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Synthetic Capabilities of Plasmolyzed Cells and Spheroplasts of *Escherichia coli*

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Effects of plasmolysis and spheroplast formation on deoxyribonucleic acid (DNA), ribonucleic acid (RNA), protein, and phospholipid synthesis by *Escherichia coli* strain THU were studied. RNA and protein synthesis were severely diminished. DNA and phospholipid synthesis were inhibited, but less so; they could be partly restored. DNA synthesis could be restored by replacing thymine in the medium with thymidine, and phospholipid synthesis, by adding back small quantities of soluble cell extract. Plasmolysis effected marked reductions in rates of growth and macromolecule synthesis, and temporarily reduced culture viability. Plasmolysis also caused an anomalous stimulation of phospholipid synthesis. Spheroplasts and plasmolyzed cells synthesized small amounts of ribosomal RNA that sedimented normally. However, this ribosomal RNA was very inefficiently packaged to ribosome subunits. Spheroplasts were unable to carry out induced synthesis of β-galactosidase, and plasmolyzed cells were delayed in this function. Radioautographs examined in an electron microscope showed that DNA synthesis in plasmolyzed cells and spheroplasts was performed by a substantial fraction of the culture populations. That DNA and membrane were associated in the spheroplasts used in this study was suggested by formation of M-bands containing membrane and most of the cell's DNA. The results are discussed in terms of alterations of membrane structure and conformation attending plasmolysis and spheroplasting.

Since it has been proposed that the bacterial chromosome is closely associated with the bacterial membrane, we wished to determine whether a complex of both could be obtained in a presumably native state, as has been reported by several workers (5, 16, and references cited therein). To do so, we hoped to lyse cells gently and obtain deoxyribonucleic acid (DNA) membrane complexes. In considering the use of *Escherichia coli* spheroplasts prepared with ethylenediaminetetraacetate (EDTA) and lysozyme for this purpose, we first sought to determine whether spheroplasts were able to carry on appreciable macromolecular synthesis or whether they were possibly so damaged in precisely the structure we wished to examine as to no longer resemble living cells.

In spite of the widespread use of EDTA-lysozyme spheroplasts, no clear answer to this question exists. They can apparently synthesize some protein (10, 11), and they have been reported to be able to produce some phage when infected with free nucleic acid (14). However, the latter might be imagined to have established a new membrane-chromosome association. Penicillin spheroplasts of *E. coli*, which (in contrast to those prepared with EDTA-lysozyme) can revert to normal, have been reported (6) to synthesize ribonucleic acid (RNA) at a rate one-half to one-quarter that of exponentially growing cells. Some other organisms, e.g., gram-positive bacteria, seem less affected by spheroplasting or protoplasting than do *E. coli* cells. Several examples have been given. Protoplasts of *Bacillus megaterium* retain significant capability for synthesis of biological macromolecules (10), and Cundliffe has obtained a preparation that is highly active metabolically (4). Lark and Lark (9) reported that cultures of *Alcoligenes faecalis* to which penicillin had been added continued to grow exponentially at a somewhat reduced rate in a rich medium. However, in a minimal medium growth slowed to a halt.

In this study, we have compared the abilities of intact *E. coli* cells and spheroplasts to synthesize nucleic acids, protein, and phospholipid. The data show that these processes are all diminished, but to different degrees. The results are discussed with regard to identifying the lesion(s) resulting from spheroplasting.

Of necessity, osmotically sensitive spheroplasts

must be prepared and maintained in a hypertonic medium. For this purpose, we used 20% sucrose solution. Suspending gram-negative bacteria such as *E. coli* in this hypertonic medium caused the cells to become plasmolyzed. The protoplast, i.e., the cytoplasmic membrane and its contents, could be seen to shrink inward from the outer layers of the envelope, which maintained their original capsular shape (2). The effect was temporary, and after 0.5 hr in 20% sucrose the cells were no longer visibly altered. As will be shown, plasmolysis has physiological as well as structural consequences.

MATERIALS AND METHODS

Chemicals. Uracil-*2-*^{14}C, thymine-*2-*^{14}C, L-histidine-*2-*^{14}C, isopropyl-β-D-thiogalactopyranoside, *O*-nitrophenyl-β-D-galactopyranoside, and lysozyme (A grade) were purchased from Calbiochem; uridine-*6-*^{3}H and thymidine-*methyl-*^{3}H, from New England Nuclear Corp.; carrier-free ^{32}P-orthophosphate ($^{32}P_i$) from R. E. Squibb and Sons; ribonuclease-free sucrose, from Mann Research Laboratories; EDTA, disodium salt, from J. T. Baker Chemical Co.; sodium *N*-lauroylsarcosinate (referred to in the text as Sarkosyl), from K & K Laboratories; and ribonuclease-free deoxyribonuclease, from Worthington Biochemical Corp.

Bacteria and media. Two strains of *E. coli*, 15 THU and K-12, were used in these experiments. The derivation of strain 15 THU has been described (15). Both strains were grown on a synthetic medium, 52, previously described (15), supplemented with 1 mg of glucose/ml. In addition, THU requires thymine, histidine, and uracil, which were supplied in concentrations of 10, 20, and 10 μg/ml, respectively. For $^{32}P_i$-uptake experiments, THU was grown in a low-phosphate medium (*p*H 8.0) consisting of 10 g of tris(hydroxymethyl)aminomethane (Tris), 2 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.5 mg of $FeSO_4 \cdot 7H_2O$, 93 mg of Na_2HPO_4, 20 mg of $CaCl_2$, and 0.2 g of KCl per liter. This medium has a P_i concentration of 0.66 mM compared with 128 mM for the high-phosphate medium. THU grew at the same rate (72-min generation time) in either medium. Viable counts were made after plating on nutrient agar.

Preparation of spheroplasts and incorporation studies. For incorporation studies, cells were grown to a density of 2×10^8 per ml and were harvested by centrifugation in the cold. The cells were washed with one-tenth the initial volume of 0.01 M Tris chloride, *p*H 8.0. A portion was resuspended in growth medium and set aside for an intact-cell control. The remainder was resuspended in 20% sucrose-0.03 M Tris, *p*H 8.0, at a density of 2×10^9 cells per ml. A portion of this suspension was set aside for the plasmolyzed-cell control. The remainder was incubated for 10 min at ambient temperature with 10 μg of lysozyme per ml and 0.001 M EDTA. During this time, the optical density at 450 nm of samples diluted 20 times with deionized water fell by about 90%. Viability decreased to <1% of the initial value. The suspensions of plasmolyzed cells and spheroplasts were pelleted and resuspended in growth medium-20% sucrose at a density of 2×10^9 cells per ml, and the resulting suspensions, along with that of the intact control cells, were inoculated into culture media containing the appropriate radioactive materials. Aeration was accomplished by swirling the cultures in cotton-plugged Erlenmeyer flasks at 75 rev/min. Samples were removed at intervals and added to equal volumes of cold 10% trichloroacetic acid. Precipitates were collected on membrane filters and counted in a liquid scintillation spectrometer. For measurements of phospholipid synthesis, the extraction method of Ames was employed (1).

RNA and ribosome gradients. Control cells, plasmolyzed cells, and spheroplasts (400 ml of each at a density of 2×10^8 cells per ml) were prepared as above and allowed to incorporate uracil-*2-*^{14}C (1 μCi/μmole) for 20 min. Cells were sedimented and washed with 40 ml of 5×10^{-3} M Tris chloride-10^{-4} M $MgAc_2$ (containing 20% sucrose in the cases of plasmolyzed cells and spheroplasts). The pellets were ground with 2.5 times their weight of alumina, and the pastes were extracted with 5 ml of the above Tris-Mg buffer per g (wet weight) of cells. Deoxyribonuclease was added (5 μg/ml), and the mixtures were centrifuged twice at 19,000 × *g* for 20 min. Portions of the supernatants were set aside for ribosome gradients, and RNA was prepared from the remaining portions by shaking them vigorously with 0.5% sodium dodecyl sulfate for 20 min. Six to eight optical density units (260 nm) of each RNA and ribosome preparation was layered onto 4.4-ml linear sucrose density gradients (4 to 20% sucrose; 5×10^{-3} M in Tris, 10^{-4} M in $MgAc_2$). Gradients were centrifuged at 38,000 rev/min (3 hr for ribosomes; 6 hr for RNA) in an SW39 rotor at 4 C. Tubes were pierced and 10-drop fractions were collected. These were diluted to 1 ml with water, and the optical density at 260 nm was measured. One drop of 1% RNA was added to each fraction. Trichloroacetic acid precipitates were prepared, collected, and counted as described above.

Preparation of cell-free extract. Cells (1 g, wet weight) were suspended in 5.5 ml of 0.1 M Tris chloride, *p*H 7.4, and broken with a French pressure cell. The suspension was centrifuged for 1 hr at 105,000 × *g* at 4 C. The supernatatant containing 9 mg of protein per ml was used without further treatment.

β-Galactosidase assay. Cultures were incubated with 1 mM isopropyl-β-D-thiogalactopyranoside. Samples of 1 ml were shaken with two drops of toluene and incubated at 37 C for 30 min. The samples were assayed for β-galactosidase by a published procedure (13).

M-band experiments. The method of Tremblay et al. (16) was employed.

Radioautography. Control cells, plasmolyzed cells, and spheroplasts were allowed to incorporate thymidine-*methyl-*^{3}H (4 μCi/ml of culture) for 30 min. Samples (5 ml) of each culture were fixed for 3 hr at 0 C by addition of 2 ml of 2% glutaraldehyde in 0.1 M phosphate buffer, *p*H 6.5. The cells were pelleted and washed twice with Ryter-Kellenberger buffer (8). The pellets were postfixed by overnight treatment with 1 ml of 2% OsO_4 in Ryter-Kellenberger buffer at room temperature, and were washed twice with the buffer.

They were suspended in agar, and the agar casts were dehydrated first by treatment with a mixture containing 70 volumes of 2% uranyl acetate and 30 volumes of ethyl alcohol, and then by successive washings with 50, 75, 95, and 100% ethyl alcohol. The castings were then embedded in Araldite, and 0.1-μm sections were cut. Sections were coated with Ilford L-4 emulsion, exposed for various periods (6 to 12 weeks), developed in Microdol-X, and stained with dilute lead hydroxide. They were examined in a Siemens electron microscope. Control experiments showed that the silver grain distribution remained unaltered when cells were extracted with cold 5% trichloroacetic acid.

RESULTS

Effects of plasmolysis and spheroplasting on macromolecular synthesis. Most of the data to be presented derive from experiments with *E. coli* strain THU, which requires thymine, histidine, and uracil (one precursor each for DNA, protein, and RNA synthesis). Thus, measurements of rates of macromolecule synthesis are free from the complications of endogenous precursor synthesis. In one experiment, strain K-12 was used. The kinetics of DNA, protein, and RNA synthesis were measured in a spheroplast culture and two control cultures (Fig. 1). In one control, intact cells were incubated in their normal growth medium; in the other, referred to as a plasmolyzed culture, the cells were treated in the same way as the spheroplasts except for lysozyme-EDTA treatment (see Materials and Methods). The dashed lines in Figure 1 represent corrections of the intact-cell control curves for multiplication; they are drawn to aid comparisons between the intact-cell and spheroplast (nondividing) curves. The data show that both plasmolysis and spheroplasting substantially retarded macromolecule synthesis. Rates of DNA, protein, and RNA synthesis by spheroplasts were about 20, 15, and 5%, respectively, of the intact-cell control rates (corrected for division). Lack of data on the actual multiplication rate of plasmolyzed cells (see below) complicates comparison of per cell synthetic rates, but at 20 min, before substantial multiplication had occurred, spheroplasts synthesized DNA, protein, and RNA at 100, 25, and 50%, respectively, of the plasmolyzed control rates. We have performed comparable experiments with *E. coli* strains B, K-12, TAUst, and TAUrel, and have obtained similar results. Therefore, the data of Fig. 1 are not peculiar to a particular strain.

Effect of plasmolysis on viability and turbidity. Plasmolysis also had substantial effects on viability, i.e., ability to generate colonies when plated on a solid medium, and turbidity (Fig. 2). In this experiment, equal numbers of cells were inoculated into media differing only in that one was 20% in sucrose. Thus, plasmolysis caused an immediate 50% drop in viability. After a further

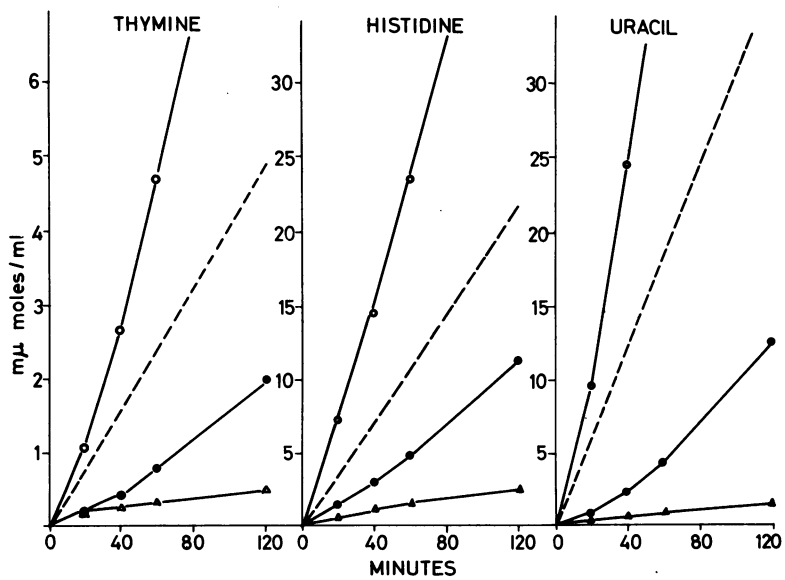

FIG. 1. *DNA, RNA, and protein synthesis by intact cells, plasmolyzed cells, and spheroplasts of E. coli strain THU. Incorporation of carbon-14 labeled thymine, histidine, and uracil (each 1 μCi per μmole) in cultures of intact cells (○), plasmolyzed cells (●), and spheroplasts (△) was measured. The dashed lines represent corrections of the intact-cell curves for growth. Initially each culture contained 2 × 10^8 bacteria per ml. Other details are given in Materials and Methods.*

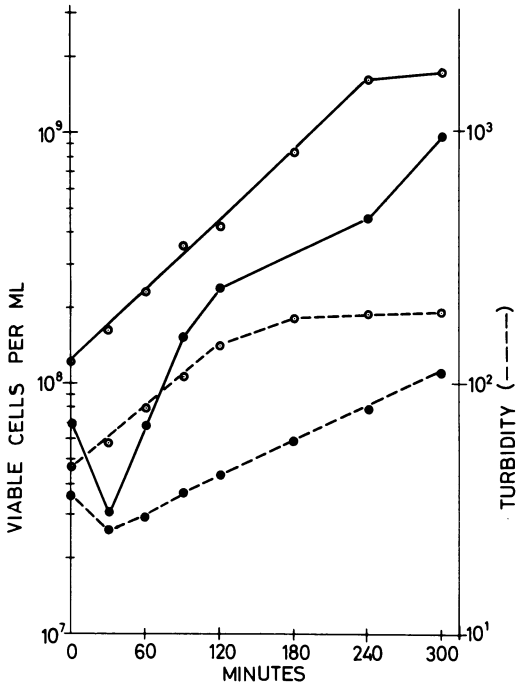

FIG. 2. *Effects of plasmolysis on growth. Viability (solid lines) and turbidity (dashed lines) of control* (○) *and plasmolyzed* (●) *cell cultures of E. coli THU. A suspension of washed cells was divided in half; one part was resuspended in growth medium containing 20% sucrose. The former was immediately inoculated into a complete culture medium; the latter, into complete medium containing 20% sucrose, both at a concentration of 1.2 × 10⁸ cells per ml.*

decrease, there was an abrupt recovery of viability, amounting to a 10-fold increase in slightly more than one generation time for the control. This indicates that division was actually occurring during the period of decreased viability. Plasmolysis, then, exerts its effect on the cell's ability to divide on solid, but not in liquid, media. Plasmolysis also causes an initial drop in turbidity, presumably owing to the formation of a vacuole. The logarithmic portion of the turbidity curve for the plasmolyzed culture corresponds to a generation time of 130 min, about twice the normal value.

Sucrose density gradient analysis of RNA synthesized by plasmolyzed cells and spheroplasts. Intact and plasmolyzed cells and spheroplasts were incubated for 20 min in a complete medium containing radioactive uracil. Radioactivity and optical density profiles of RNA and ribosome preparations are shown in Fig. 3 and 4. In two experiments with THU, the ratio of the total 23*S* RNA to 16*S* RNA was low in the controls but not in the plasmolyzed or spheroplasted preparations. However, in each instance, the radioactivity profile followed closely the optical density profile. Thus, the RNA made by both plasmolyzed cells and spheroplasts has the same sedimentation properties as normal ribosomal RNA. Packaging of even this little RNA into ribosome subunits is apparently blocked (Fig. 4) in both plasmolyzed cells and spheroplasts. In both cases, most of the radioactivity sedimented in a broad band between the 30*S* position and the top of the tube.

We next sought to determine whether significant degradation of RNA was occurring in spheroplasts. Cells in logirithmic growth were prelabeled by growing them in medium containing radioactive uracil for one generation, after which they were grown for another half generation in excess cold uracil to chase label from rapidly de-

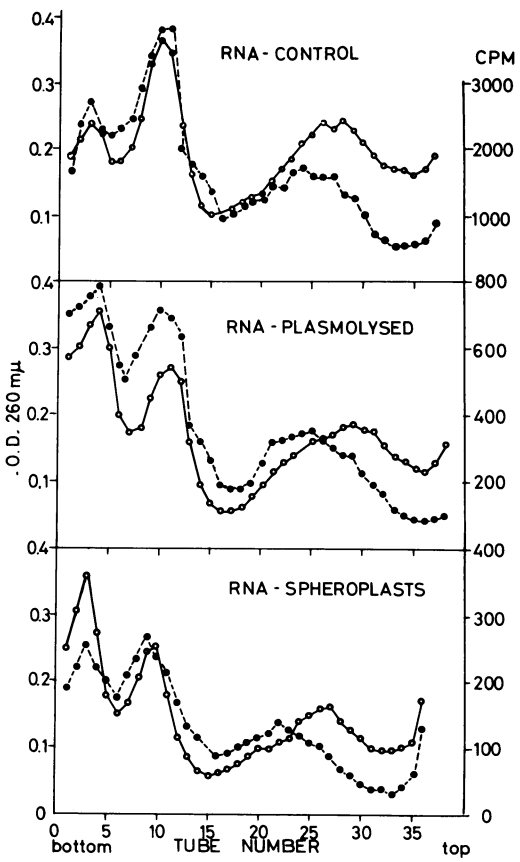

FIG. 3. *Sucrose gradient centrifugations from RNA preparations of control cells, plasmolyzed cells, and spheroplasts of E. coli THU. Bacteria were allowed to incorporate radioactive uracil (1 μCi/μmole) for 20 min.* (○) *Optical density at 260 nm;* (●) *trichloroacetic acid-precipitable radioactive material. The peaks of ribosomal RNA are at the left of the gradients; the first is 23S ribosomal RNA, the second 16S.*

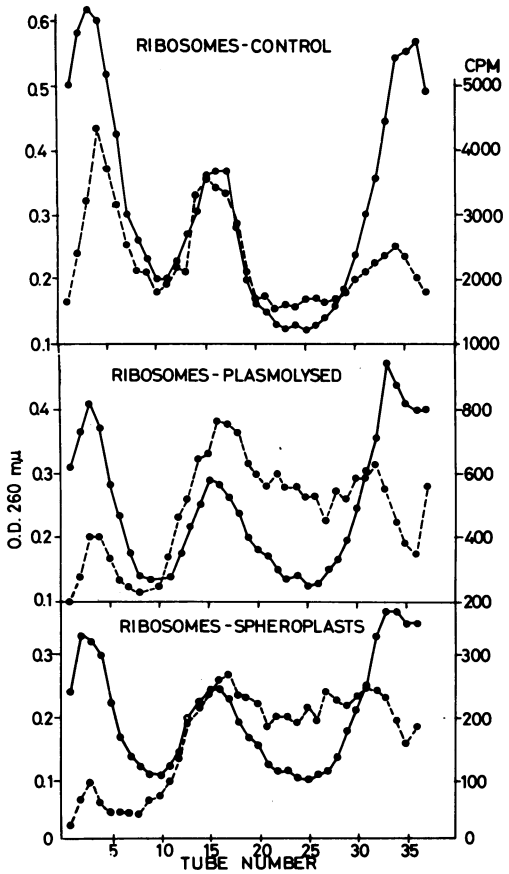

FIG. 4. *Sucrose gradient centrifugations from cell extracts of control cells, plasmolyzed cells, and spheroplasts of E. coli THU. Bacteria were allowed to incorporate radioactive uracil (1 μCi/μmole) for 20 min. (○) Optical density at 260 nm; (●) trichloroacetic acid-precipitable radioactive material. The peaks of ribosome subunits are in the left-hand portion of the gradient; the first is the 50S subunit, the second 30S.*

graded RNA species. Degradation of RNA was monitored by taking cold trichloroacetic acid-insoluble fractions and determining alkali-soluble counts. It was found that, during incubation of the cells with EDTA and lysozyme, 15% of the radioactivity originally present in RNA was lost. During 20 min of incubation of the spheroplasts in growth medium, 10% of the original radioactivity was lost. This demonstrates that the low rate of RNA synthesis observed in spheroplast cultures is not in large measure due to rapid degradation of RNA.

Inducibility for β-galactosidase synthesis. Inducibility of spheroplasts and plasmolyzed cells for β-galactosidase synthesis was also studied (Fig. 5). The inducer was added at zero time; samples were taken at intervals, treated with toluene, and assayed for β-galactosidase. Spheroplasts were unable to synthesize appreciable quantities of the enzyme. Plasmolysis delayed the appearance of enzyme by about 0.5 hr.

Differential effects of thymine and thymidine on DNA synthesis. It is highly probable that nucleoside pools are depleted during spheroplasting, because it is known that Tris-EDTA treatment alone causes leakage of bases and nucleosides (12). In that case, availability of deoxyribose and ribose donors might limit the rates of nucleic acid synthesis. Consequently, thymine and thymidine were compared as precursors for DNA synthesis (Fig. 6). There was no effect on the intact cell control (not shown). Substantial effects, however, were observed in the cultures of plasmolyzed cells and spheroplasts. In each instance, there was an initial enhancement of the rate of DNA synthesis by thymidine, after which the rates for thymine and thymidine incorporation were equal. For spheroplasts, there was a sixfold enhancement, and for 15 min they synthesized DNA at 60% of the intact-cell rate. These data tend to support the

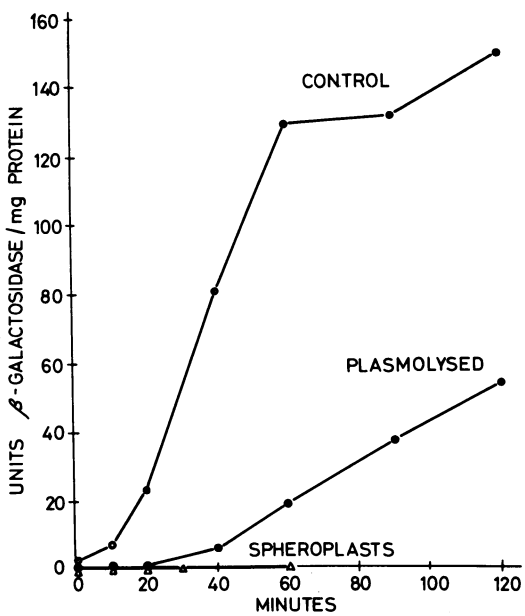

FIG. 5. *Inducible β-galactosidase synthesis in control cells, plasmolyzed cells, and spheroplasts of E. coli K-12. The technique is described in Materials and Methods. The curves for control (○) and plasmolyzed cells (●) refer to cultures prepared as described in the caption to Fig. 2. The spheroplast curve (△) derives from a separate experiment. Equal numbers of cells were present initially in each case (1.5 × 10⁸ per ml). The enzyme unit is defined as that amount of substrate that will hydrolyze 0.01 μmole of substrate per min.*

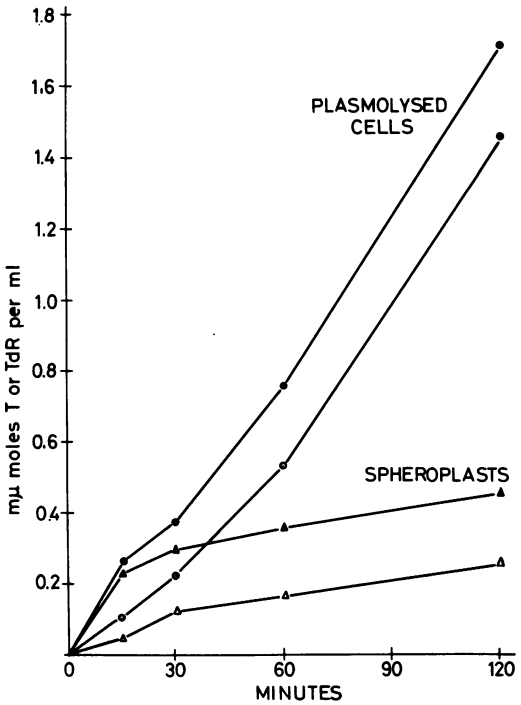

FIG. 6. *Comparison of thymine and thymidine as precursors for DNA synthesis by plasmolyzed cells and spheroplasts. Uptakes of radioactive thymine (closed symbols; 0.58 nmoles per 10^3 counts per min) and thymidine (open symbols; 1.40 nmoles per 10^3 counts per min), both 7.2 μmoles per ml of culture, by plasmolyzed cells (●, ○) and spheroplasts (▲, △) of E. coli THU were measured. The initial cell concentration in each culture was 2.5 × 10^8 per ml.*

belief that leakage of metabolites is at least in part responsible for the reduced synthetic capabilities of spheroplasts and, it would seem, of plasmolyzed cells. However, replacement of uracil by uridine had no appreciable effect on RNA synthesis in either spheroplasts or plasmolyzed cells.

Phospholipid synthesis. Since spheroplasting and plasmolysis exert a primary effect on the cell periphery, it was of interest to determine their effects on phospholipid synthesis. The latter was measured as conversion of radioactive phosphate to material that was soluble in chloroform-methanol. Phospholipid synthesis in intact cells, plasmolyzed cells, and spheroplasts is shown in Fig. 7a, in which the dashed line represents a correction of the intact-cell curve for multiplication. The rate of phospholipid synthesis for the spheroplast culture was about one-third of the intact control rate. Thus, spheroplasts retain the capacity to synthesize substantial amounts of phospholipid. The effect of plasmolysis on phospholipid synthesis was unexpected and somewhat dramatic. After the first hour of growth, during which the plasmolyzed-cell rate lagged behind the control, plasmolyzed cells actually synthesized phospholipid at a rate greater than that for the intact control cells. This is so despite the fact that the plasmolyzed cells multiply and synthesize macromolecules more slowly than the control.

Additionally, it was found that phospholipid synthesis in spheroplasts could be stimulated by adding small amounts of a crude, undialyzed, soluble cell extract (Fig. 7b and c). Adding extract inhibited phospholipid synthesis in intact control cells (Fig. 7b), but stimulated it in spheroplasts to about 50% of the control rate (Fig. 7c). The stimulation was roughly proportional to the amount of extract added. The largest amount of extract added derives from only twice the number of cells present in the spheroplast culture. The extract had no effect on plasmolyzed cells or on nucleic acid synthesis in spheroplasts. Identification of the stimulating substance awaits further investigation.

The effects of various additions to the medium on RNA synthesis by spheroplasts were studied. It was shown initially that spheroplasts synthesize RNA at 6% of the control rate. Addition of 1 mM spermidine doubled that rate. Smaller stimulations were noted (about 20%) when purine ribonucleosides were added and when the potassium ion content of the medium was increased 10-fold.

DNA-membrane association. The data presented thus far have no direct bearing on the question of whether spheroplasting disrupts the presumptive DNA-membrane association. If such an association is required for DNA synthesis to occur, then this association must remain intact, because spheroplasts do synthesize substantial quantities of DNA. Schaechter and his co-workers (5, 16) recently presented strong evidence for an intact DNA-membrane association in protoplasts of *B. megaterium* and spheroplasts of *E. coli*. Since they used a different stain of *E. coli* and a somewhat different method for preparing spheroplasts, we have repeated their experiments. In the so-called M-band technique, a spheroplast suspension to which Mg^{++} has been added is layered onto a sucrose density gradient. The spheroplasts are then lysed with the detergent Sarkosyl. Crystals of magnesium Sarkosyl form and, by virtue of possessing hydrophobic surfaces, combine with membrane fragments. The crystals are then banded at their buoyant density. The resulting M-band is found to contain most of the cell's DNA and a substantial portion of the phospholipid. Since the magnesium Sarkosyl crystals do bind membrane constituents but not DNA alone, it was concluded that DNA is present in the M-band by virtue of its association with the mem-

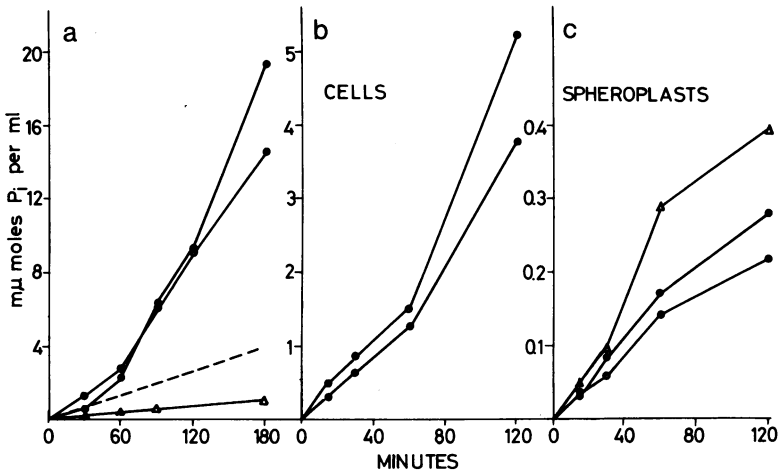

FIG. 7a. *Phospholipid synthesis by intact cells, plasmolyzed cells, and spheroplasts. Uptake of radioactive phosphate (3.4 × 10⁶ counts per min per ml of culture) into phospholipid by intact cells (○), plasmolyzed cells (●), and spheroplasts (△) of E. coli THU as described in Materials and Methods. The dashed line represents a correction of the intact-cell curve for growth. The initial cell concentration in each case was 1.7 × 10⁸ per ml.*

FIG. 7b and c. *Effect of soluble cell extract on phospholipid synthesis. Phospholipid synthesis was measured in cultures of control cells (b) and spheroplasts (c) of E. coli THU to which had been added soluble cell extract (○, none; ●, 7.5 μliters per ml; △, 25 μliters per ml). The extract had 9.9 mg of protein per ml. Initial cell concentrations were 1.5 × 10⁸ per ml. The radioactive phosphate had 3.76 × 10⁵ counts per min per μmole.*

brane. A compendium of our results from eight experiments is shown in Table 1. When labeled T5 phage DNA was added to the top layer, either before or after lysis, less than 1% of the label appeared in the M-band. The presence of DNA in the M-band was found not to depend on the presence of Mg^{2+} in the lysis medium. Magnesium ions could be added after lysis without affecting the quantity of DNA found in the M-band.

We would like to report another method for obtaining DNA-membrane complexes. It had been found that, when cells were disrupted and extracted in the presence of spermidine, one of the polyamines normally found in *E. coli*, the DNA was not extracted from bacterial structures (3). Extracts made in magnesium ion contained bacterial DNA (3). When 10^{-2} or 10^{-3} M spermidine was added to a spheroplast suspension prior to lysing with 0.1% Sarkosyl, a low viscosity lysate formed. Centrifugation at 38,000 × *g* for 15 min gave a discrete pellet that was found to contain 96% of the total DNA, 98% of the lipid phosphorus, 22% of the protein, and 13% of the RNA. If spermidine was omitted from the above procedure, a viscous lysate was obtained which upon centrifugation gave a gel at the bottom of the tube. With spermidine at concentrations of 10^{-4} M or less, pellets varying in consistency between a discrete pellet and gel were formed.

It was considered possible that the presence of DNA in the pellet resulted from its precipitation by spermidine rather than its association with the membrane. Labeled T5 DNA was added to a spheroplast suspension prior to lysing in the presence of 10^{-3} M spermidine, and the resulting pellet was found to contain less than 2% of the added DNA. This indicates that DNA is attached to the membrane prior to lysis. At 10^{-2} M spermidine, 40% of the added DNA entered the pellet.

This method of obtaining DNA-membrane complexes appears promising, but will not be further explored at this time.

TABLE 1. *Distribution of cellular constituents in M-band gradients*

Constituent	Distribution (%)	
	M-band	Top fraction
DNA[a]	86 ± 1 (5)[e]	14 ± 1
RNA[b]	16 ± 3 (5)	84 ± 3
Protein[c]	15 ± 5 (5)	85 ± 5
Phospholipid[d]	45 ± 1 (3)	55 ± 1

[a] Cells were prelabeled with radioactive thymine for two generations; label in cold trichloroacetic acid-insoluble material was determined.

[b] Orcinol method.

[c] Lowry method.

[d] Cells were prelabeled with $^{32}P_i$ for two generations. Lipid phosphorus was extracted and counted as described in Materials and Methods.

[e] Numbers in parentheses indicate number of trials.

Autoradiography of thymidine incorporation. For much of the data presented thus far, there is the possibility that the apparent synthetic capacities remaining to spheroplasts represent active synthesis by a small cell population rather than reduced synthesis by the entire population. The question of population homogeneity was approached by conducting a radioautographic study of DNA synthesis. Cultures of intact cells, plasmolyzed cells, and spheroplasts were allowed to incorporate tritiated thymidine for 30 min. Cells were fixed, and 0.1-μm sections were made; radioautographs were prepared and scored with an electron microscope. The results (Table 2) show that the proportion of labeled cells was somewhat less for plasmolyzed cells and spheroplasts than for the intact-cell control. In the case of spheroplasts, this figure may be an underestimate of the synthesizing population, because the increased cell volume leads to a decreased probability of encountering a grain in a given section. In any event, as compared with control cells, at least about two-thirds of the plasmolyzed cell and spheroplast populations were demonstrably active in synthesis. Additionally, grains were often seen at the cell periphery in sections from all three cultures, suggesting that synthesis occurs at the membrane (Fig. 8). This would probably not be the case for repair synthesis.

DISCUSSION

It can be concluded from these data that the metabolism of spheroplasts is not completely arrested, as might be the case, for example, if they could neither synthesize nor retain adenosine triphosphate. Synthetic capacities are reduced but not to equal extents; DNA and phospholipid synthesis are considerably more active than RNA and protein synthesis, and can be stimulated by specific treatments. Plasmolysis in some respects mimics spheroplasting. Capacities for multiplication on agar and induced enzyme synthesis are lost temporarily to plasmolyzed cells, permanently to spheroplasts. Synthesis of macromolecules is retarded by plasmolysis; phospholipid synthesis is temporarily reduced, but eventually exceeds the control.

TABLE 2. *Proportions of cell populations engaged in DNA synthesis*

Population	No. of cells	No. of labeled cells
Control	561	362 (65%)
Plasmolyzed cells	776	354 (46%)
Spheroplasts	840	334 (40%)

Reduced DNA synthesis in plasmolyzed cells and spheroplasts is due at least in part to leakage of deoxyribose donors, because thymidine is a more effective DNA precursor than thymine. Plasmolysis as well as spheroplasting is known to alter cell permeability (7). That permeability is not the whole story is indicated by the fact that uridine is no more effective an RNA precursor than uracil. The possibility that shortages of phosphorylated metabolites may be responsible for the reduced synthetic capacities cannot be tested, because spheroplasts, like intact cells, seem impermeable to such substances. It was found that some substance normally found in cells can in part restore phospholipid synthesis to spheroplasts. The data offer no clue as to its nature or mode of action.

We have shown by a radioautographic experiment that the bulk of the spheroplast population in a culture is actively engaged in DNA synthesis, rather than some small osmotically resistant fraction of the population. The fact that grains are often seen at the cell periphery suggests that this synthesis is of a normal rather than a repair nature. Ribosomal RNA with normal sedimentation properties is synthesized in both plasmolyzed cells and spheroplasts. Conversion of this RNA to ribosome subunits, however, is markedly impaired. Whether this is due to a shut-off of ribosomal protein synthesis or to interference with the assembly process, the present data give no clue.

A possible, but purely speculative, explanation for the reduced synthetic capacities independent of permeability involves changes in membrane conformation associated with the destruction of the rigid peptidoglycan layer and with the increase in cell surface area accompanying spheroplast formation. Thus, if some particular conformation of the membrane were necessary for the cell to achieve optimal rates of macromolecule synthesis (assuming these functions are localized at the membrane), spheroplasting could cause a destructive disorganization. Perhaps it is the cell wall that holds the membrane in its optimal conformation. Disorganization attending temporary separation of wall and membrane in plasmolysis could then explain those effects.

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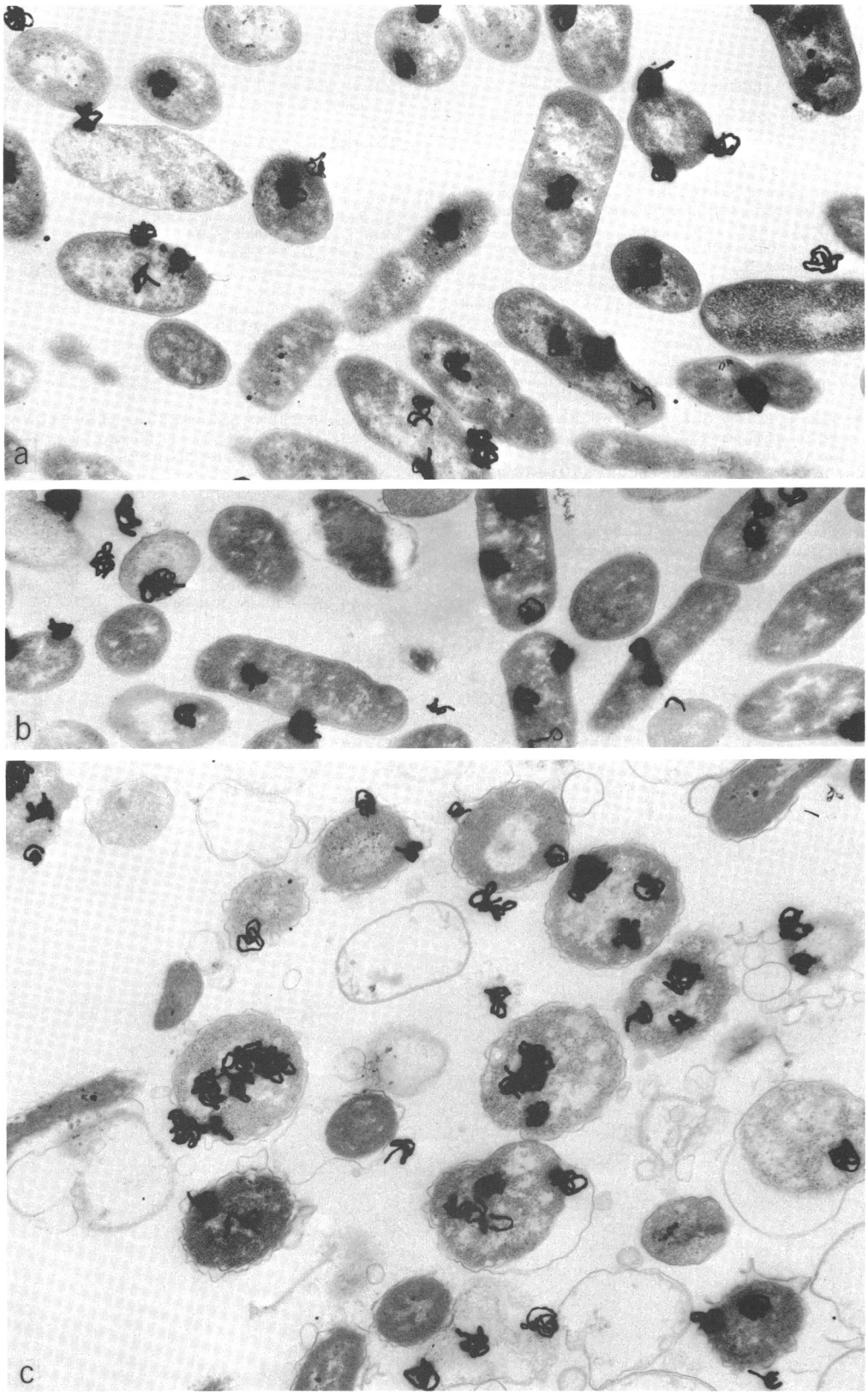

FIG. 8. *High-resolution radioautograph of spheroplasts incubated in the presence of tritiated thymidine. The technique is described in Materials and Methods. Grains are seen near the spheroplast periphery.*

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