

*Figure 22.* A structure often seen in nuclear preparations liberated by lipase at pH 7.4. Magnification as shown in figure 21.

*Figures 23, 24, 24 and 25.* Material from nuclear fraction liberated by lipase at pH 6.1 to 6.2.

*Figure 20.* A strand of chromatin attached to a more dense mass of lipase resistant material—the nuclear core (see also figure 10).

*Figures 22-24.* Liberated chromatin bodies in various stages of organization; figure 22 a nuclear core largely free of chromatin; figure 24 presumably a large strand of chromatin material winding off a core. Magnification as shown in figure 21.

*Figure 25.* A small rounded accumulation of fibrous material found in well organized preparations of liberated nuclear bodies.
TABLE 4
Ratios of nucleic acid components in protoplasts
initial, and purified nuclear fractions

The numbers are representative values found in experiments employing the succinate, and the
succinate-citrate medium in washings to constant
composition. DNA content is taken as unity and
the numbers are rounded out to the nearest integer. The initial pellet is the first 10 GSP pellet
from the lipase lysate. The purified fraction has
gone through at least 2 washings.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>DNA</th>
<th>RNA</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protoplasts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial nuclear pellets</td>
<td>1</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Purified nuclear fractions</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

TABLE 5
Complement fixation titer of antiproteoplast serum
(APS) with protoplast membrane and
nuclear bodies

The preparation of antiprot plast serum
(APS) and protoplast membranes are as described
in Vennes and Gerhardt (1956). Nuclear bodies
were prepared in succinate-citrate medium and
purified by several centrifugations. This was
followed by two washes with 10 per cent NaCl to
remove contaminating lipase which interfered
with the serological tests.

<table>
<thead>
<tr>
<th>APS × protoplast membrane</th>
<th>Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS × nuclear bodies</td>
<td>1:1280</td>
</tr>
<tr>
<td>APS adsorbed with protoplast membrane</td>
<td>1:1280</td>
</tr>
<tr>
<td>APS adsorbed with protoplast membrane × nuclear bodies</td>
<td>1:40</td>
</tr>
<tr>
<td>APS adsorbed with nuclear bodies × protoplast membrane</td>
<td>1:1280</td>
</tr>
<tr>
<td>APS adsorbed with nuclear bodies × nuclear bodies</td>
<td>1:1280</td>
</tr>
</tbody>
</table>

The preceding observations on intact protoplasts, partially digested preparations, and isolated nuclear fractions suggest certain conclusions about the structural arrangement of the protoplast nucleus. The nuclear structure of an intact succinate-citrate stabilized protoplast consists of a phase-light zone containing a delicate phase
dense body or core. On liberation from the digested protoplast the nuclear body is detected in phase contrast microscopy only by its core; the
mass of the structure is a phase invisible gel. The latter can be identified as chromatinic material
by suitable stains. That the chromatin is attached to the central body is further supported by evidence obtained from electron microscopy to be detailed below.

(2) Electron microscopy:—Partial lipase digests as well as purified nuclear fractions were subjected to electron microscopic observation. The partial digests were contaminated with protoplast membranes in various states of disorganization (figure 18). The electron scattering of these membranes and any undigested membranes make up the bulk of the bottom yellow pellets of digested preparations previously referred to (table 1). These yellow fractions and associated beads correspond presumably to the particles found by others in combination with the fraction containing the cytochrome oxidase activity of the bacterial cells (Georgi et al., 1955; Mitchell and Moyle, 1956).

The electron micrographs of purified nuclear fractions show the presence of two types of material intimately connected with each other. One is fibrous strands of low electron density in various stages of dispersion and the other structures of approximately 1 μ in size composed of more dense material, (figures 19, 20, and 22).

Electron microscopy and the chemical data reveal that the fiber strands make up the phase transparent gel and correspond to the DNA. The more dense plaques and discs seen by electron microscopy are identifiable as the delicate phase dense central cores in whole fixed protoplasts as well as the bodies seen in the nuclear fractions at pH 7.4 (figures 13 and 23), and at pH 6.1 (figures 16, 20, 22, and 24).

The electron transparent chromatin can be clearly seen in shadowed grids. It tends to spread out as a major strand some 400 A in diameter (figures 20–22) and is always found associated with an electron dense central body. This strand is more continuous in preparations made at pH 6.1 than those prepared at pH 7.4. At the higher pH subfibrils tended to form a netlike pattern (figure 19).

DNase digestion (40 μg/ml 30 min at 37 C) of suspension of such nuclear preparations resulted in a marked loss of their viscosity. In the dark-phase microscope there was no change in the phase visible bodies or cores. Examination of the DNase digested residues in the electron microscope revealed either an absence of fibrous structures or their replacement by short lengths of fibers. Further electron microscopic studies of protoplasts and isolated fractions will be published separately.

**DISCUSSION**

The chemical and cytological evidence discussed in the present paper indicate that the enzymatic dissolution of protoplasm membranes releases a pre-existent nuclear structure which can be isolated and purified by several cycles of low speed centrifugation in a suitable medium. The data accumulated show that the nuclear apparatus is a bipartite structure consisting of a core and chromatin. On liberation these two remain closely associated with each other. They can be readily isolated together and differentiated from other components of the protoplasts.

When the central bodies or cores are freed of chromatin, these objects remain discrete as has been noted in electron photomicrographs of them. This indicates that they are not, as might perhaps be supposed, occluded cytoplasm or cytoplasm modified by close association with chromatinic material. The data suggest rather that they are independent structures. On initial isolation, they are in general found as relatively phase contrasting and electron dense bodies within a chromatinic mass which is phase light and relatively electron transparent. Materials with similar electron properties as these are evident in the electron micrographs of whole cells of *Escherichia coli* (Kellenberger, 1953) and thin sections of cells of *Salmonella typhimurium* possessing aggregated nuclear bodies (Maaløe and Birch-Anderson, 1956). In these sections, the nuclear cores appear as well outlined closed systems and are considered to be the site of DNA. However, the cytological and chemical studies presented here of isolated nuclear bodies have disclosed that similar centrally located material gives rise to discrete cores which do not contain the DNA enclosed within them. Rather this important biological polymer makes up the outer less dense zone of such aggregated bacterial nuclear bodies. In agreement with previous work, the chromatin was found not bounded by a membrane and thus on liberation it may stream off the core as a strand some 10 to 20 times longer than the original cell. This explains the ease with which the DNA can be
physically separated from the structure to which it is initially attached.

The presence of a core or nonchromatinic center of the nuclear bodies is in agreement with the observations of nuclear structures in disrupted and sectioned spores (Fitz-James, 1953; Robinow, 1953a, 1953b) and in young vegetative cells of Bacillus cereus—following methanol-formalin fixation (Murray, 1953). The arrangement of chromatin on the surface of a central body has also been observed in the vegetative nuclei of several fungi (Pontefract, 1956; Robinow and Bakerspigil, personal communication).

There remains, however, some uncertainty among bacterial cytologists regarding the detailed nature and chemical composition of the nuclear cores (Robinow, 1956, 1957). The data reported in the present paper on the analysis of repeatedly washed nuclei suggest that the core is composed principally of ribonucleoprotein, the protein being comparatively poor in aromatic amino acids.

DeLamater and Minnsavage (1957) have briefly reported on a method for the liberation of nuclear bodies from B. megaterium by a procedure involving treatment of cells with sodium deoxycholate. When the relevant data are made available in detail, it will be interesting to compare the chemical composition and structural details of the bodies liberated by this procedure with the purified preparations described in the present paper.

ACKNOWLEDGMENTS

We are grateful to Dr. C. F. Robinow for the use of his optical equipment for the light photomicrographs. We should also like to thank Drs. J. W. Vennes and P. Gerhardt for running the complement fixation tests on material sent to them.

SUMMARY

Lipase digestion of protoplasts of Bacillus megaterium leads to the liberation of material which has been shown in the course of the present investigation to be composed of the nuclear bodies of the protoplast. These bodies can be collected by 5 min centrifugation at 10,000 G. Chemical, immunological and density gradient fractionation data are provided demonstrating that the nuclear structures are distinct from residues of the protoplast membranes.

The released nuclear body is similar in all respects to that which pre-exists in the protoplast. It was found to be a bipartite structure composed of a nonchromatinic center or core, visible in dark phase contrast, on which the chromatin, invisible by phase microscopy, lies. Chemical analyses of purified nuclear bodies indicate that they are composed of deoxyribonucleic acid, ribonucleic acid and protein in a ratio of 1:1:3.

REFERENCES


MURRAY, R. G. E. 1953 Symposium on bacterial


**Pontefract, R. D.** 1956 Structure and mode of division of nuclei in vegetative hyphae of penicillium. M.Sc. thesis. Faculty of Medicine, University of Western Ontario, London, Canada.


**Spiegelman, S.** 1957 *The chemical basis of heredity*. Johns Hopkins Press, Baltimore.
