MACROMOLECULE SYNTHESIS IN LONG FORMS OF *MYCOBACTERIUM BOVIS* INDUCED BY SULFATE DEFICIENCY

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Spitznagel and Sharp (1959) reported that mycobacteria starved of sulfate in a chemically defined medium increased in length and width but did not divide. Basing their conclusions on cytological studies, they suggested that ribonucleic acid (RNA) had continued to increase in these cells whereas deoxyribonucleic acid (DNA) had not.

There has been considerable interest in the differential rates of synthesis of macromolecules in bacterial systems distorted by a variety of means (reviewed by Roberts, McQuillen, and Roberts (1959)). This has been due to the utility of such systems in studying interactions between the synthesis of nucleic acids and proteins. These systems have depended upon the treatment of cultures with antibiotics or antimetabolites, the culture of auxotrophs and wild-type organisms on specifically deficient media, bacteriophage infection, physical disturbances, and combinations of these methods.

This paper describes the changes which occur in the nucleic acid and protein metabolism of *Mycobacterium bovis* growing in a sulfate-deficient medium. These findings are of interest because they provide additional information concerning events which may occur during the development of bacterial long forms. Mycobacterial long forms possess intrinsic interest because they occur in certain tuberculous lesions (Spitznagel and Sharp, 1959). The condition to be described is unique in that it is created by starving the bacilli of inorganic sulfur. It is lethal for the bacteria but leads to continued net synthesis of RNA without continued net synthesis of protein or DNA.

MATERIALS AND METHODS

The Phipps strain (BCG) of *M. bovis* (obtained originally from the Henry Phipps Institute, Philadelphia) was used.

The media used have been described previously (Spitznagel and Sharp, 1959). Stock cultures were grown in a liquid Tween 80-albumin medium. The experimental cultures were grown in a liquid medium containing asparagine, salts, Tween 80, and glucose. The experimental medium is markedly deficient in sulfur sources. This medium was also used to wash and dilute the bacterial inocula.

Pyrex brand glassware was used and was washed by procedures found satisfactory for glassware preparation in studies on magnesium and sulfate deficiency (Spitznagel and Sharp, 1959).

The results are derived from multiple observations made upon two sets of replicate cultures. The work was planned to obtain simultaneously quantitative data concerning cell size, cell viability, and macromolecule synthesis.

A culture was grown for 7 days in the liquid Tween 80-albumin medium and then washed three times in the sulfate-deficient medium. The organisms were suspended and inoculated into 1,000 ml of the sulfate-deficient medium. The medium so inoculated contained 3.2×10^7 bacterial units/ml and had a turbidity of 15 Klett units at 543 m μ . The inoculated medium was kept thoroughly agitated, divided into 50-ml samples, and distributed into steel-capped, 300-ml flasks equipped with side arms suitable for turbidity measurements in a photoelectric colorimeter. One-half of the flasks were selected at random and their contents supplemented with sterile K₂SO₄ (0.4 mM/liter). The other one-half of the flasks received an equal volume of sterile deionized distilled water.

The turbidity of each flask was measured and recorded daily. Immediately and at 24-hr intervals flasks were selected randomly from each group for analysis. Viable bacterial counts, and direct bacterial counts were determined, and the

residual sample of each culture was frozen at -20°C to permit chemical analyses to be performed on all specimens simultaneously. The direct bacterial counts were done by standard methods using the Petroff-Hauser counting chamber and a phase microscope. The viable counts were done by the surface-plate count technique (Fenner and Leach, 1958). Clumps of bacteria were counted as one. In doing the viable counts the cultures were diluted to contain 2×10^8 cells/ml according to the direct count. Ten drops of this dilution each containing 0.025 ml were deposited on each of two duplicate plates which were then incubated at 37°C for 2 weeks. Thus the total number of cells sampled was approximately 1,000 for each culture. Air-dried smears of the cultures were prepared for morphological studies. In the case of the cultures which failed to show division of the organisms, it was found that repeated shaking was reducing the average number of bacteria per clump. Thus an increase in the number of bacterial units was produced. A correction was therefore applied by counting the frequency of numbers of bacteria per clump, and calculating from this the number of bacterial cells actually present. The method used was that of Fenner and Leach (1958). It was found that the number of bacterial units in the sulfate-deficient cultures increased during the experiment but the actual number of cells did not change. The clumps had indeed been broken up into smaller aggregates. It was not feasible to apply such correction to the sulfate-replete cultures because both the number of clumps and the number of bacterial cells per clump increased.

Bacterial dry weights were determined. Ten milliliters or more of each frozen sample were measured and dried to constant weight after three washings in distilled water. Weighings were done on a Mettler analytical balance (series B). The determinations of dry weights on sulfate-deficient cells from days 4 through 8 were not entirely satisfactory. These organisms formed unstable pellets during centrifugation, making it very difficult to remove the supernatant without disturbing them.

Measurements of bacterial size were made on no fewer than 100 cells per culture, using a calibrated ocular micrometer. Randomly selected cells were measured to the nearest micron. The microscope used was a Leitz Ortholux equipped with a $100\times$ achromatic oil immersion lens

na 1.30 and $10\times$ perplanatic oculars. Total magnifications were 1,250.

The bacterial smears were prepared for examination by air drying and staining with Kinyoun's stain (Spitznagel and Sharp, 1959).

RNA and DNA were determined photometrically by means of the orcinol (Mejbaum, 1939) and diphenylamine reactions (Burton, 1956), respectively, in perchloric acid extracts prepared by a modification of the method of Schneider (1945). The results of the orcinol determinations were corrected for color due to the presence of DNA. Protein nitrogen was determined by digestion and nesslerization of the residues from the hot perchloric acid extractions (Lanni, Dillon, and Beard, 1950).

RESULTS

The effects of sulfate starvation on the length of *M. bovis* are shown in Fig. 1. The average length of the bacilli at the beginning of the experiment (not shown) was $2\ \mu$. Both sulfate-replete and sulfate-deficient organisms increased in length although the increase was greatest in the sulfate-deficient cultures. This increase in length continued exponentially in the sulfate-deficient cultures for an additional 48 hr and then slowed considerably but still continued until the sixth day. The diameter of the sulfate-deficient bacteria also increased, but this was not measured. The stage of increased cell size was only transient in the sulfate-replete cultures, as might have been expected (Fenner and Leach, 1958), and by the third day the organisms had returned to the average length of $2\ \mu$.

Morphological changes which characterize these events have been described and illustrated

Fig. 1. Effect of sulfate deficiency on the length of *Mycobacterium bovis* grown in a chemically defined liquid medium.

Fig. 2 (A, left). Effects of sulfate deficiency on nucleic acid synthesis by *Mycobacterium bovis* grown in a chemically defined medium. (B, right) Effects of sulfate deficiency on the accumulation of protein in cultures of *M. bovis* grown in a chemically defined medium.

(Spitznagel and Sharp, 1959). The acid fastness of the sulfate-deficient bacilli was less intense than that of the controls. There tended to be a bulbous enlargement in the center of the organisms. This central structure gave a positive stain reaction for lipids, chromatin, and volutin arranged in a distinctive manner. The distal portions of the organism were basophilic. The basophilia was diminished by ribonuclease digestion. Electron microscopy revealed that the central structure appeared as a large vacuole or vacuoles with very dense electron-scattering granules adjacent. In unsectioned organisms the structure of the distal portions appeared rather homogeneous but unpublished studies which have been made in this laboratory using ultrathin sections have revealed that complex structure may be visualized in the distal areas of the deficient bacilli.

In the sulfate-deficient cultures net synthesis of ribonucleic acid occurred (Fig. 2A). RNA increased at a constant rate throughout the course of the experiment. Its concentration was increased approximately 5-fold in 8 days.

The RNA content per cell was not calculated because of the tendency of these bacteria to clump. Since the increase in numbers of bacterial cells was negligible in the sulfate-deficient cultures, it is evident that the RNA content per cell also increased.

The DNA (Fig. 2A) did not undergo net synthesis with sulfate-deficient cultures. There was not even a doubling of this material and the concentration fell relative to the size and mass of the cells (compare Figs. 2A, 3A, and 4). The sulfate-replete cultures behaved quite differently. Net synthesis of both RNA and DNA took place and the rates of synthesis were greater at all stages of the experiment than was the rate of synthesis of RNA in the sulfate-deficient cultures. The ratio between RNA and DNA concentrations showed much less variation in the sulfate-replete organisms. The final ratio was 6:1. In the sulfate-deficient cultures the final ratio of RNA to DNA was 12:1.

Net protein synthesis was not detected in sulfate-deficient cultures (Fig. 2B). In the sulfate-replete cultures, however, net protein syn-

Fig. 3 (A, left). Effects of sulfate deficiency on the turbidity increase in cultures of *Mycobacterium bovis* grown in a chemically defined medium. (B, right) Effects of sulfate deficiency on the viability and cellular integrity of *M. bovis* grown in a chemically defined medium.

thesis proceeded at a rate similar to that for the nucleic acids in the same cultures. The turbidities of the cultures (Fig. 3A) followed curves similar to those for dry weight (Fig. 4). The time course for the turbidity increases in the sulfate-deficient cultures was parallel with the time course for changes in bacillary length. The time course for the turbidity increases in the sulfate-replete cultures bore no relation to the bacillary length but was similar to the nucleic acid and protein synthesis curves, as well as the direct and viable counts.

The viability of the long forms induced by sulfate deficiency was determined (Fig. 3B) and compared with the viability of the sulfate-replete organisms. The sulfate-deficient forms continued to grow in size and increase their RNA content but they rapidly lost their viability. Beginning on day 3 of the culture cycle, there was a rapid decline in the viable count in sulfate-deficient cultures, although the viable count in

the sulfate-replete cultures was increasing rapidly at this time.

The direct bacterial counts were also compared. The direct counts remained constant in the sulfate-depleted cultures, indicating that no autolysis occurred. In the sulfate-replete cultures, the direct bacterial counts increased parallel with the viable counts.

DISCUSSION

The induction of long forms in cultures of mycobacteria, by means of chemically defined nutritional manipulations, and the role of inorganic sulfur in the morphogenesis of bacteria have been described and the pertinent literature reviewed (Spitznagel and Sharp, 1958, 1959). Winder and Denny (1959) reported that iron or zinc deficiency caused *Mycobacterium smegmatis* to become unusually long in liquid culture. They stated that net protein and DNA synthesis continued, whereas net RNA synthesis appeared

Fig. 4. Dry weight increase in cultures of *Mycobacterium bovis* growing in sulfate-replete and sulfate-deficient, chemically defined media. The broken line indicates instability of pellets formed by centrifugation during washing procedures.

to be suppressed, but did not report on the viability of the starved bacteria. Other than this, reports concerning macromolecule synthesis in long forms appear to have been concerned with other genera.

The behavior of sulfate-deficient *M. bovis* resembled that of thymineless *Escherichia coli* (Cohen and Barner, 1954) in that unbalanced growth occurred which was accompanied by imbalance in the synthesis of nucleic acid and protein and was lethal for the bacteria. A striking difference was noted in that net synthesis of protein was suppressed, as well as net synthesis of DNA, whereas net synthesis of RNA continued when *M. bovis* was starved of sulfate. Both conditions were lethal. The effects of sulfate starvation on BCG differed from the effects of chloramphenicol treatment on *E. coli* (Wissemann et al., 1954) in that sulfate starvation inhibited net DNA synthesis as well as net protein synthesis. The effects of sulfate depriva-

tion in *M. bovis* most closely resembled effects produced by Borek, Rockenbach, and Ryan (1956) in a unique methionineless *E. coli*. Borek found that methionine deprivation caused *E. coli* strain K12W-6 to continue RNA synthesis, whereas DNA and protein synthesis were inhibited. This condition, unlike the sulfateless condition in *M. bovis*, was reported to be non-lethal. Other systems, characterized by continued net RNA synthesis and absence of net DNA and protein synthesis, have been reported. Gros and Gros-Doulcet (1958) produced such a system by growing amino acid auxotrophs of *E. coli* in media which contained chloramphenicol but were deficient for the required amino acids. Kerridge (1958) found that *Saccharomyces carlsbergensis* grown in Actidione performed net synthesis of RNA but not of DNA or protein.

The absence of a sulfur source would be expected to cause a deficiency of sulfur-containing amino acids and a general disturbance of protein synthesis. It was indeed observed that net synthesis of protein did not occur. It was apparent, however, that net synthesis of molecules other than protein continued in the sulfate-deficient cells. Presumably this activity was carried on by enzymes and other factors synthesized prior to or very soon after the *M. bovis* culture was made sulfate deficient. Hogness, Cohn, and Monod (1955) have shown that little or no amino acid turnover occurs in growing *E. coli*. If the same is true of *M. bovis* then sulfur-containing amino acids would not be available for synthesis of new proteins. The failure of protein synthesis would then be manifest principally in the systems requiring new proteins. For this reason, and by analogy with the findings of McQuillen, Roberts, and Britten (1959) in *E. coli*, it seems likely that the RNA synthesized by sulfateless *M. bovis* would be either soluble RNA or the nucleic acid components ordinarily destined to become ribosomes. In any case it appears that some RNA synthesis can take place in the absence of net protein synthesis and possibly in the absence of any new protein synthesis.

The mechanisms by which sulfate deficiency interferes with net DNA synthesis are much less easy to envision owing to the limited scope of our data and to the less advanced state of general knowledge in this area. At present it

seems reasonable to assume that net DNA synthesis cannot proceed without the concomitant synthesis of some substance which requires sulfur. It is hardly useful to speculate as to whether this factor would be enzyme protein, structural protein, or a cofactor of some type. However, since net RNA synthesis took place in the absence of net DNA synthesis, it seems unlikely that the missing substance would be an enzyme or a cofactor. Such an enzyme or cofactor would be quite different from those involved in RNA synthesis and its activity would not be shared by the synthetic pathways common to both RNA and DNA. It would be rapidly exhausted and require to be synthesized practically simultaneously with DNA. It seems more likely that sulfur deficiency prevents synthesis of a nonenzymatic protein essential to the synthesis of DNA.

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SUMMARY

Mycobacterium bovis grown in a chemically defined medium deficient in sulfate increased in size but did not divide, thus becoming long forms. The growth of the long forms was accompanied by changes in the patterns of macromolecule synthesis. Net synthesis of ribonucleic acid continued in a linear fashion. Net synthesis of deoxyribonucleic acid and protein was not detected. The dry weight of the bacteria and the turbidity of the cultures increased parallel to the increase in cell length. The viability of the long forms decreased rapidly beginning about the third day of the culture cycle, although direct bacterial counts remained constant.

REFERENCES

- BOREK, E., J. ROCKENBACH, AND A. RYAN 1956 Studies on a mutant of *Escherichia coli* with unbalanced ribonucleic acid synthesis. *J. Bacteriol.*, **71**, 318-323.
- BURTON, K. 1956 A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.*, **62**, 315-323.
- COHEN, S. S., AND H. D. BARNER 1954 Studies on unbalanced growth in *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S.* **40**, 885-893.
- FENNER, F., AND R. LEACH 1958 The growth of mammalian tubercle bacilli in Tween-albumin liquid medium. *Am. Rev. Tuberc. Pulmonary Diseases*, **63**, 321-339.
- GROS, F., AND F. GROS-DOULCET 1958 Role des acides amines dans la synthèse des acides nucléiques chez *Escherichia coli*. *Exptl. Cell Research*, **14**, 104-131.
- HOGNESS, D. A., M. COHN, AND J. MONOD 1955 Studies on the induced synthesis of β -galactosidase in *Escherichia coli*. The kinetics and mechanism of sulfur incorporation. *Biochim. et Biophys. Acta*, **16**, 99-116.
- KERRIDGE, D. 1958 The effect of Actidione and other antifungal agents on nucleic acid and protein synthesis in *Saccharomyces carlsbergensis*. *J. Gen. Microbiol.*, **19**, 497-506.
- LANNI, F., M. L. DILLON, AND J. W. BEARD 1950 Determination of small quantities of nitrogen in serological precipitates and other biological materials. *Proc. Soc. Exptl. Biol. Med.*, **74**, 4-7.
- MCQUILLEN, K., R. B. ROBERTS, AND R. J. BRITTEN 1959 Synthesis of nascent protein by ribosomes in *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S.*, **45**, 1437-1447.
- MEJBAUM, W. 1939 Über die Bestimmung kleiner Pentosemengen, insbesondere in Derivaten der Adenylsäure. *Z. physiol. Chem.*, **258**, 117-120.
- ROBERTS, R. B., K. MCQUILLEN, AND I. Z. ROBERTS 1959 Biosynthetic aspects of metabolism. *Ann. Rev. Microbiol.*, **13**, 1-48.
- SCHNEIDER, W. C. 1945 Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. *J. Biol. Chem.*, **161**, 293-303.
- SPITZNAGEL, J. K., AND D. G. SHARP 1958 Effects of magnesium sulfate on the growth and morphology of *M. tuberculosis*. *Bacteriol. Proc.*, **1958**, 81.
- SPITZNAGEL, J. K., AND D. G. SHARP 1959 Magnesium and sulfate ions as determinants in the growth and reproduction of *Mycobacterium bovis*. *J. Bacteriol.*, **78**, 453-462.
- WINDER, F. G., AND J. M. DENNENY 1959 Effect of iron and zinc on nucleic acid and protein synthesis in *Mycobacterium smegmatis*. *Nature*, **184** (Suppl. no. 10), 742-743.
- WISSEMAN, C. L., J. E. SMADEL, F. E. HAHN, AND H. E. HOPPS 1954 Mode of action of chloramphenicol on assimilation of ammonia and on synthesis of proteins and nucleic acids in *Escherichia coli*. *J. Bacteriol.*, **67**, 662-673.