INFECTION AND IMMUNITY, June 1977, p. 967-973

Vol. 16, No. 3
Printed in U.S.A.

# Factors Regulating Cell Wall Thickening and Intracellular Iodophilic Polysaccharide Storage in *Streptococcus mutans*

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Received for publication 14 February 1977

The effects of a series of different antibiotics on the synthesis and accumulation of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), protein, cell wall peptidoglycan (PG), and intracellular iodophilic polysaccharide (IPS) in *Streptococcus mutans* FA-1 were examined. D-Cycloserine, penicillin G, or vancomycin treatment resulted in rapid inhibitions of PG synthesis and a consequent decrease in the relative amount of lysine found in PG fractions. Decreases in culture turbidity, an indicator of gross cellular lysis, were not observed. Secondary inhibitions of the rates and extent of syntheses of DNA, RNA, and protein were observed. With all three inhibitors of PG synthesis, IPS synthesis continued for varying time intervals but, at most, resulted in only relatively small and transient increases in cellular IPS content. Chloramphenicol inhibited protein synthesis but permitted continued synthesis of RNA and PG. After 6 h, the cells contained 42% of their [$^3$H]lysine in the PG fraction compared with 25% in exponential-phase cells, a good indication of thickened cell walls. In the presence of chloramphenicol, cellular IPS content increased about 2.5-fold during the first 45 min and then decreased to a level (13%) at 6 h very similar to that of exponential-phase cells (about 10%). Rifampin inhibition of RNA (and, consequently, also protein) synthesis resulted in accumulation of cellular PG and IPS. After 6 h, IPS accounted for 38% of the cellular dry weight, and the cells contained 43% of their lysine in PG. Thus, rifampin-inhibited cells appear to have both thickened walls and a high IPS content. The correlation between inhibition of RNA synthesis and IPS accumulation was confirmed by exposing cultures to rifampin for 60 min and then removing the drug, thus permitting the cells to regrow. Upon removal of rifampin and resumption of RNA synthesis, cellular IPS content rapidly decreased to the level expected for exponential-phase cells.

Van Houte and Saxton (20) observed that coccal-shaped cells with thickened gram-positive walls, which were located in the deeper regions of dental plaque and close to the tooth surface, contained many intracellular iodophilic polysaccharide (IPS) granules. However, cocci located in the outer portions of plaque had thinner cell walls and fewer IPS granules and, in general, appeared to be more representative of actively growing bacterial cells. Thickening of the cell wall has been observed to occur in streptococci and lactobacilli upon nitrogen starvation (3, 13). Bacteria with thickened walls are known to be resistant to many chemical and physical agents such as lysozyme (15), antibiotics (12), and cold shock (14), and to survive longer under non-nutrient conditions at 37°C (14). This physiological resistance is similar to that of bacterial spores except for the fact that the thick-walled cells can continue to metabolize and, for example, produce acid (19).

Mattingly et al. (8) recently examined the effect of withholding essential amino acids on macromolecular synthesis (deoxyribonucleic acid [DNA], ribonucleic acid [RNA], protein, and peptidoglycan [PG]) and IPS accumulation in *Streptococcus mutans* FA-1. When cells from the balanced, exponential growth phase were washed and suspended in medium lacking required amino acids (glutamate/glutamine or lysine) that are precursors of PG, further synthesis of DNA, RNA, protein, and PG rapidly terminated. However, IPS continued to accumulate at a rapid rate, and, within 2 to 3 h, the cellular IPS content increased from 10 to 37% of the cellular dry weight. Deprivation of amino acids (leucine and cystine) that are not precursors of PG resulted in significant increases in

all macromolecules over extended periods of incubation, including a doubling in the cellular content of IPS. However, in contrast to results obtained with *S. faecalis* 9790 (when required, non-wall amino acids were withheld [13]), little, if any, cell wall thickening was detected either by isotopic or electron microscopic analysis. Wall thickening was only observed in *S. mutans* when protein synthesis was inhibited by tetracycline treatment (8). However, in this latter case, little accumulation of IPS was observed.

In the present study, we examined the effect of a variety of antibiotics on wall thickening and IPS accumulation and found that inhibition of RNA synthesis resulted in cells with both thickened walls and large amounts of IPS.

## MATERIALS AND METHODS

**Growth conditions.** *S. mutans* FA-1 was obtained from A. Bleiweis, Department of Microbiology, University of Florida, Gainesville, Fla. Cultures were stored in the lyophilized state. Routinely, lyophilized cultures were rehydrated and grown for 24 h at 37°C on Todd-Hewitt agar (BBL) supplemented with 2% D-glucose. This initial culture was used to inoculate a series of slants, which were stored at 4°C for no longer than 4 weeks. These slants, only one transfer away from the lyophilized culture, were used to inoculate cultures in a chemically defined liquid medium that was described previously (17). Purity of the cultures was monitored by plating on mitis salivarius and blood agar plates, as well as by determining the ability of the cultures to ferment mannitol and sorbitol and to clump in the presence of 2% sucrose. All experiments were performed with cultures growing "aerobically" (16) in the chemically defined medium to which freshly prepared, filter-sterilized (Millipore Corp.; 0.45 μm) 0.01 M sodium bicarbonate was added just prior to the start of the experiment. Inocula were grown into the mid-exponential phase of growth (0.23 mg/ml, dry weight) in the chemically defined medium prior to use. In experiments in which the incorporation of radioactively labeled precursors was determined, the specific activity of each precursor was increased by decreasing the normal concentration of that component in the growth medium. For example, lysine and valine were reduced from 100 to 30 μg/ml; leucine was reduced from 100 to 10 μg/ml; glutamate was reduced from 300 to 30 μg/ml; cystine was reduced from 200 to 1 μg/ml; and uracil was reduced from 30 to 20 μg/ml. In all cases, the reduced concentrations of the various nutrients were sufficient to insure a more-than-adequate supply of that component and did not significantly affect either growth rate or yield. When [$^{14}$C]thymidine was used as a labeled precursor of DNA, 15 μg of non-radioactive thymidine per ml was added to the medium. Also, to prevent catabolism of exogenous thymidine, 200 μg of 2'-deoxyadenosine per ml was added to the cultures (7).

Growth was measured turbidimetrically at 675 nm and expressed in adjusted optical density (AOD) units (18). One AOD unit is equivalent to 0.39 μg of cellular dry weight per ml (17).

**Determinations of DNA, RNA, protein, PG, and IPS contents.** Radioisotopic methods were used to determine DNA, RNA, protein, and PG (9). Exponential-phase cultures were grown for at least eight generations in the presence of the appropriately radiolabeled precursor, so that the measured radioactivity would reflect the relative cellular level of these macromolecules. For DNA and RNA determinations, cultures were grown in the presence of 0.1 μCi of [2-$^{14}$C]thymidine per ml or [2-$^{14}$C]uracil per ml, respectively, and 0.5-ml samples were precipitated with 5 ml of ice-cold 10% trichloroacetic acid. For protein determination, cultures were grown in the presence of 1.0 μCi of L-[4,5-$^{3}$H]valine per ml and 0.5-ml samples were precipitated with 10% trichloroacetic acid and heated at 95°C for 30 min. PG was determined by a modification of the procedure of Boothby et al. (4) as follows. Instead of heating the acid precipitates for 30 min at 95°C, the heating time was decreased to 10 min. L-[$^{3}$H]lysine (1.0 μCi/ml) was used as the labeled precursor of PG. Relative amounts of PG were determined by comparing the levels of [$^{3}$H]lysine incorporated into the PG fraction (4) with those incorporated into hot trichloroacetic acid precipitates (protein plus PG). All trichloroacetic acid precipitates were collected on glass-fiber filters (Reeve Angel, no. 984-H), and the filters were washed three times with cold 10% trichloroacetic acid and twice with absolute alcohol. Filters were transferred to scintillation vials, and precipitates were dissolved in 0.5 ml of 90% NCS (Amersham/Searle Corp.) for 2 h at 50°C. After the mixture cooled to room temperature, 5 ml of a toluene-based scintillation cocktail (10) was added, and the samples were counted in a Searle Mark III scintillation counter equipped with computer conversion of counts per minute to disintegrations per minute. IPS was determined as previously described (5).

**Studies on the effect of antibiotic treatment of macromolecular synthesis.** Exponential-phase cultures were grown to an AOD of 600 (0.23 mg of cellular dry weight per ml). The cells were harvested on a 0.65-μm membrane filter, washed with two 20-ml portions of prewarmed (37°C) medium, and suspended in the chemically defined medium at a cellular turbidity of 150 to 200 AOD (0.06 to 0.08 mg of cellular dry weight per ml) containing individually, or in combination, the following antibiotics: chloramphenicol, 20 μg/ml; rifampin, 0.5 μg/ml; D-cycloserine, 100 μg/ml; penicillin G, 0.1 μg/ml; and vancomycin, 5.0 μg/ml. When D-cycloserine was employed, the DL-alanine concentration was reduced from 110 to 30 μg/ml. The concentration of each antibiotic employed was that which prevented an increase in turbidity of cultures of *S. mutans* FA-1 inoculated at an AOD of 1 after 18 h at 37°C.

## RESULTS

**Inhibition of PG synthesis.** At the concentrations used, D-cycloserine (100 μg/ml), peni-

cillin G (0.1 μg/ml), and vancomycin (5 μg/ml) all rapidly inhibited PG synthesis (Fig. 1). Inhibition of incorporation of [$^3$H]lysine into PG, with continued incorporation into protein, resulted in a decrease in the relative amount of [$^3$H]lysine in PG of from 25 to about 19% of the total trichloroacetic acid-precipitable cellular [$^3$H]lysine (Fig. 2A). In contrast to observations with several other gram-positive species, such as *S. faecalis* (13), treatment of *S. mutans* FA-1

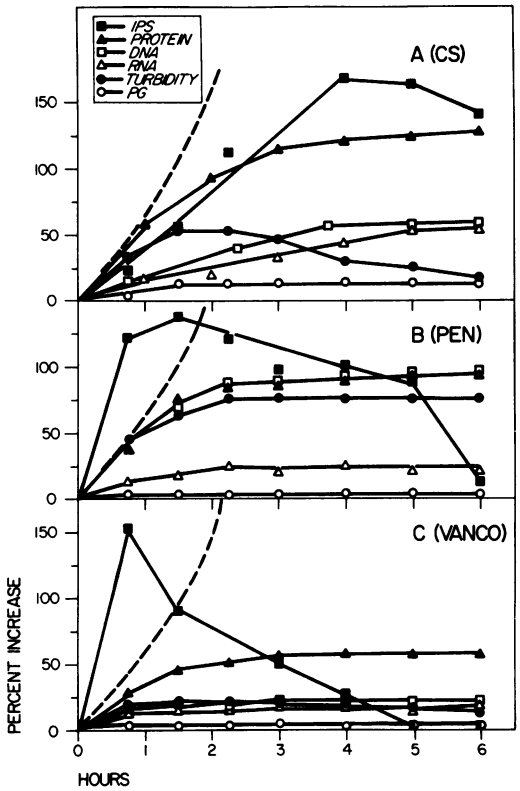


FIG. 1. *Effect of (A) D-cycloserine (CS) (100 μg/ml), (B) penicillin G (PEN) (0.1 μg/ml), and (C) vancomycin (VANCO) (5.0 μg/ml) on macromolecular synthesis in S. mutans FA-1. Increases in cellular mass (●) were determined turbidimetrically; DNA (□) was measured by the incorporation of [$^{14}$C]thymidine into cold 10% trichloroacetic acid precipitates; RNA (△) was measured by the incorporation of [$^{14}$C]uracil into cold 10% trichloroacetic acid precipitates; protein (▲) was measured by the incorporation of [$^3$H]valine into hot (95°C), 10% trichloroacetic acid precipitates; PG (○) was measured by the incorporation of [$^3$H]lysine into the PG fraction; and IPS (■) was determined chemically as previously described (5). The data are expressed as percent increases from time zero of antibiotic treatment. The dashed line represents the theoretical data for synthesis of each type of macromolecule during balanced exponential growth (by definition).*

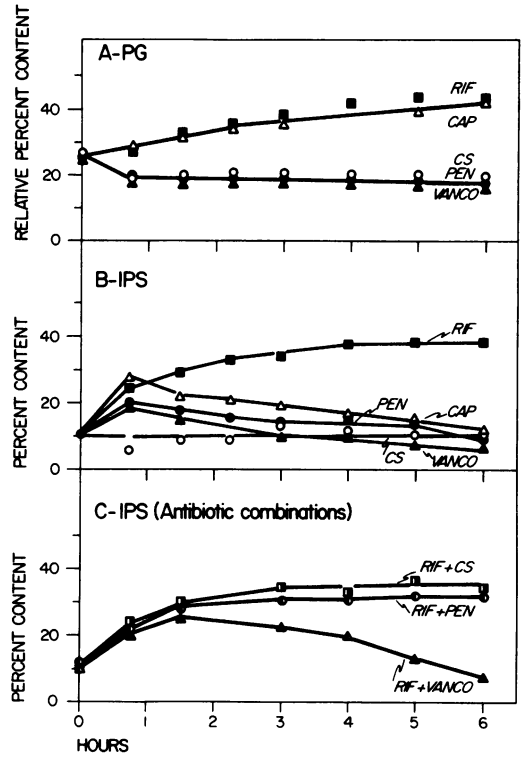


FIG. 2. *Effect of antibiotics and combinations of antibiotic on the percent cellular content (A) of [$^3$H]lysine in PG (B) and (C) IPS. Relative cellular PG content was determined by dividing [$^3$H]lysine in the PG by total trichloroacetic acid-precipitable [$^3$H]lysine (× 100). Percent content of IPS was calculated from chemical determinations on cell suspensions of known turbidity (and mass). Symbols: ○, D-cycloserine (CS); ●, penicillin G (PEN); ▲, vancomycin (VANCO); △, chloramphenicol (CAP); ■, rifampin (RIF); ◨, rifampin plus cycloserine; ◐, rifampin plus penicillin; and ◭, rifampin plus vancomycin.*

with any of these three inhibitors of PG synthesis failed to result in gross lysis of the cultures, as manifested by a substantial decrease in culture turbidity. In the case of D-cycloserine treatment (Fig. 1A), culture turbidity increased by about 50% in 90 min and then slowly decreased. After 6 h, culture turbidity was still 25% higher than at zero time. This turbidity increase was accompanied by an increase in protein at nearly the exponential rate for the first hour and then at a slowly decreasing rate for the remainder of the incubation, reaching a 125% increase over the zero-time level at 6 h. The syntheses of DNA and RNA were more severely affected than was protein synthesis by cycloserine. Slower rates of accumulation of DNA and RNA resulted in net increases of 65

and 60%, respectively, at 6 h. Cellular IPS accumulation continued (Fig. 1A) but at a rate substantially slower than that expected for exponential-phase cultures, and, after 4 h, reached a level 165% higher than that at zero time. When expressed in terms of percent of cellular dry weight (Fig. 2B), the IPS content of cycloserine-treated cultures remained at about the same level (about 10%) as untreated, exponential-phase cells. Thus, the increases in other cellular constituents (e.g., DNA, RNA, and protein) combined to increase cellular mass, resulting in a relative constant percent content of cellular IPS.

The consequences of penicillin G (0.1 μg/ml) and vancomycin (5 μg/ml) treatment were very similar to that of D-cycloserine. In fact, with both antibiotics, inhibition of PG synthesis was even more rapid and severe than that with cycloserine (Fig. 1B and C), and relative cellular content of PG decreased in a manner very similar to that induced by D-cycloserine (Fig. 2A). Quantitative differences in the secondary inhibitory effects of these antibiotics on DNA, RNA, and protein syntheses were noted. However, the extent and kinetics of these secondary effects are probably related to antibiotic concentration (M. Mychajlonka and G. D. Shockman, in preparation). Worth noting are the rapid increases in IPS levels accompanying penicillin G (Fig. 1B) and vancomycin (Fig. 1C) treatment, which were reflected as transient increases in percent content of cellular IPS (Fig. 2B).

**Inhibition of protein or of RNA and protein synthesis.** Chloramphenicol (20 μg/ml) treatment of exponentially growing cultures immediately inhibited protein synthesis (Fig. 3A). Continued DNA, RNA, PG, and IPS accumulations resulted in a substantial increase in cellular turbidity, which, at 6 h, reached a level 180% of the zero-time level. Continued PG accumulation, to a level 140% of the zero-time value, resulted in cells that contained 42% of their cellular lysine as PG as compared with 25% in exponential-phase cells. DNA continued to accumulate and, at 6 h, reached a level 190% of the zero-time level. RNA accumulated at the rate expected for exponential-phase cultures for 2 h, after which time the rate of accumulation decreased so that a level 375% of the zero-time level was attained at 6 h. These results are consistent with a "relaxed" type of response, also observed previously after tetracycline treatment of this organism (8). Significant was the observation that IPS accumulated at a rate faster than that expected for exponentially growing cultures. At 45 min, a 180% increase in

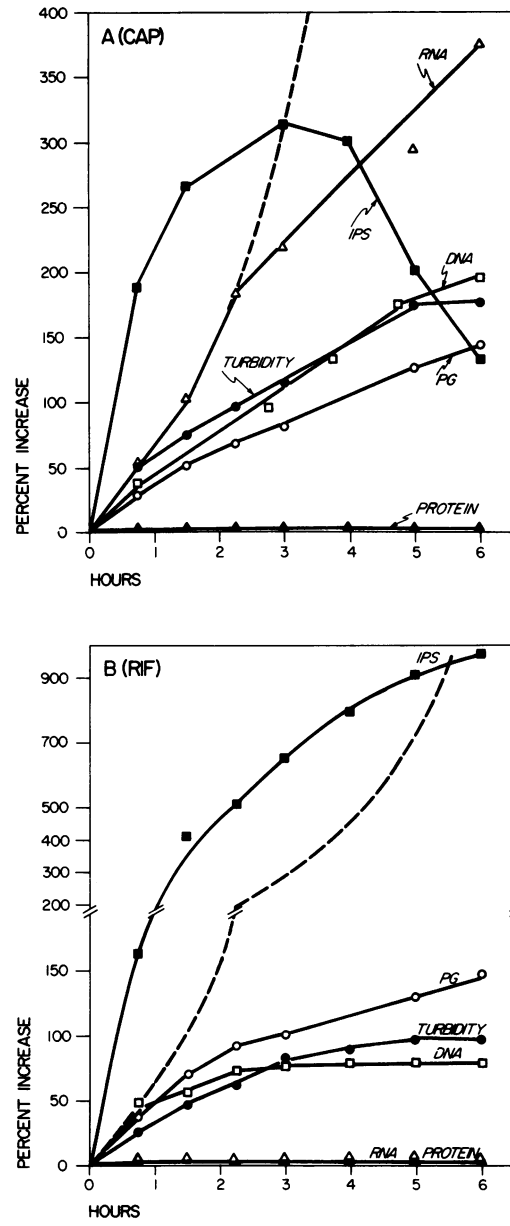


FIG. 3. *Effect of (A) chloramphenicol (CAP) (20 μg/ml) and (B) rifampin (RIF) (0.5 μg/ml) on macromolecular synthesis in S. mutans FA-1. See legend of Fig. 1 for symbols. Note the expanded scale in (B).*

IPS was observed, which yielded a cellular IPS content of 28% of cellular dry weight (Fig. 2B). After 3 h in chloramphenicol, cellular IPS content decreased to 13% of the cellular dry weight at 6 h—close to the same level measured in exponential-phase cells.

Rifampin (0.5 μg/ml) proved to be a potent

and rapid inhibitor of both RNA and protein syntheses in this organism (Fig. 3B). DNA and PG accumulations continued and, at 6 h, reached levels 76 and 140%, respectively, higher than those at zero time. Six hours after rifampin addition, cells contained 43% of their total cellular lysine in the PG fraction (Fig. 2A). Although culture turbidity did not increase as much as in the chloramphenicol-treated cultures (a 95% increase over the zero-time level at 6 h), IPS accumulated at a very rapid rate and reached the very high level of 975% of the zero-time level at 6 h. Expression of these results in terms of percent of cellular dry weight (Fig. 2B) showed that rifampin-treated cells contained 38% IPS at 4 h and remained at this high level for the next 2 h. This cellular content of IPS was nearly the same as the maximum reached in the stationary phase by this strain of *S. mutans* (5). Cells from such stationary-phase cultures were shown to contain very large numbers of IPS granules (5).

**Effects of the simultaneous inhibition of RNA, protein, and PG syntheses on IPS accumulation.** As described above, antibiotic inhibition of PG synthesis was accompanied by, at most, only small and transient increases in cellular IPS content (Fig. 2B). Therefore, the effect of simultaneous addition of rifampin plus D-cycloserine, penicillin, or vancomycin was examined (Fig. 2C). Both D-cycloserine and penicillin additions resulted in substantial increases in cellular IPS content. After 6 h, the cells contained 34 and 32%, respectively, of their dry weight as IPS. Somewhat less IPS was accumulated by cultures treated with rifampin plus vancomycin. Maximum cellular IPS content (23%) was observed after 1.5 h, after which the IPS content decreased to reach the levels observed in either exponential-phase cells or cells treated for 5 to 6 h with only vancomycin.

**IPS levels during recovery from rifampin treatment.** Further information on the relationship between continued RNA synthesis and IPS accumulation was obtained by exposing exponentially growing cultures to rifampin for 60 min and then removing the rifampin, thus permitting growth of the culture to resume (Fig. 4). As expected, rifampin addition resulted in an immediate cessation of RNA synthesis and a rapid increase in cellular IPS content from 10 to 26% of cellular dry weight. Removal of rifampin was accompanied by rapid resumption of RNA synthesis and a rapid decrease in cellular IPS content to the 10% level expected for exponential-phase cells, which was maintained during further growth. An exponential increase in culture turbidity was observed only after the attainment of the lower IPS content.

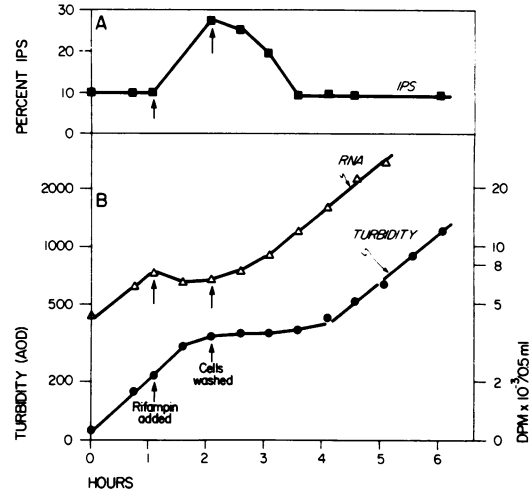


FIG. 4. *Regrowth of rifampin-treated cells of S. mutans FA-1. Rifampin (0.5 μg/ml) was added to a balanced exponential-phase culture (arrows) for 60 min. RNA synthesis was inhibited (B), but IPS increased from 10 to 26% of the cellular dry weight (A). The cells were washed in antibiotic-free complete growth medium (arrows), suspended in complete medium, and allowed to regrow. Symbols as in Fig. 1.*

## DISCUSSION

Addition of antibiotics that inhibit cell wall PG synthesis (D-cycloserine, penicillin, or vancomycin) to exponentially growing cultures of *S. mutans* FA-1 resulted in rapid inhibition of further net accumulation of PG (Fig. 1A) and, consequently, in a reduction of about 20% in relative cellular PG content (Fig. 2A). Although the method of labeling used would not detect a balance between continued PG accumulation and loss (e.g., turnover), culture turbidities remained relatively constant and at least somewhat above zero-time levels. Thus, in contrast to the rapid lysis induced by inhibition of cell wall synthesis in other streptococcal species (13), indications of cellular lysis or, for that matter, of a net loss in cellular PG were not obtained. The observed maintenance of intact cells upon treatment with cell wall antibiotics is consistent with previously reported data (8), which showed that deprivation of lysine or glutamate, two amino acids that are required for growth of *S. mutans* FA-1 (16) and that are components of its cell wall PG (2), was not followed by gross cellular autolysis observable by turbidimetric measurements (13). Furthermore, in cultures treated with any of the three antibiotic inhibitors of PG synthesis, continued accumulations of protein and IPS and, to a lesser extent, of DNA and RNA are consistent with the maintenance of metabolically

active cells for at least a portion of the observation period. The reduced rates of accumulation of the informational macromolecules upon specific inhibition of PG synthesis suggests that, at least in this species in which gross cellular autolysis does not seem to be a factor, syntheses of DNA, RNA, and protein may be more closely coupled to continued PG synthesis than previously considered.

From the studies presented here and earlier (8), the factors that regulate IPS and PG syntheses appear to be independently regulated. Inhibition of continued PG, RNA, and protein syntheses via lysine or glutamate deprivation (8) was accompanied by large accumulations of IPS and an increase in cellular IPS content to about 38% of the cellular dry weight. In contrast, as shown above, relatively specific antibiotic inhibition of PG synthesis under conditions that permitted RNA and protein syntheses to continue, although at reduced rates, was accompanied by, at most, relatively small and transient increases in cellular IPS content (Fig. 2B). Also, as shown above, inhibition of protein synthesis by chloramphenicol, which permitted continued RNA synthesis, was not accompanied by maintenance of increased IPS content. Thus, the magnitude of increase in IPS content and its maintenance do not appear to be related to inhibition of PG or of protein synthesis but appear to be more closely linked to inhibition of RNA synthesis.

In all cases examined in the previous (8) and current studies, inhibition of protein synthesis was accompanied by continued PG synthesis (e.g., Fig. 2A) and, presumably, wall thickening. Wall thickening appears to occur in both the presence (e.g., chloramphenicol treatment) and absence (rifampin treatment) of continued RNA synthesis. A major difference between the presence and absence of continued RNA synthesis seems to be in the continued accumulation of IPS. The maximum cellular content of IPS was observed when RNA (and protein) synthesis was inhibited with rifampin (Fig. 2B). Also, accumulation of high levels of IPS occurred when RNA, protein, and PG syntheses were all inhibited (Fig. 2C). Except in the case of treatment with vancomycin, which may exert secondary effects, high levels of cellular IPS content were maintained for 6 h.

Synthesis of bacterial glycogen is known to be catalyzed by two enzymes (6). The first enzyme, adenosine-5′-diphosphate (ADP)-glucose synthase, results in the synthesis of ADP-glucose from adenosine-5′-triphosphate (ATP) and glucose-1-phosphate (ATP + glucose-1-P $\rightleftarrows$ ADP-glucose + inorganic pyrophosphate). The second enzyme, ADP-glucose:$\alpha$-glucan-4-glucosyltransferase, catalyzes the transfer of glucose from ADP-glucose to glycogen (ADP-glucose + glucan primer $\rightleftarrows$ $\alpha$-1,4-glucosylglucan + ADP). Allosteric regulation of bacterial glycogen synthesis is known to occur at the level of ADP-glucose synthase (6). The most important effectors in the regulation of ADP-glucose synthase in *Escherichia coli* B are the energy charge ([ATP] + 0.5 [ADP]/[ATP] + [ADP] + [AMP]), which is a measure of the metabolic energy available from adenine nucleotides (1), and the level of fructose-1,6-diphosphate. An increase in the level of adenosine-5′-monophosphate (AMP) has been demonstrated to inhibit ADP-glucose synthase (11), while accumulation of ATP and subsequent formation of fructose-1,6-diphosphate result in activation of the enzyme.

The relationship of IPS accumulation to the synthesis of other cellular macromolecules can be explained, in part, on the basis of the regulation of ADP-glucose synthase. *S. mutans* FA-1 accumulated IPS (a glycogen-amylopectin type of polymer) in a two-step fashion during a typical culture growth cycle (5). When the stationary phase of growth was entered, the IPS level increased from about 10% of the cellular dry weight maintained during balanced exponential growth to 37 to 40% of the cellular dry weight. These results can be interpreted in terms of a substantial requirement for ATP in the synthesis of the other macromolecules (DNA, RNA, protein, and PG) during exponential growth, leaving only a low level of ATP for IPS synthesis. During the stationary phase of growth and reduced rates of macromolecular synthesis, the ATP drain is minimal and excess ATP can be used for the synthesis and storage of large amounts of IPS. Continued RNA and protein syntheses at reduced rates after antibiotic inhibition of PG synthesis resulted in continued consumption of ATP and permitted little or, in the case of D-cycloserine, no increase in cellular IPS content. Inhibition of both RNA and protein syntheses with rifampin spared large amounts of ATP, which could then be used for IPS accumulation. The addition of an inhibitor of PG synthesis to rifampin-treated cultures failed to increase the near-maximal level of IPS accumulation seen with rifampin alone. This relationship of sparing of ATP consumption, especially for RNA synthesis, is consistent with previous data (8) obtained with glutamate- or lysine-starved cultures. In these latter cases, RNA, protein, and PG syntheses were all severely inhibited, and again cells with a high content of IPS were obtained. Finally, the negative correlation between the on-

going level of RNA synthesis and IPS accumulation was demonstrated in the cultures released from rifampin inhibition (Fig. 4). Resumption of RNA synthesis was accompanied by a drop in cellular IPS content to the level characteristic of exponentially growing cultures.

The amounts of IPS found in various strains of *S. mutans* and other oral streptococci (5) grown under the usual conditions of culture appear to range from very high (e.g., *S. mutans* FA-1) to very low (e.g., *S. mutans* OMZ-176). Although such data provide information on IPS content of these organisms, in the in vitro growth conditions used, they fail to provide information on the capacity of each strain to accumulate IPS. It seems clear that the environment in the oral cavity is substantially different from that in the test tube. Since varying the conditions of unbalanced growth in vitro influences the cellular IPS content of a strain that produces a high level of IPS when grown conventionally, it seems reasonable to postulate that other growth conditions may increase the level of IPS produced by other cariogenic strains, which apparently produce low levels of IPS. Perhaps rifampin treatment would be a useful tool to survey the capacity of various cariogenic strains to accumulate IPS.

The observation that, in dental plaque, coccal-shaped cells with thickened walls contained numerous IPS granules can be readily reproduced in vitro by rifampin inhibition of both RNA and protein syntheses in *S. mutans* FA-1. Studies are currently in progress to determine the physiological conditions that result in this resistant and potentially cariogenic state.

## ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant DE-03487 from the National Institute of Dental Research. Stephen J. Mattingly was supported by a National Institute of Dental Research postdoctoral fellowship (1F22 DE-0474-01) at Temple University School of Medicine, Philadelphia, Pa., and by an Institutional Research grant from the University of Texas Health Science Center at San Antonio, San Antonio, Tex.

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