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Mannitol Sensitivity

PAMELA JENSEN, CHARLOTTE PARKES, AND DAVID BERKOWITZ

Department of Biochemistry, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

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The addition of mannitol to cultures of *Salmonella typhimurium* mutants missing mannitol-1-phosphate dehydrogenase causes stasis or lysis. Mannitol-1-phosphate accumulates intracellularly to concentrations of 20 mM. The incorporation of acetate into lipid is inhibited before cell wall, protein, or nucleic acid synthesis.

Mutants of *Salmonella typhimurium* which have low levels of mannitol-1-phosphate dehydrogenase are sensitive to D-mannitol (2). Concentrations of D-mannitol as low as 2×10^{-5} M cause inhibition of growth or lysis of the D-mannitol-sensitive mutants. This paper describes some of the biochemical events resulting from the addition of D-mannitol to a mannitol-sensitive culture. The incorporation of ^{14}C-acetate into lipid was inhibited before protein, nucleic acid, or cell wall synthesis and may be the primary cause of the sensitivity.

MATERIALS AND METHODS

Media. Nutrient broth (Difco) supplemented with 0.5% NaCl was used for all the growth experiments described.

Strains. The strains were derived from SL751 as described previously (2). The mannitol-sensitive strains (DB76, DB79, DB80, and DB82) had no detectable mannitol-1-phosphate dehydrogenase activity (2).

Incorporation studies. The incorporation of radioactive precursors into nucleic acid, protein, and cell walls was followed by plating samples of the cultures onto Whatman no. 1 filter paper discs. The discs were transferred to ice-cold 5% trichloroacetic acid, washed with trichloroacetic acid, dried, and counted. This is similar to the method of Bollum (4) as modified by Smith and Pizer (12).

Determinations of intracellular diethylaminoethyl (DEAE) cellulose-adsorbable ^{14}C-mannitol. Samples of cultures incubated with ^{14}C-mannitol were centrifuged, washed twice with nutrient broth, and suspended in 5% perchloric acid. The precipitate was removed and the supernatant fluid was neutralized with potassium carbonate. After removal of the potassium perchlorate precipitate, samples of the supernatant fluid were plated onto DEAE-filter paper discs (DE81, Reeve Angel). The discs were washed twice with distilled water, dried, and counted.

Identification of mannitol-1-phosphate. A perchlorate extract prepared as described above was divided in half. One half was chromatographed directly and the other half was treated with *Escherichia coli* alkaline phosphatase (Sigma type III). Nonradioactive mannitol was added to each fraction as carrier before chromatography. A descending system was used with 50-cm strips of Whatman no. 54 paper. The solvent system was ethylmethylketone-glacial acetic acid-boric acid-saturated water (9:1:1) (11). Resolution was improved by allowing the solvent to drip off the ends of the strips. The running time was 16 hr. Mannitol, sorbitol, fructose, and mannose were clearly resolved. Spots were located with a periodate-benzidine spray (8). Radioactivity was detected by counting 1-cm slices of the paper strips in a scintillation counter. The chromatogram from the alkaline phosphatase-treated extract had only one radioactive spot. This was coincident with the chemically detected mannitol spot. On the chromatogram from the untreated extract, 20% of the radioactivity was coincident with the mannitol spot, and the remainder was in a spot which had the same mobility as mannitol-1-phosphate.

Lipid extraction. Samples of the culture were extracted by the procedure of Bligh and Dyer (3) as adapted by Ames (1). The chloroform phase was transferred to scintillation vials, dried, and counted after addition of the fluor.

Growth rates. Growth was measured in a Spectronic 20 spectrophotometer at 420 nm (except for results presented in Fig. 1). An absorbance of 0.100 corresponded to a cell density of approximately 3×10^7 cells per ml.

Chemicals. D-Mannitol was purchased from Mann Research Laboratories. D-Mannitol-*1*-^{14}*C* (47 μCi/μmole) was obtained from New England Nuclear Corp. L-Phenylalanine-*U*-^{14}*C* (477 μCi/μmole), acetate-*1*-^{14}*C* (59 μCi/μmole), uracil-*2*-^{14}*C* (60 μCi/μmole), and 2,6-diaminopimelic acid-*G*-^{3}*H* (200 μCi/μmole) were obtained from Amersham-Searle.

RESULTS

The response of three mannitol-sensitive mutants to 2×10^{-4} M mannitol is shown in Fig. 1. During the time interval shown the

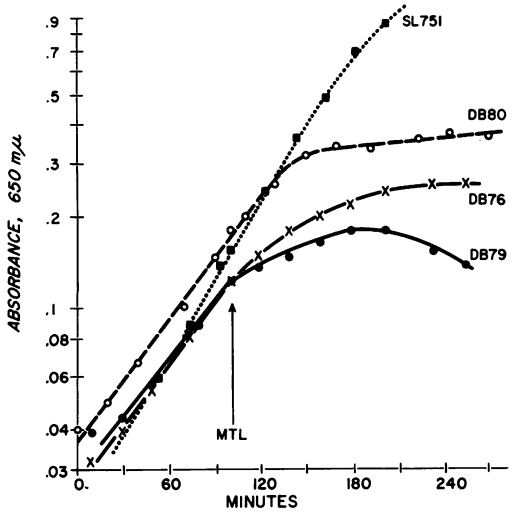

FIG. 1. *Mannitol inhibition of mannitol-sensitive mutants. Mannitol at a final concentration of 2 × 10^{-4} M was added to nutrient broth cultures where indicated by the arrow. An absorbance of 0.100 corresponded to a cell density of approximately 10^8 per ml.*

growth rates of DB80 and DB76 decreased, and DB76 began to slowly lyse. Growth of the parental strain, SL751, was not affected by mannitol. Figure 2 illustrates the effect of 5 × 10^{-4} M mannitol on DB82. Approximately 1 hr after the addition of mannitol, the absorbance peaked, and a rapid lysis phase followed. Mannitol concentrations as low as 2 × 10^{-5} M caused the rapid lysis of DB82. The dramatic response of DB82 to mannitol may be the result of a more complete loss of mannitol-1-phosphate dehydrogenase activity than in the other strains, but this difference could not be detected by enzyme assay (2).

Some events accompanying the lysis of DB82 are also shown in Fig. 2. The absorbance decrease which occurred 1 hr after the addition of mannitol to the culture was paralleled by a decrease in the viable cell count. Further evidence that the absorbance decrease corresponded to lysis is the concomitant release of nucleic acid to the culture supernatant (Fig. 2).

Most of the ^{14}C-mannitol which accumulated inside the cells prior to lysis was ^{14}C-mannitol-1-phosphate. Seventy-five to 100% of the radioactivity in perchlorate extracts (see Materials and Methods) of cultures incubated with ^{14}C-mannitol was retained by columns of Dowex-1. The radioactive material co-chromatographed with authentic mannitol-1-phosphate and, after treatment with alkaline phosphatase, the radioactivity co-chromatographed with authentic mannitol (see Materials and Methods). Perchlorate extracts of the parental strain (SL751) contained only 7% of the radioactivity present in DB82 extracts, and none of this radioactivity co-chromatographed with mannitol or mannitol-1-phosphate. Perchlorate extracts of DB82 served as a source of substrate for mannitol-1-phosphate dehydrogenase. With mannitol-induced extracts of the parental strain (SL751) as the source of enzyme, the concentration of intracellular mannitol-1-phosphate accumulated by DB82 in 1 hr was estimated at 25 mM, in agreement with estimates made from the amount of radioactivity accumulated. From the experiment shown in Fig. 2, the concentration of mannitol-1-phosphate accumulated at 150 min was 20 mM. Approximately 10% of the accumulated radioactive material was charcoal adsorbable and was released from the charcoal by treatment with 1 N HCl for 7 min at 100 C. This result is consistent with the presence of small amounts of a compound such as a nucleoside

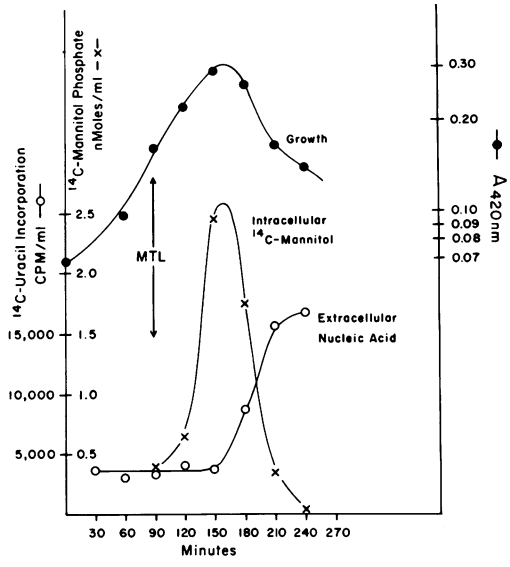

FIG. 2. *Mannitol-induced lysis of strain DB82. An overnight nutrient broth culture of DB82 was diluted 1:100 into each of two flasks containing 50 ml of nutrient broth. For the determination of nucleic acid released to the culture supernatant fluid, ^{14}C-uracil, 6 μCi/μmole, was added to one flask. The final concentration of the added ^{14}C-uracil was 1 × 10^{-5} M. At 90 min nonradioactive mannitol was added at a final concentration of 5 × 10^{-4} M. For the determination of DEAE cellulose-adsorbable ^{14}C- mannitol inside the cells, 5 × 10^{-4} M ^{14}C-mannitol, 4 μCi/μmole, was added to the other nutrient broth culture at 90 min. Nucleic acid and DEAE cellulose-adsorbable mannitol were determined as described in Materials and Methods.*

diphosphate mannitol. Although most of the radioactivity which accumulated was in mannitol-1-phosphate, it is not clear that this compound is the immediate cause of the sensitivity.

To identify the metabolic processes affected by the addition of mannitol to sensitive cultures, we measured the incorporation of radioactive precursors into cell wall, nucleic acid, protein, and lipid. The results of these experiments are given below.

The effect of mannitol on the incorporation of diaminopimelic acid into DB82 is shown in Fig. 3. Under the conditions used, 95% of the radioactivity from diaminopimelic acid incorporated is incorporated into the cell envelope fraction (10). Diaminopimelic acid incorporation was not inhibited by mannitol until the onset of lysis. A decreased rate of diaminopimelate incorporation occurs very late in the sequence of events following mannitol addition and is not a primary cause of the sensitive response. Figure 3 also shows that there is not an extensive loss of diaminopimelate during the lysis. This was true of both recently incorporated (curve A) and older (curve B) diaminopimelate, indicating that the lysis is not accompanied by extensive muramidase activity.

Figure 4 shows that the rate of incorporation of ^{14}C-phenylalanine paralleled the growth curve for about 1 hr after the addition of mannitol and then began to level off. Protein synthesis stopped about the time the culture density reached a maximum. Incorporation of ^{14}C-uracil also followed the growth curve for the first hour after mannitol addition, but was stimulated before the onset of lysis. This nucleic acid effect was reproducible. The effects of mannitol on protein and nucleic acid synthesis occurred before the inhibition of cell wall synthesis.

The earliest process found to be inhibited by the addition of mannitol to nutrient broth cultures of DB82 was the incorporation of ^{14}C-acetate into lipid. Figure 5 shows that ^{14}C-ac-

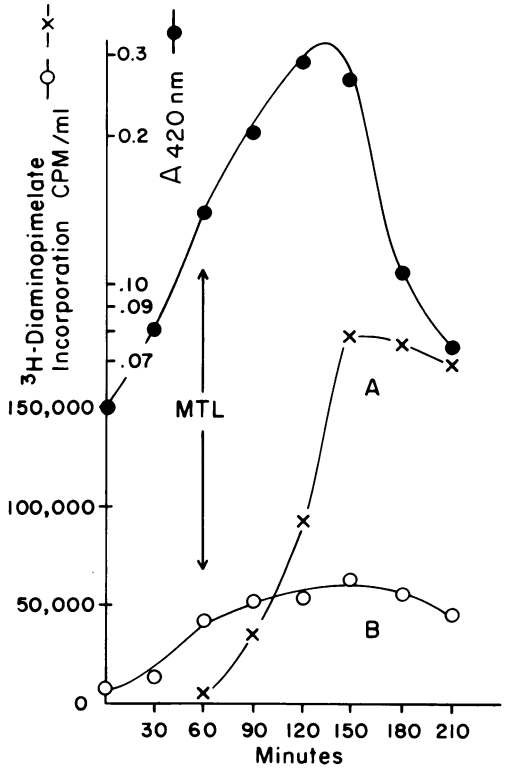

FIG. 3. *The effect of mannitol on the incorporation of diaminopimelic acid. Tritiated diaminopimelic acid (200 mCi/mmole) was added to both cultures at a concentration of 1.7 μCi/ml. The ^{3}H-diaminopimelic acid was added to culture A at 60 minutes with the mannitol (10^{-3} M). At zero time, culture B got ^{3}H-diaminopimelic acid and at 60 min got mannitol and nonradioactive diaminopimelic acid, each at a final concentration of 10^{-3} M.*

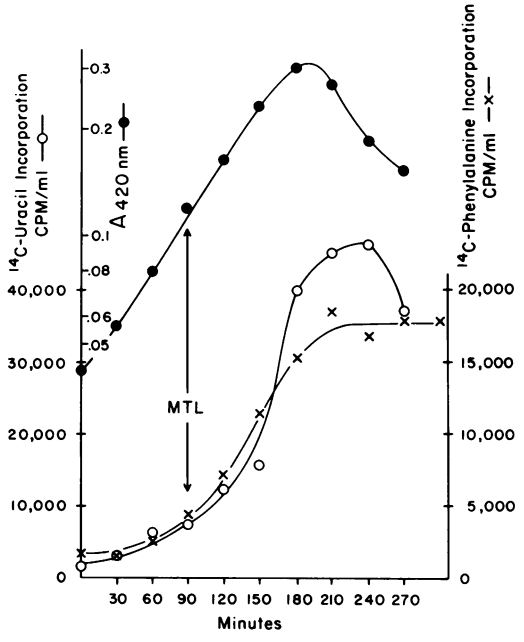

FIG. 4. *The effect of mannitol on the synthesis of protein and nucleic acid. Protein synthesis was measured as the trichloroacetic acid-precipitable radioactivity from ^{14}C-phenylalanine. The culture contained 0.2 μCi of U-^{14}C-phenylalanine (477 mCi/mmole) per ml. Nucleic acid synthesis was measured as acid-precipitable radioactivity derived from ^{14}C-uracil. The culture contained 0.2 μCi/ml of uracil-2-^{14}C (20 μCi/μmole). Mannitol was added at a final concentration of 10^{-3} M at 90 min.*

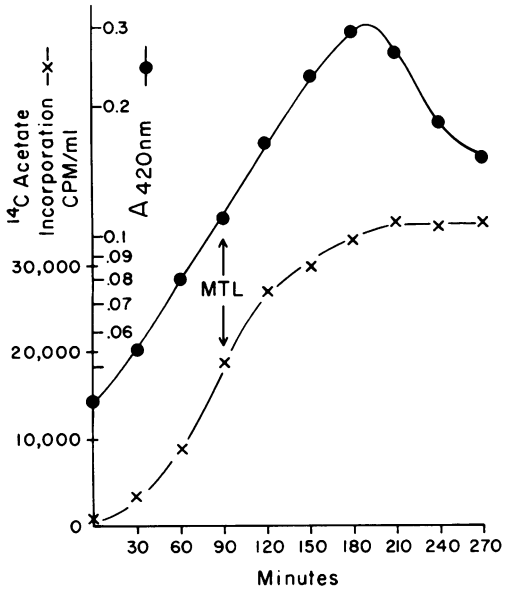

FIG. 5. *The effect of mannitol on the incorporation of ^{14}C-acetate into lipid. Strain DB82 was grown in nutrient broth containing 1 μCi of ^{14}C-acetate per ml (59 μCi/μmole). Mannitol, 10^{-3} M final concentration, was added 90 min after inoculation. Radioactivity in the lipid fraction was determined as described in Materials and Methods.*

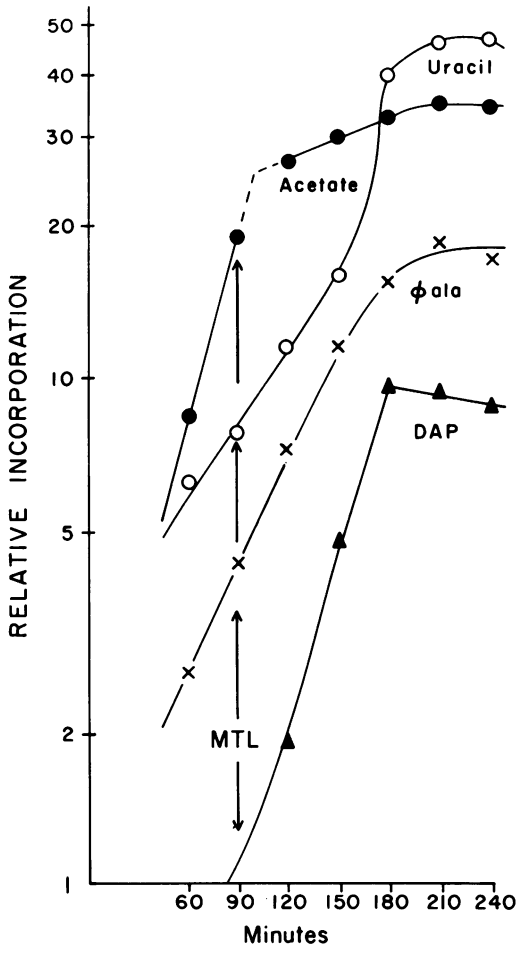

FIG. 6. *Semilog plot of incorporation data. Incorporation data from Fig. 3–5 are shown in semilog form. The data from Fig. 3 have been displaced along the horizontal axis. The maximal cell titer for all data in this figure occurred at 180 min. Mannitol (MTL) was added at 90 min. DAP, diaminopimelic acid; φ ala, phenylalanine.*

etate incorporation was inhibited within 30 min after the addition of mannitol. Similar results were obtained by using $^{32}P_i$. At 30 min after the addition of mannitol to a culture of DB82 the doubling time for $^{32}P_i$ incorporation increased from 30 to 160 min. This inhibition of radioisotope incorporation into lipid was not observed when mannitol was added to cultures of the parental strain (SL751) or when the mannitol-sensitive mutant (DB82) was grown in the absence of mannitol. This alteration of lipid metabolism is an early consequence of mannitol sensitivity and is under further investigation.

DISCUSSION

The temporal sequence of events following the addition of mannitol to a sensitive strain (DB82) is shown more clearly in Fig. 6, which gives the incorporation data from Fig. 3, 4, and 5 in semilog form. Acetate incorporation was inhibited at least 30 min before any of the other effects were seen. Nucleic acid synthesis continued at at least the normal rate until lysis, even though protein synthesis was inhibited. Diaminopimelic acid incorporation was not inhibited until the time of lysis.

The relationship of the inhibition of acetate incorporation to the slowing of growth and eventual lysis of the strain is not clear. Acetate incorporation was inhibited long before the intracellular mannitol-1-phosphate concentration was maximal (Fig. 2). The accumulation of high concentrations of metabolites of mannitol may lead to as yet undetected events which are more directly responsible for the inhibition and lysis. An early inhibition of acetate incorporation was also seen after the addition of mannitol to DB80, a strain which is inhibited but does not lyse (Fig. 1). Inhibition of lipid synthesis, caused by faulty acylating enzymes (5) or by starvation for unsaturated fatty acids (9), does not lead to the rapid lysis seen with DB82. However, these are only

quantitative differences which may be dependent upon the growth medium. The cause of the inhibition of acetate incorporation and its relationship to growth inhibition and lysis are being examined.

The causes of glycerol and glucose sensitivities in *E. coli* have been described. Glycerol is toxic in certain strains, due to the production of methylglyoxal (7). The glucose sensitivity results from the inhibition of fructose diphosphatase by accumulated glucose-6-phosphate (6).

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LITERATURE CITED

1. Ames, G. F. 1968. Lipids of *Salmonella typhimurium* and *Escherichia coli:* structure and metabolism. J. Bacteriol. **95**:833–843.
2. Berkowitz, D. B. 1971. D-Mannitol utilization in *Salmonella typhimurium*. J. Bacteriol. **105**:232–240.
3. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**:911–917.
4. Bollum, F. J. 1959. Thermal conversion of nonpriming deoxyribonucleic acid to primer. J. Biol. Chem. **234**: 2733–2734.
5. Cronan, J. E., T. K. Ray, and P. R. Vagelos. 1970. Selection and characterization of an *E. coli* mutant defective in membrane lipid biosynthesis. Proc. Nat. Acad. Sci. U.S.A. **65**:737–744.
6. Fraenkel, D. G. 1968. The accumulation of glucose-6-phosphate from glucose and its effect in an *Escherichia coli* mutant lacking phosphoglucose isomerase and glucose-6-phosphate dehydrogenase. J. Biol. Chem. **243**:6451–6457.
7. Freedberg, W. B., W. S. Kistler, and E. C. C. Lin. 1971. Lethal synthesis of methylglyoxal by *Escherichia coli* during unregulated glycerol metabolism. J. Bacteriol. **108**:137–144.
8. Gordon, H. T., W. Thornberg, and L. N. Werum. 1956. Rapid paper chromatography of carbohydrates and related compounds. Anal. Chem. **28**:849–855.
9. Henning, U., G. Dennert, K. Rehn, and G. Deppe. 1969. Effects of oleate starvation in a fatty acid auxotroph of *Escherichia coli* K-12. J. Bacteriol. **98**:784–796.
10. Mauck, J., L. Chan, and L. Glaser. 1971. Turnover of the cell wall of gram-positive bacteria. J. Biol. Chem. **246**:1820–1827.
11. Rees, W. R., and T. Reynolds. 1958. A solvent for the paper chromatographic separation of glucose and sorbitol. Nature (London) **181**:767–768.
12. Smith, H. S., and L. I. Pizer. 1958. Abortive infection of *Escherichia coli* strain W by T2 bacteriophage. J. Mol. Biol. **37**:131–149.