REVERSION OF L FORMS AND SPHEROPLASTS OF *PROTEUS MIRABILIS*

ROBERT A. ALTENBERN

U.S. Army Chemical Corps Biological Laboratories, Fort Detrick, Frederick, Maryland

Received for publication 18 August 1962

ABSTRACT

ALTENBERN, ROBERT A. (U.S. Army Chemical Corps, Fort Detrick, Frederick, Md.). Reversion of L forms and spheroplasts of *Proteus mirabilis*. J. Bacteriol. **85**:269–272. 1963.—Spheroplasts or 3-B type L forms of *Proteus mirabilis* formed by growth in the presence of penicillin are able to revert to the bacillary form, as judged by regain of resistance to osmotic shock. Reversion takes place in the absence of exogenous nutrients, although at a lesser rate than that observed in a nitrogen-rich medium. Either chloramphenicol or 6-azauracil can completely inhibit reversion of spheroplasts in a penicillin-free medium. By direct measurement, there was no detectable change in total protein content throughout the period of reversion of a spheroplast suspension. Assay for acid-insoluble diaminopimelic acid (DAP) revealed that a large part of the DAP of the cell wall of normal cells was lost when the cells were converted to spheroplasts by exposure to penicillin. Upon transfer to penicillin-free medium, there was a rapid increase in acid-insoluble DAP in the wall of the reverting spheroplast. When spheroplasts were transferred to penicillin-free medium containing chloramphenicol, the acid-insoluble DAP in the wall of the spheroplast continued to decrease. The significance of these results in regard to sites of activity of the antibiotics involved is discussed.

Reversion of 3-B type L forms or spheroplasts to bacterial cells of normal morphology has been recorded by photomicrographs in many investigations (Dienes and Weinberger, 1951; Lederberg and St. Clair, 1958; Zinder and Arndt, 1956), although the general biochemical aspects of reversion of these spherical bodies lacking a rigid wall remained virtually unknown. Both L forms and spheroplasts are readily induced in many enteric organisms by exposure of the cells to high concentrations of penicillin in hypertonic medium. Some of the detailed knowledge of the biochemistry of the cell wall and of the action of penicillin gained in recent years was applied to an examination of the reversion of 3-B type L forms and spheroplasts of a strain of *Proteus mirabilis*.

MATERIALS AND METHODS

A strain of *P. mirabilis* employed in previous investigations (Altenbern, 1961) was used throughout this work. It was maintained on nutrient agar slants at 4 C and transferred every 8 weeks.

The 3-B type L bodies were grown by the methods described in an earlier paper (Altenbern and Landman, 1960). After 7 days of incubation, the L bodies were harvested by suspending in fresh, hypertonic medium composed of Penassay Broth (Difco) plus 10^{-2} M $MgSO_4$ and 0.3 M sodium succinate (medium I). Spheroplasts were produced by mixing 400 ml of an overnight culture of cells in medium I with 400 ml of fresh medium I containing 400 units/ml of penicillin G. Further incubation of this suspension at 37 C for 3 hr resulted in nearly 100% conversion of the cells to spheroplasts.

For determination of osmotic sensitivity, 1-ml amounts of L body or spheroplast suspension were added to a small flask, and 20 ml of sterile distilled water were added by rapid pouring. The flask contents were briefly mixed by swirling and then diluted decimally in medium I and plated by spreading onto the surface of plates containing a medium composed of Penassay Broth plus 0.8% agar, 10^{-2} M $MgSO_4$, and 0.5 M sodium succinate (medium II). The viable counts obtained before and after such "water shock" were used to calculate the per cent viability loss as it appears on Fig. 1 to 3. The degree of osmotic resistance so determined is taken as a direct measure of the reversion of these spherical forms.

Cell walls of whole cells, spheroplasts, or L bodies were prepared readily by the method of

Park and Hancock (1960). These walls were hydrolyzed by autoclaving in 6 N HCl for 4 hr. The hydrolysates were then evaporated to dryness three times under reduced pressure. The residue was finally taken up in a small amount of water, and the whole volume spotted onto Whatman no. 1 chromatography paper. The papers were developed for 30 to 36 hr in the solvent recommended for diaminopimelic acid (DAP) determinations by Rhuland et al. (1955). The papers were then dried at 60 C, sprayed with 0.1% ninhydrin in *n*-butanol, and heated for 5 min in an oven at 100 C. The characteristically green spots of DAP were then excised, and the quantities were determined by the method of Housewright and Thorne (1950). Total protein was determined by the method of Weichselbaum (1946).

RESULTS

Reversion in nitrogen-free medium. A suspension of 3-B L bodies in liquid medium obtained as described above was centrifuged, and the pellet of L forms was washed twice with and finally suspended in a solution containing 10^{-2} M $MgSO_4$, 0.3 M sodium succinate, and 0.06 M NaCl in 0.067 M phosphate buffer (pH 7.0). A comparison between reversion in the nitrogen-free menstruum just described and in fresh medium I is presented in Fig. 1. It is obvious that the reversion can occur in the absence of exogenous nutrients, although at a somewhat slower rate than that occurring in a rich medium. After 4 hr of incubation at 37 C, reversion in medium I was essentially complete, inasmuch as the viability loss by water shock was no more than that observed for normal cells.

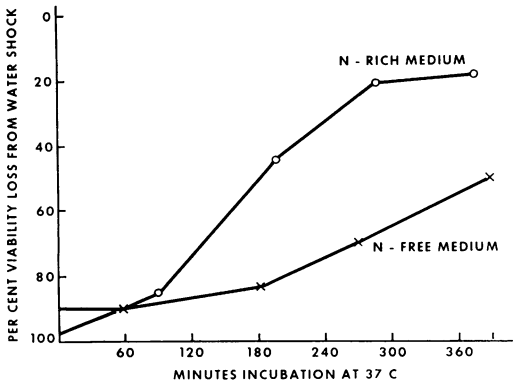

FIG. 1. *Reversion of 3-B type L bodies at 37 C in Penassay Broth and in a nitrogen-free salts solution.*

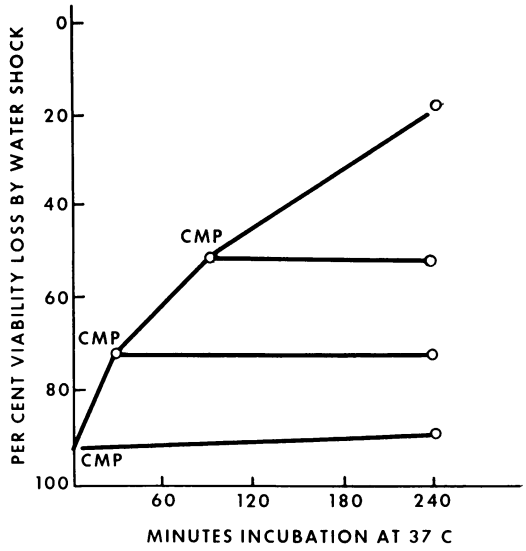

FIG. 2. *Inhibition of reversion of 3-B type L bodies at 37 C in Penassay Broth by addition of chloramphenicol at various times.*

Supplementation of this medium with various concentrations of DAP or uridine or both failed to accelerate the rate of reversion of the L bodies. The kinetics of reversion of freshly prepared spheroplasts were identical to those of the classical 3-B type L body, and support the contention that 3-B bodies are merely multiplying spheroplasts.

Inhibition of reversion. Since endogenous reserves of the L body or spheroplasts apparently furnish all the substrates necessary for reversion, the action of some inhibitors in this system was determined. Both chloramphenicol (40 μg/ml) and 6-azauracil (100 μg/ml) completely prevented reversion of L bodies or spheroplasts. Figure 2 presents some data concerning the effect of chloramphenicol added at various times during reversion of 3-B type L bodies in medium I. Addition of the antibiotic at any time during reversion immediately blocked any further reversion, although total viability remained the same. The strong inhibition of reversion by chloramphenicol illustrates the necessity for some protein synthesis as a part of the reversion process. 6-Azauracil was employed as an inhibitor of ribonucleic acid (RNA) synthesis, although the specificity of this inhibitor is less certain than that of chloramphenicol. There is evidence that one uracil analogue may block synthesis of the basal cell wall by interfering with

the metabolism of the mucopeptide unit (Tomasz and Borek, 1960). Therefore, the action of 6-azauracil in preventing reversion of spheroplasts may represent a penicillinlike inhibition of reversion rather than any blockade of RNA synthesis.

The magnitude of the protein synthesis required for reversion of spheroplasts to osmotically resistant forms was determined by measurement of total protein on samples of a spheroplast suspension in the nitrogen-free solution described above. The results (Fig. 3) show that throughout the period of change from osmotic sensitivity to osmotic resistance there was no measurable change in total protein. This result was found with both freshly prepared spheroplasts and 3-B type L forms.

Determination of acid-insoluble DAP in spheroplasts before and during reversion. After formation of spheroplasts by exposure of cells to penicillin in medium I, the amount of DAP found in cell-wall preparations obtained by the method of Park and Hancock (1960) was approximately 30% of the amount present in cells at the time of addition of penicillin. Such results are in agreement with those of Weidel, Frank, and Martin (1960). If such spheroplasts are then centrifuged and resuspended in an equal volume of penicillin-free medium I, the amount of DAP in cell-wall preparations increases rapidly and reaches a maximum within 60 min (Fig. 4). Resuspension of spheroplasts in medium I containing chloramphenicol resulted in a gradual and progressive decline in the amount of DAP found in cell-wall preparations obtained from samples taken at various times after resuspension.

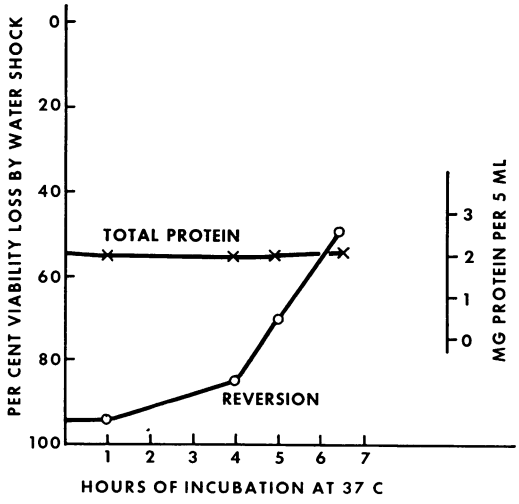

FIG. 3. *Measurement of total protein content of spheroplast suspension reverting in a nitrogen-free medium at 37 C.*

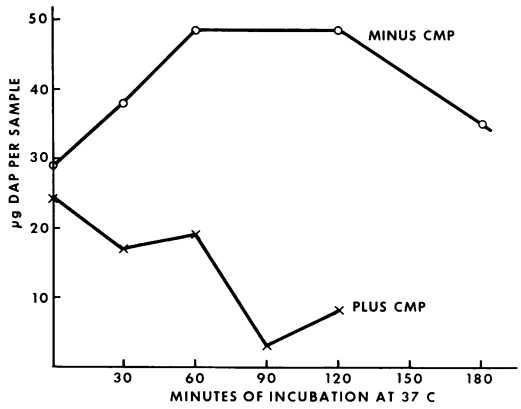

FIG. 4. *Determinations of diaminopimelic acid* (DAP) *content of cell-wall preparations from spheroplasts reverting with or without chloramphenicol* (CMP) *in Penassay Broth. At each time interval, 80 ml of the spheroplast suspension containing about 10^9 spheroplasts/ml were removed, chilled rapidly in an ice-water bath, and centrifuged. The pellet of spheroplasts was then extracted by the method of Park and Hancock (1960) for cell-wall preparation.*

Of incidental interest is an analysis of the quantity of amino acid present in cell-wall preparations of normal *Proteus* cells. Glutamic acid, alanine, and DAP were present in acid hydrolysates along with traces of other unidentified amino acids. The ratio of DAP to glutamate to alanine obtained in one such analysis was 1.0:1.14:3.4. Such results indicate that the degree of contamination of the wall preparation with other nitrogenous materials is slight, and that there are three alanine residues in the mucopeptide monomer in contrast to the speculation that the basic mucopeptide unit in *Escherichia coli* contains only two alanine residues (Primosigh et al., 1961).

Discussion

Extensive literature on the mode of action of penicillin describes, in great detail, the irreversible binding of penicillin to certain sites on the periphery of the cell (Cooper, 1956). It has always been assumed that the binding sites are the specific areas for basal cell-wall synthesis and that the synthesis of basal cell wall ceases when the sites are bound by penicillin. The data pre-

sented in this paper concerning the rate of deposition of acid-insoluble DAP are of considerable interest in regard to the role of "bound" penicillin. There is a very rapid rate of synthesis of basal wall (Fig. 4) immediately upon removal of penicillin from the suspending medium. This phenomenon implies that the "bound" penicillin does not prevent polymerization of mucopeptide units to form the basal wall, since bound penicillin should have retained its activity even after removal of the penicillin in solution. The further implication is that the exchangeable penicillin blocks polymerization of mucopeptide units, and that polymerization resumes immediately after spheroplasts are transferred to penicillin-free medium.

It appears, at present, most unlikely that the spheroplast synthesizes a large number of polymerizing sites so rapidly after removal from penicillin-containing medium since there is no apparent increase in the number, size, and protein content of the spheroplasts. There is, however, an accumulation of acid-soluble DAP within the spheroplast during penicillin exposure, which probably is the source of the DAP deposited in an acid-insoluble state after removal of the spheroplasts from penicillin-containing medium.

The inhibition by chloramphenicol of manufacture of basal cell-wall material was unexpected and is at variance with results of Hancock and Park (1958) and Nathenson and Strominger (1961). Therefore, the resumption of synthesis of basal wall material, specifically mucopeptide polymer, by a spheroplast seems to be a qualitatively different system from synthesis of wall material by a normal cell. Chloramphenicol, however, affects another process concerned with regain of osmotic resistance of spheroplasts (Fig. 2). This activity seems to be separable, on a time scale, from prevention of basal wall synthesis, since the inhibition of reversion can be demonstrated by addition of chloramphenicol long after maximal synthesis of basal wall material has occurred. Perhaps this phenomenon reveals the need for some small amount of protein synthesis to proceed in order to regain osmotic resistance.

LITERATURE CITED

ALTENBERN, R. A. 1961. Reversion of 3A type L forms of *Proteus mirabilis*. J. Bacteriol. **81**:762–767.

ALTENBERN, R. A., AND O. E. LANDMAN. 1960. Growth of L-forms of *Proteus mirabilis* in liquid media. J. Bacteriol. **79**:510–518.

COOPER, P. D. 1956. Site of action of radiopenicillin. Bacteriol. Rev. **20**:28–48.

DIENES, L., AND H. J. WEINBERGER. 1951. The L forms of bacteria. Bacteriol. Rev. **15**:245–288.

HANCOCK, R., AND J. T. PARK. 1958. Cell-wall synthesis by *Staphylococcus aureus* in the presence of chloramphenicol. Nature **181**:1050.

HOUSEWRIGHT, R. D., AND C. B. THORNE. 1950. Synthesis of glutamic acid and glutamyl polypeptide by Bacillus anthracis. I. Formation of glutamic acid by transamination. J. Bacteriol. **60**:89–100.

LEDERBERG, J., AND J. ST. CLAIR. 1958. Protoplasts and L-type growth of *Escherichia coli*. J. Bacteriol. **75**:143–160.

NATHENSON, S. G., AND J. L. STROMINGER. 1961. Effects of penicillin on the biosynthesis of the cell walls of *Escherichia coli* and *Staphylococcus aureus*. J. Pharmacol. Exptl. Therap. **131**:1–6.

PARK, J. T., AND R. HANCOCK. 1960. A fractionation procedure for studies of the synthesis of cell-wall mucopeptide and of other polymers in cells of *Staphylococcus aureus*. J. Gen. Microbiol. **22**:249–258.

PRIMOSIGH, J., H. PELZER, D. MAASS, AND W. WEIDEL. 1961. Chemical characterization of mucopeptides released from the *E. coli* B cell wall by enzymic action. Biochim. Biophys. Acta **46**:68–80.

RHULAND, L. E., E. WORK, R. F. DENMAN, AND D. A. HOARE. 1955. The behavior of the isomers of α-diaminopimelic acid on paper chromatograms. J. Am. Chem. Soc. **77**:4844.

TOMASZ, A., AND E. BOREK. 1960. The mechanism of bacterial fragility produced by 5-fluorouracil: the accumulation of cell wall precursors. Proc. Natl. Acad. Sci. U.S. **46**:324–327.

WEICHSELBAUM, T. E. 1946. An accurate and rapid method for the determination of proteins in small amounts of blood serum and plasma. Am. J. Clin. Pathol. Tech. Sec. **16**:40–49.

WEIDEL, W., H. FRANK, AND H. H. MARTIN. 1960. The rigid layer of the cell wall of *Escherichia coli* strain B. J. Gen. Microbiol. **22**:158–166.

ZINDER, N. D., AND W. F. ARNDT. 1956. Production of protoplasts of *E. coli* by lysozyme treatment. Proc. Natl. Acad. Sci. U.S. **42**:586–590.