in vivo avec les protéines,[1] ne provoque pas de lésions des mitochondries ou du réticulum endoplasmatique,[4] ne modifie pas la teneur du plasma en enzymes normalement concentrés à l'intérieur des cellules.

Cette concordance ne prouve pas que l'hypothèse est exacte du "shock biochimique" proposée par Bacq et Alexander soit exacte; (11, voir aussu 1 et 12) mais il fallait la démontrer.

Summary—Intraperitoneal injection of 160 mg/kg of β-mercaptoethanol in rats does not change the plasma levels of various enzymes (TGO, LDH, MDH, GlDH, β-glucuronidase) which are much increased by β-mercaptoethylamine. This amine protects against ionizing radiation; mercaptoethanol does not.

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On the inhibition of ribonucleic synthesis in *Bacillus subtilis* cells by steffimycin

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PREVIOUS papers dealing with steffimycin (U-20, 661) described its preparation, isolation and characterization,[1] its primary mechanism of action,[2, 3] and its interactions with di- and trivalent cations.[4] The present paper describes the inhibitory effect of steffimycin on the synthesis of RNA and the effect of the antibiotic upon ribosome stability and upon the assembly of ribosomes in *Bacillus subtilis* cells.

B. subtilis UC-564 cells were grown in the medium described by Nakada.[5] Shaken flasks containing 100 ml of medium were inoculated with 5 ml of seed and the flasks were incubated on a rotary shaker at 37° untill the cell suspensions reached an optical density of 0·9 to 1·0 at 570 mμ (4–5 hr). Cells from 400-ml culture aliquots were recovered by centrifugation and washed in 12 ml of 0·12 M Tris-HCl buffer, pH 7·5, resuspended in 10 ml of the same buffer and reincubated with 90 ml of culture medium. The cultures were preincubated for 10 min before radioactive precursors and antibiotic were added. The cells were harvested by pouring them onto crushed ice, followed by centrifugation. They were washed once in 20 ml of 0·01 M Tris-HCl buffer, pH 7·4, containing 0·0001 M magnesium acetate and 40 mg polyethylene sulfonate per liter (ribosomal buffer). The frozen cell pellets were ground with twice their weight of alumina for 10–15 min. The resulting homogenate was extracted with 2 ml ribosomal buffer per gram wet wt. of cells. Unbroken cells and large debris were removed by centrifugation at 20,000 *g* for 20 min. Deoxyribonuclease (electrophoretically purified, Worthington Biochemical Corp., Freehold, N.J.) was added to the supernatant solution to a final concentration of 5 μg/ml and the cell extract was again centrifuged at 20,000 *g* for 20 min. RNA was obtained by shaking an aliquot of cell extract containing 0·5% sodium dodecyl sulfate and 0·25 mg pronase (Calbiochem) per ml cell extract for 15 min. Aliquots (0·2 ml) of cell extract containing ribosomes or RNA after sodium dodecyl sulfate treatment were layered on 4·6 ml of 4–20% linear sucrose gradients prepared with ribosomal buffer (see above). The ribosomal preparations were centrifuged for 180 min and the RNA preparations for 6·5 hr at 38,000 rpm in an SW39 rotor in a Spinco model L-2 centrifuge at 4°. Samples were collected and processed as described by Leive.[6]

Steffimycin acts as a potent and specific inhibitor of RNA synthesis in cell-free *E. coli* systems. It was, therefore, of interest to determine to what extent the antibiotic inhibits synthesis of ribosomal, messenger and soluble RNA in whole cells. Five μc of uniformly labeled ^{14}C-uridine (290 mc/m-mole, Nuclear Chicago) and 10 μg/ml of steffimycin were added per flask. The flasks were incubated for 30 min and the cells were recovered and extracts prepared. Portions of these extracts were fractionated directly on sucrose density gradients (Fig. 1). The patterns obtained show that ^{14}C-uridine was

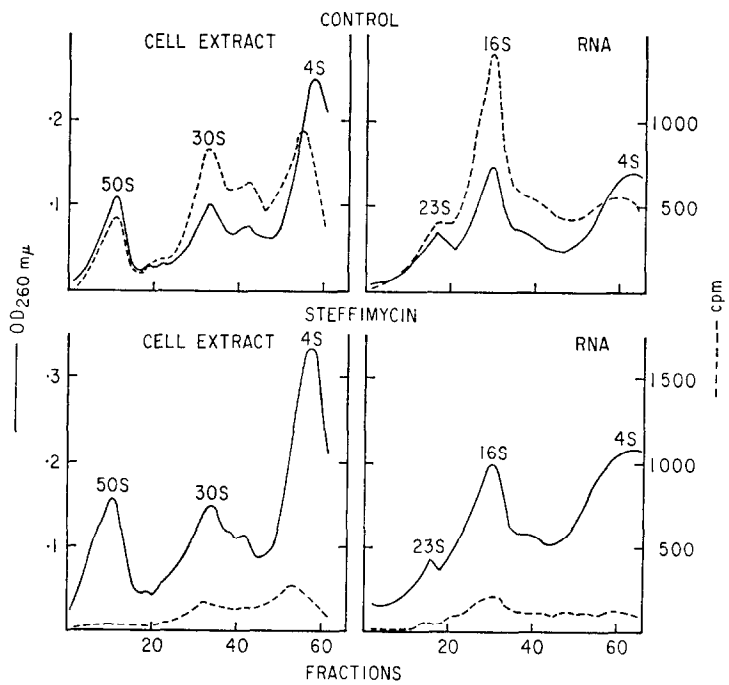

FIG. 1. Sedimentation patterns of cell extracts and RNA from cells treated with steffimycin. Five μc ^{14}C-uridine was added per 100 ml culture medium; steffimycin, 10 μg/ml. The cultures were incubated for 25 min.

not incorporated into the 50S ribosomal particles in the presence of steffimycin, and a drastic reduction of label was apparent within the 30S ribosomal peak, the 5–29S and 4S regions. The 4S peaks in the cell extract gradients, shown in Fig. 1, as well as in all further cell extract gradients discussed in this paper are partially masked by pigmented materials contained in the extracts. A drastic reduction of ^{14}C-uridine incorporation into the cellular RNA fractions comprised of ribosomal RNA in the 23S and 16S peaks, messenger RNA in the 5–15S region and soluble RNA in the 4S region of the gradient is apparent in the presence of steffimycin. These data indicate that synthesis of all three main cellular RNA fractions in *B. subtilis* is inhibited by the antibiotic.

Actinomycin D causes total cessation of RNA synthesis within 15 sec or less after addition of the drug to an *E. coli* culture. Since no RNA is synthesized, destruction of preexisting RNA can be estimated.[6] It was of interest to determine if steffimycin has similar properties. Therefore, cells were prelabeled with uracil-2-^{14}C (40·6 mc/m-mole, Nuclear Chicago) for various periods of time followed by the addition of antibiotic (Fig. 2). The radioactivity precipitable with TCA at the time of drug addition was equated as 100 per cent and all subsequent activities after drug addition were expressed in per cent of initial activity present to allow calculation of gain or loss of radioactivity with time. The results shown in Fig. 2 indicate that with short prelabeling periods ^{14}C-uracil continues to be

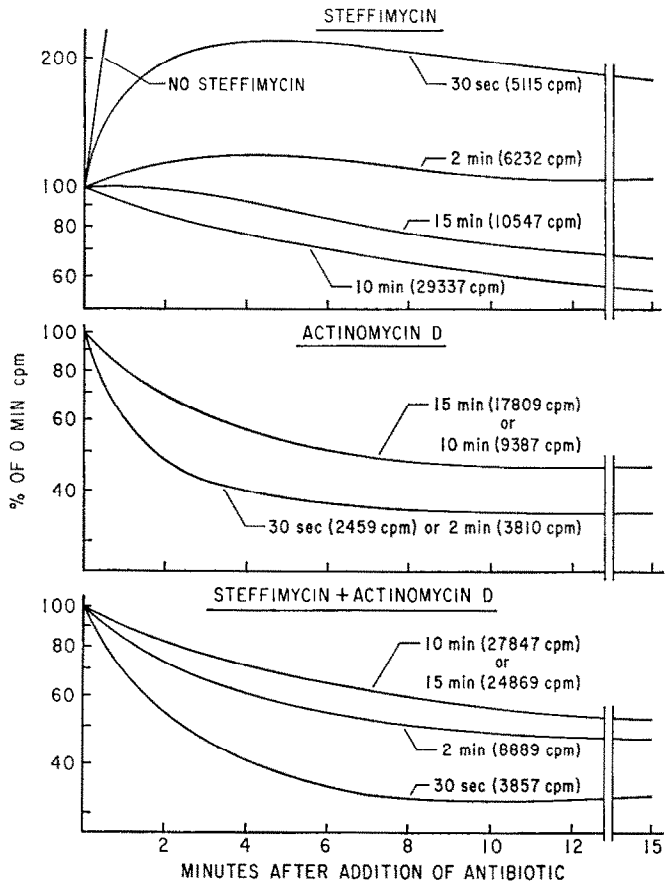

FIG. 2. Kinetics of inhibition of RNA synthesis by steffimycin. Cells were labeled with 20 μc uracil-2-^{14}C per 100 ml culture medium. At various times specified in the figure, steffimycin, actinomycin D or a combination of both was added. The concentration of both antibiotics was 10 μg/ml. Sample aliquots (0·5 ml) were taken at appropriate times and precipitated with an equal volume of cold 10% TCA. The precipitates were collected on 0·45 μ millipore filters, thoroughly washed and the radioactivity was counted by liquid scintillation spectrometry. Actual counts per minute at 0 time are given in the figure in parenthesis.

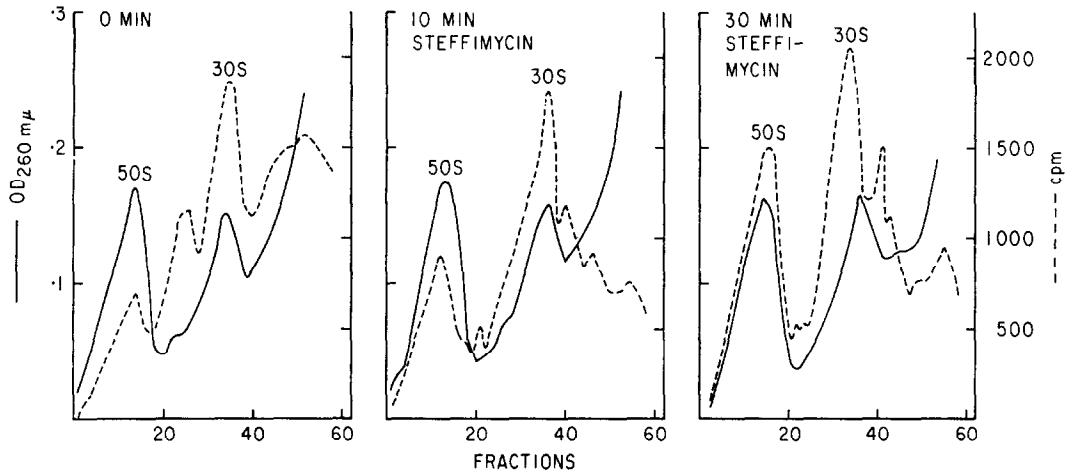

FIG. 3. Stability of ribosomes. Sedimentation patterns of cell extracts from cells prelabeled with 20 μc ^{3}H-uridine per 100 ml culture for 10 min. The control culture was harvested at the end of the labeling period (t = 0); additional cultures were treated with steffimycin (10 μg/ml) for 10 or 30 min.

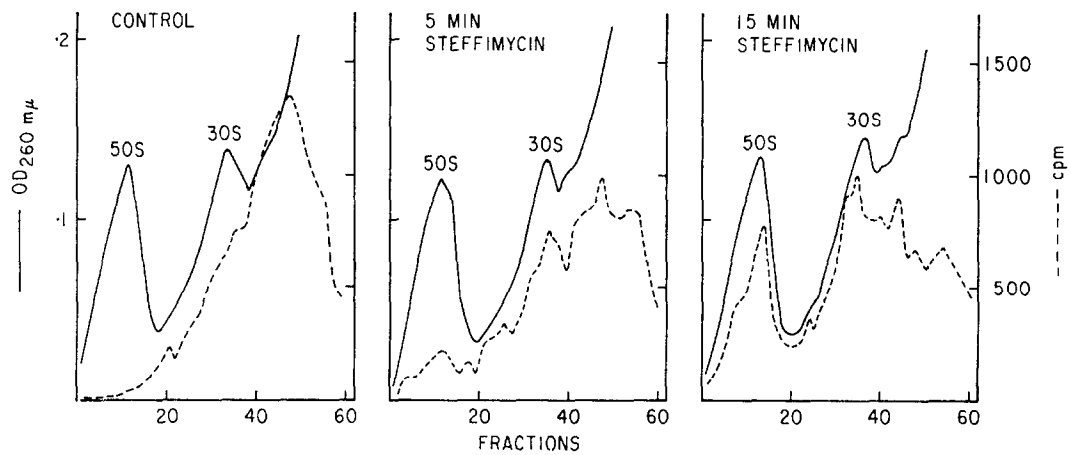

FIG. 4. Assembly of ribosomes. Sedimentation patterns of cell extracts from cells pulse-labeled with 100 μc ^{3}H-uridine per 100 ml culture for 2 min. The control culture was harvested at the end of the labeling period (t = 0); additional cultures were treated with steffimycin (10 μg/ml) for 5 or 15 min.

incorporated at a reduced rate for a period of 3–5 min after addition of steffimycin. A time-dependent decrease of radioactivity reflecting destruction of labeled RNA is apparent only after prolonged prelabeling periods (10–15 min). These observations suggest that exertion of inhibitory activity by the antibiotic is relatively slow. An alternate explanation of these results might be that some RNA fractions were stabilized in the presence of the drug. This latter possibility was tested by adding actinomycin D alone and in combination with steffimycin to the culture (Fig. 2). In the presence of actinomycin D alone, ^{14}C-uracil incorporation ceases immediately. A half-life of slightly less than 2 min is obtained for the material prelabeled for 30 sec or 2 min. Increased prelabeling times lead to a decrease of the labile RNA fraction. In the presence of both actinomycin D and steffimycin, labile RNA synthesized during a 30-sec ^{14}C-uracil pulse decays with an average half-life of approximately 2 min, which compares favorably with the corresponding value obtained with actinomycin D alone. Longer prelabeling periods yield RNA with a longer average half-life than that observed with the 30-sec material. The continued limited increase of RNA synthesis after addition of steffimycin to the system is thus due to a slow expression of the drug's effect on RNA synthesis. At least 3–5 min are required before RNA synthesis ceases completely.

To determine the effect of steffimycin on ribosome stability during prolonged exposure, cells were labeled with ^{3}H-uridine (2730 mc/m-mole, Nuclear Chicago). Twenty μc was added per 100 ml culture and the cells were labeled for 10 min. Control cultures were harvested at the end of the labeling period. Other cultures received steffimycin (10 μg/ml final concentration) and the cells were harvested after 10 or 30 min of exposure to the drug. Sucrose density gradient patterns of cell extracts derived from these cells are shown in Fig. 3. In the gradient obtained from the control sample, both the 50S and 30S ribosomal particles as well as the 5–29S and 4S regions contain significant amounts of radioactivity. The radioactive peak located within the 40S region probably consists of 50S precursor material similar to that described by Leive.[6] Treatment with steffimycin for 10 or 30 min results in a slight progressive increase of radioactivity in the 50S peak and an almost constant amount of label in the 30S peak. The radioactive peak in the 40S region had almost disappeared. Material within the 5–29S region shows significant loss of radioactivity, presumably due to both decay and gradual incorporation into ribosomes. These results demonstrate that matured ribosomes remain quite stable in the presence of steffimycin over periods of at least 30 min.

Having demonstrated that ribosomes remain stable when exposed to the antibiotic, studies pertaining to the assembly of ribosomes from preformed precursor particles in the presence of the drug appeared feasible, although the stability studies described above provided substantial evidence that ribosome maturation probably remained unaffected. Cells were pulse-labeled for 2 min with 100 μc ^{3}H-uridine (3330 mc/m-mole, Nuclear Chicago) per 100 ml culture and were then treated for 5 and 15 min with steffimycin (10 μg/ml, final concentration). Control cells were harvested immediately after the labeling period. The cell extracts prepared from these cells were fractionated on sucrose density gradients. The results assembled in Fig. 4 show that the control sample contains the bulk of the label within the 4–29S region, some label within the 30S peak and none above 30S. Treatment with steffimycin for 5 and 15 min results in gradual displacement of the label from the slower sedimenting region (4–29S) into the 30S and particularly the 50S ribosomal peaks. Unquestionably a small portion of the label contained in the sample treated for 5 min with the drug arises by continued incorporation of ^{3}H-uridine after addition of the antibiotics, since RNA synthesis ceases only after 3–5 min of exposure to the antibiotic, as shown above. However, the significant translocation of label into the heavier region of the gradient (50S ribosomes), which had taken place between 5 and 15 min of steffimycin treatment, demonstrates clearly that the antibiotic does not interfere with the assembly of ribosomal particles from preformed ribosomal precursors.

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3-Methoxy-4-hydroxyphenylglycol sulfate in brain and cerebrospinal fluid

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THE MAJOR urinary metabolites of catecholamines are the products of two enzymatic reactions, deamination and *O*-methylation. The major metabolite of dopamine, 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid), has been found in brain and in cerebrospinal fluid,[1, 2] but attempts to find the corresponding metabolite of norepinephrine (3-methoxy-4-hydroxymandelic acid) have failed.[3, 4]

We have recently demonstrated that the sulfate conjugate of 3-methoxy-4-hydroxyphenylglycol (MHPG) is the major metabolite formed in brain when norepinephrine-^{3}H or normetanephrine-H^3 is injected into the cisterna magna of rats.[5] These findings led us to examine brain and spinal fluid of several species for endogenous 3-methoxy-4-hydroxyphenylglycol sulfate.

Brains of animals killed by decapitation were rapidly removed and homogenized in 4 volumes of ice-cold 0·4 N perchloric acid. Proteins in cerebrospinal fluid obtained by lumbar or ventricular puncture were precipitated by addition of 0·04 vol. of perchloric acid (60 per cent). After centrifugation, aliquots of the clear supernatant equivalent to 1·5–2·0 g brain or 5 ml cerebrospinal fluid were adjusted to pH 5·5 with 1 N sodium hydroxide; 1 ml of 1 N sodium acetate buffer (pH 5·5) and 0·4 ml of a sulfatase (Glusulase, Endo Products, New York) were then added. Free MHPG was extracted and assayed without incubation, while total MHPG was determined after incubation at 37° for 24 hr. The procedures for isolation and assay by gas chromatography of MHPG have been described by Wilk *et al.*[6] When possible, samples were assayed in duplicate and samples with 1 or 2 μg MHPG added were assayed in parallel to assess recovery. In some experiments, a trace amount of MHPG-sulfate-^{3}H (previously isolated from the urine of rats treated with norepinephrine-7-^{3}H) was added to correct for completeness of hydrolysis as well as for recovery of the free MHPG. (Hydrolysis was 40–80 per cent effective, while recovery of hydrolyzed MHPG was 80–90 per cent.)

In other experiments, pooled ethyl acetate extracts of the products of hydrolysis were evaporated *in vacuo* and the dry residue was taken up in a small volume of ethyl acetate and applied to Whatman No. 3 mm filter paper. After chromatography in butanol:ethanol:water (4:1:1), elution and rechromatogramming on thin-layer silica gel (isopropanol:ammonia:water, 8:1:1), a compound was found which had the same R_f value and the same color after treatment with diazotized *p*-nitroaniline as authentic MHPG.

Free or conjugated MHPG was present in the brains of all species examined (Table 1). In the cat there appeared to be little if any conjugated MHPG, while in the rat and guinea pig only MHPG