In all previous in vitro experiments[3-7] antibody formation could be demonstrated only because of the high potency of the antigens used and because of the very sensitive immunological methods available for the detection of antibodies against particulate antigens and toxins.

The method of incubation described here would seem to hold promise for other experiments requiring the maintenance of differentiated cells in a functional state in vitro.

SUMMARY

A technique has been developed which permits incubation at rest of large quantities of rabbit spleen cells in vitro. Under these conditions net synthesis of precipitating antibody against bovine serum albumin occurs. The amount of antibody formed corresponds closely to the amount obtained after transfer of spleen cells to nonimmunized recipients in vivo. It seems that the success of this technique is due to the prevention of damage by agitation and to provisions for adequate nutrition and aerobic metabolism of the cells.

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[1] T. N. Harris and S. Harris, *Am. J. Med.*, **20**, 114, 1956.
[2] H. Green and H. S. Anker, *Biochim. et biophys. acta*, **13**, 365, 1954.
[3] R. C. Parker, *Methods of Tissue Culture*, 2nd ed.; New York: P. B. Hoeber, 1950).
[4] S. Roberts *et al.*, *J. Immunol.*, **62**, 155, 1949.
[5] A. Fagraeus, *J. Immunol.*, **58**, 1, 1948.
[6] G. J. Thorbecke and F. J. Keuning, *J. Immunol.*, **70**, 129, 1953.
[7] A. B. Stavitsky, *J. Immunol.*, **75**, 214, 1955.
[8] M. Heidelberger and C. F. C. MacPherson, *Science*, **97**, 405, 1953.
[9] O. R. Lowry *et al.*, *J. Biol. Chem.*, **193**, 265, 1951.
[10] N. Nelson, *J. Biol. Chem.*, **153**, 375, 1944.
[11] H. Eagle, *Science*, **122**, 501, 1955.
[12] O. A. Trowell, *Exptl. Cell Research*, **9**, 258, 1955.
[13] J. F. Morgan, H. J. Morton, and R. C. Parker, *Proc. Soc. Exptl. Biol. Med.*, **73**, 1, 1950.
[14] W. H. Taliaferro and D. W. Talmadge, *J. Infectious Diseases*, **97**, 88, 1955.

PRODUCTION OF PROTOPLASTS OF ESCHERICHIA COLI BY LYSOZYME TREATMENT

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Recently, investigators of some biochemical problems have made use of subcellular elements of bacteria in an attempt to study systems more highly integrated than soluble extracts but more amenable to external manipulation than intact cells. Cells which either have been sonically disrupted[1] or have had their cell walls removed (protoplasts)[2] have been used. Protoplasts have hitherto been prepared only from gram-positive organisms. Treatment of *Bacillus megaterium* with lysozyme in hypertonic media brings about a change from its normal rodlike form

to a spherical one and results in its susceptibility to lysis upon suspension in hypotonic media.[3] These altered properties are presumed to result from dissolution of the cell wall, the cytoplasmic contents then being retained by the cell membrane. Unfortunately, little is known of the genetics of the gram-positive organisms, and the fruitful concomitant biochemical and genetic attack is thus restricted. For this reason it would be of obvious advantage to obtain protoplasts of such organisms as *Escherichia coli*, which has been the subject of detailed genetic and physiological investigation. This report details the development of such a technique which also involves the use of lysozyme.

Our thinking on the problem of obtaining protoplasts of *E. coli* was influenced by the report of Hirsch[4] that some material isolated by him from rabbit leukocytes (phagocytin) would kill *E. coli* strains. Phagocytin had chemical properties similar to, but not identical with, those of lysozyme. When *E. coli* cells were treated with crude phagocytin, (kindly provided by Dr. Hirsch) in hypertonic media at pH 5, they became susceptible to lysis upon dilution into distilled water. However, only a rare cell was morphologically identifiable as a protoplast. The difficulty in obtaining phagocytin in sufficient quantity for detailed investigation caused us to seek another source of active material. On the hypothesis that phagocytin was a lysozyme of different specificity, hen egg albumen, a rich source of lysozyme, was tested. Treatment of cells with a one-to-four dilution of fresh albumen resulted in the rapid conversion of cells to protoplasts at pH 5 and 9. At pH 5 the spheres were badly agglutinated. All further experiments, unless otherwise noted, were therefore done at pH 9. No activity of albumen was detected at pH 6–8. Crude fractionation of egg albumen revealed that the activity was associated with the lysozyme fraction. Commercial lysozyme (Armour Chemical Company) was equally effective.

CONVERSION OF CELLS TO PROTOPLASTS

The following procedures for the efficient conversion of cells of *E. coli* strains K12 and B, and their derivatives, were empirically developed. Overnight broth cultures of cells are diluted tenfold in Difco Penassay broth and incubated with aeration for 90 minutes. This takes the culture just out of logarithmic growth phase (cells less fragile) and gives a titer of 4–5 $\times$ 10^8 cells per milliliter. The culture is washed once in saline by centrifugation and resuspended in saline. After 3 minutes of incubation at 37° C., sufficient tris buffer (Sigma Chemical Company) and lysozyme are added to make the final concentrations 1 *M* and 200 μg/ml., respectively. Further incubation for 5 minutes converts the cells to protoplasts (Fig. 1). Other supporting media that can be used are sucrose 0.6 *M* with 0.1 *M* carbonate buffer, pH 9, or *M*/2 NaCl with carbonate buffer, or 0.3 *M* NH_4Cl brought to pH 9 with NaOH. In these latter two media the cells will cytolyze upon dilution into hypotonic media but do not assume a spherical shape. Centrifugation of the preprotoplasts and resuspension in 0.6 *M* sucrose will cause their immediate swelling into spheres.

With regard to these procedures, some points need be mentioned: (1) At pH 8–8.8, protoplasts are formed but are badly agglutinated. This agglutination can be prevented by the addition of 0.1 per cent Mg^{++}; however, some 10 per cent of the cells retain their rodlike shape and are not subject to cytolysis. (2) It is requi-

site that the cells be washed and starved slightly for quantitative conversion to occur. Perhaps they can heal the lysozyme-induced lesions if they are able to grow. (3) Certain strains will not form spheres in the treatment medium but will do so when resuspended in other media. In order for the cells to form spheres, they apparently must imbibe some water and swell—in a sense, hatch out of their cell walls. As yet there is no rationale for the preferential use of any medium, other than its empirical success with the strain in question.

PROPERTIES OF PROTOPLASTS OF *E. coli*

1. *General.*—Growing cells of *E. coli* are generally in a doublet condition. Each doublet gives rise to two protoplasts, with separation occurring at the point of incipient division. Motile strains lose their motility when converted to protoplasts. The spheres do not detectably adsorb bacteriophage. The protoplasts readily reduce methylene blue.

When protoplasts are suspended in broth media, they lyse no matter how high the osmotic pressure of the medium and whether it is maintained by sucrose, NaCl, or dextran. Addition, to a final concentration of 2 per cent, of bovine serum albumin prevents their lysis and makes further study possible. The following medium (P-broth) was used to study the growth of bacteriophage by, and the viability of, the protoplasts: Difco Penassay broth was supplemented with 0.6 *M* sucrose, 2 per cent serum albumin, and 0.1 per cent Mg^{++} (this prevents agglutination at the lower pH of this medium and may be required for growth processes).

2. *Growth of Bacteriophage.*—Protoplasts of *E. coli* B will grow bacteriophage T4. Cells were grown as described, and suspended, after washing, in saline containing 100 μg/ml. *l*-tryptophan (necessary for phage adsorption). Phage was added so as to give one particle per hundred bacteria, and after 5 minutes of incubation for adsorption and injection the cells were converted to protoplasts in tris buffer. Infective center titers (plaques) were obtained for protoplasts, lysed protoplasts, and cells by plating in *M*/2 NaCl phage agar (8 per cent tryptone, 0.1 per cent glucose, and agar) with an excess of *E. coli* B. Suitable samples were incubated in P-broth to determine final yields. Table 1 shows the results of such an experiment. About 20 per cent of protoplast infective centers are recovered directly. Only 1 per cent of infective centers are recovered from lysed protoplasts. These probably represent unadsorbed and desorbed phage, as there is little rise in the titer with time. Protoplasts give almost as large a yield as the untreated cells, indicating that the initial loss of protoplast infective centers is due to their premature lysis on the plates rather than to inability to grow phage.

TABLE 1

GROWTH OF BACTERIOPHAGE T4 IN PROTOPLASTS AND CELLS OF *E. coli* B

(Number of Plaques per Plate, Had Equivalent Volumes of Equivalent Dilutions Been Plated)

	Incubation Time			Incubation Time	
	0 Min.	90 Min.		0 Min.	90 Min.
Cells	350	21,100	Lysed protoplasts	3	12
Protoplasts	83	15,500			

3. *Viability.*—For 4 hours after suspension and incubation in P-broth, protoplasts exhibit no gross morphological changes as observed by phase-contrast microscopy. At this time, the majority of the protoplasts start to shrink, become highly refractile at their periphery, and assume a coccobacillus form. Their fate is still uncertain, but they probably represent a degenerate state. On the other hand, about 10 per cent of the protoplasts increase in volume and form a large vacuole. From the side distal to the vacuole, finger-like processes appear which grow into rods (Fig. 2). The mother protoplast becomes extremely distorted,

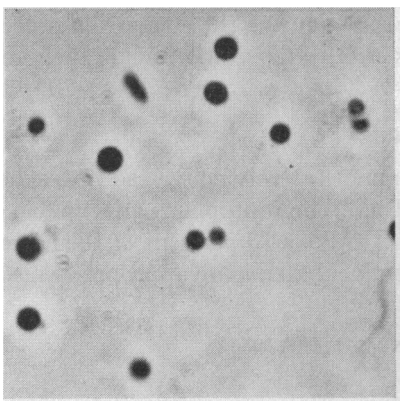

FIG. 1.—Phase-contrast microphotograph of wet mount of protoplasts. Note nonswollen rod for size comparison between the rods and spheres. ×1,700.

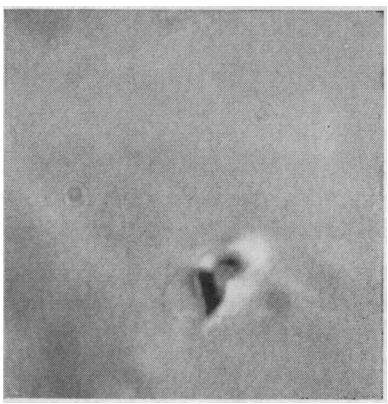

FIG. 2.—Phase-contrast microphotograph of wet mount of protoplast budding off a rod. ×1,700.

and a wide variety of morphological abberations are observed, reminiscent of the growth of L-forms from large bodies.[5] Direct demonstration of the viability of the protoplasts has been obtained by plating them in P-broth to which sufficient agar to just solidify the mixture was added. The equivalent of from 2 to 20 per cent (different experiments) of the treated cells formed normal colonies. On no other solid media have the protoplasts returned to the actual bacillary form.

DISCUSSION

Although lysozyme has been claimed to be without effect on *E. coli* cells, it was known to be bound by them.[6] In our studies it was clear that at pH 6–8 lysozyme was without effect, whereas its activity was extremely rapid at pH 8 and above. It is interesting to note that suspension of *E. coli* in the alkaline treatment medium, in the absence of lysozyme, brings about, within 30 minutes, the slow lysis of the cells upon their suspension in hypotonic media. This action may be analogous to, though less efficient than, that of lysozyme, which is itself a basic protein. Certain classes of bacteriolytic agents may have as their primary site of activity the cell-wall substance, with the lysis of the cell being due to secondary osmotic effects following the dissolution of the cell wall. In the course of these investigations it was noted that the lysis of *E. coli* by phage ghosts (phage membranes obtained by osmotic shock) went through a protoplast stage. However, no suitable stabilizing medium for such protoplasts was found. Thus three biological reagents—phagocytin,

lysozyme, and phage ghosts—are available for efficient lysis of *E. coli* cells for preparation of bacterial enzymes, nucleic acids, cell membranes, etc.

The protoplasts of *E. coli* have retained—as had their predecessors, the protoplasts of *B. megaterium*[7]—sufficient of their synthetic capacities to synthesize bacteriophage. This may be taken as a general indication of their biochemical integrity. The difference in the viability of *E. coli* and *B. megaterium* protoplasts may be only a reflection of the different media used in the different studies. As pointed out, there has been considerable variation in the viability of the *E. coli* protoplasts. This may depend on the amount of residual cell-wall substance left on the protoplast and/or the soluble materials lost during the conversion process.

With the ready accessibility of protoplasts of *E. coli*, investigation of their genetic potentialities is under way.

SUMMARY

The cells of two strains of *E. coli* can be quantitatively converted into spherical protoplasts by the action of lysozyme at pH 9. The protoplasts are capable of all the biosyntheses necessary for phage growth. About 10 per cent of them are also capable of reverting to normal rod forms when incubated in an appropriate medium.

[1] E. F. Gale and J. Folkes, *Nature*, **173**, 1223, 1954.
[2] O. Landman and S. Spiegelman, these PROCEEDINGS, **41**, 698, 1955.
[3] C. Weibull, *J. Bacteriol.*, **66**, 688, 1953.
[4] J. G. Hirsch, *J. Exptl. Med.*, **103**, 598, 1956.
[5] L. Dienes and H. I. Weinberger, *Bacteriol. Revs.*, **15**, 245, 1951.
[6] E. H. Boasson, *J. Immunol.*, **34**, 281, 1938.
[7] S. Brenner and G. S. Stent, *Biochim. et biophys. acta*, **17**, 473, 1955; M. R. J. Salton and K. M. McQuillen, *Biochim. et biophys. acta*, **17**, 465, 1955.

ION ASSOCIATION IN AQUEOUS SOLUTIONS OF 1-1 ELECTROLYTES AT 25°

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According to Onsager's theory, the conductance of an unassociated electrolyte in aqueous solution decreases with concentration in accordance with the equation

$$\Lambda = \Lambda_0 - (\alpha\Lambda_0 + \beta)\sqrt{C}. \quad (1)$$

It has been found, however, that, while the conductance approaches the theoretical value in this solvent as C approaches zero, the observed values of Λ at higher concentrations are somewhat higher than those predicted by theory. The deviations from theoretical values increase with concentration approximately as a linear function of C. We thus have Shedlovsky's empirical extension[1] of Onsager's equation.

$$\Lambda = \Lambda_0 - (\Lambda_0\alpha + \beta)\sqrt{C} + BC, \quad (2)$$

where B is an empirical constant.