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Uptake of Dihydrostreptomycin by Intact Splenic Cells Cultured from Immunized Rabbits and its Binding to the 40S Ribosomal Subunit.* (31415)

ROBERT G. KRUGER† (Introduced by William Burrows)
Department of Microbiology, University of Chicago, Chicago, Ill.

Streptomycin (SM) bound to the 30S subunit of ribosomes from SM-sensitive *Escherichia coli*(1) and, with polyuridylic acid as a messenger, caused decreased incorporation of phenylalanine and increased incorporation of isoleucine, thus suggesting that the SM bound to ribosomes altered translation of the information of the messenger(2). Pestka *et al*(4) and Kaji and Kaji(5) showed that SM affects the binding of transfer RNA to the ribosomal template complex and changes the recognition of RNA codons. In support of this hypothesis, Schwartz(3) demonstrated that in cell-free extracts of *E. coli* SM modified the information contained in the RNA of the bacteriophage F_2 in such a way as to cause synthesis of altered viral coat protein, and Bissell(6) found that in the presence of SM or neomycin *E. coli* produced an altered β-galactosidase that had lost its enzymatic activity but still cross-reacted with antiserum to the normal enzyme. SM affected the synthesis of antibody in cultures of splenic and lymph node cells from rabbits immunized with the RNA bacteriophage MS-2 by causing the production of 7S antibody with altered immunological specificity in that it did not combine with complete infectious phage but still combined with incomplete noninfectious phage(7,8). It was suggested that SM may bind to the ribosomes of splenic cells and cause the synthesis of altered antibody by mechanisms similar to those described for bacteria. This paper reports that dihydrostreptomycin (DHSM), which acts similarly to SM in both *E. coli*(9) and splenic cells (7,8), readily penetrates intact splenic cells from immunized rabbits *in vitro* and binds to the 40S subunit of the 76S splenic ribosome. This is the first demonstration that antibiotics of the SM family can bind to similar intracellular sites in mammalian and bacterial cells.

Methods. The preparation of MS-2 phage antigen, immunization of rabbits, and preparation of splenic cell cultures have been previously described(7). Cells from immunized animals were used because they contain more ribosomes than cells from unimmunized ones(10). Tritiated DHSM was the generous gift of Dr. Charles Rosenblum of Merck, Sharp and Dohme Research Laboratories, Rahway, N. J. It was used in preference to SM because it was less likely to exhibit nonspecific binding(11).

For measuring uptake of H^3-DHSM by intact cells, splenic cells were removed from the main culture, washed twice in minimal essential medium (MEM)(12), and resuspended in MEM to a cell count of 2 × 10^6/ml. H^3-DHSM was added to a final

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† Present address: Dept. of Microbiology, New York Medical College, New York.

concentration of 200 μg/ml (1.4 μc/ml) and the cell suspension was incubated at 37°C. Two-ml samples were taken at appropriate intervals; 1 ml was diluted with 1 ml of cold phosphate-buffered saline minus Mg^{++} (PBS)(13) and the remaining 1 ml was diluted with 1 ml cold 10% trichoracetic acid (TCA). Both samples were kept for 30 minutes at 4°C, filtered through a Millipore filter (size HAWP, 0.45 μ, 22 mm diameter), and washed with 10 ml cold PBS or 10% TCA. The filters were dried, placed in 15 ml Bray's(14) scintillation mixture and counted in a Packard Tri-Carb liquid scintillation counter.

For demonstration of the binding of H^3-DHSM by splenic ribosomes, splenic cell suspensions containing 200 μg/ml (1.4 μc/ml) H^3-DHSM were made as just described. Five-ml volumes were incubated at 37°C for 5 minutes, immediately diluted with 10 ml cold PBS, and centrifuged for 10 minutes at 10,000 × g and 4°C. The cells were resuspended in 2 ml of either buffer A (0.01 M KCl, 0.002 M $MgCl_2$, 0.01 M mercaptoethanol, 0.26 M sucrose, and 0.01 M tris, pH 7.4) or buffer B (as buffer A but with only 0.0005 M $MgCl_2$). They were then disrupted in a glass tissue homogenizer with a loose-fitting Teflon pestle, and the homogenate was centrifuged for 10 minutes at 12,000 × g and 4°C to remove nuclei and cell debris. The supernatant was centrifuged for 2 hours at 100,000 × g and 4°C, and the microsomal pellet was suspended in 1 ml buffer A or B containing 0.5% sodium deoxycholate. Volumes of 0.2 ml in 4% sucrose were immediately layered on linear 5%-20% (w/w) sucrose gradients in either buffer A or B and centrifuged for 2 hours at 37,000 rpm and 4°C in the Spinco SW-39 rotor. Ten-drop fractions were collected from the bottom of the tubes, diluted to 1 ml, and assayed for optical density at 260 mμ and cold 10% TCA-insoluble radioactivity as in the intact cell experiments.

Splenic cell homogenates contain a highly active ribonuclease which, if not controlled, will destroy polysomes(15) and ribosomes. Mercaptoethanol partially inhibited this enzyme and was incorporated into all buffers used. Addition of deoxycholate had to be delayed until just before zonal centrifugation in order to obtain reproducible profiles of the ribosomes and their subunits. However, even with these precautions, the splenic ribosomal preparations still contained ribonuclease.

TABLE I. Reversible Uptake and Binding of H^3-DHSM by Splenic Cells Cultured from Immunized Rabbits.

	Treatment of cells before exposure to H^3-DHSM		
		Cultured with—	
Cells washed* with	Cultured without DHSM	200 μg/ml DHSM	200 μg/ml DHSM and washed* with MEM
Unwashed			
PBS insoluble†	256‡	222	256
TCA " ‡	145	57	173
PBS			
PBS insoluble	232	224	—
TCA "	139	53	—
PBS + 10^{-3} M Mg^{++}			
PBS insoluble	37	19	—
TCA "	3	2	—
MEM (10^{-3} M Mg^{++})			
PBS insoluble	32	15	—
TCA "	2	1	—

* Washed at 26°C by centrifugation 2 times with 10 volumes of the indicated suspending medium.

† H^3-DHSM not removed by washing whole cells with PBS or TCA as described in text.

‡ Counts/min/10^6 splenic cells.

Results. Synthesis of antibody against phage MS-2 in splenic cell cultures from immunized rabbits was clearly altered 10 minutes after addition of 200 μg/ml DHSM(7). Under these conditions, H^3-DHSM was readily taken up by splenic cells and was not removed by washing with cold PBS on Millipore filters. In a typical experiment, when the cells had been cultured in the absence of DHSM, about 70% of the H^3-DHSM was so firmly bound that it was not removed by washing with cold 10% TCA (Table 1). When the splenic cells exposed to H^3-DHSM were from a culture maintained in the presence of 200 μg/ml DHSM, approximately the same amount of H^3-DHSM was taken up, but only 16% was bound in TCA-insoluble form. This reduced binding in TCA-insoluble form suggests that the intracellular sites which firmly bind DHSM were already occupied by unlabeled DHSM. The failure of previous ex-

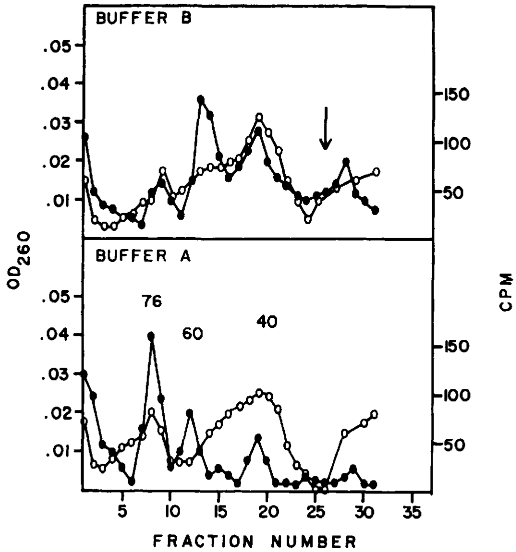

FIG. 1. Sucrose density gradient centrifugation of ribosomal fractions from splenic cells of immunized rabbits incubated with H^3-DHSM. See text for details. Closed circles: optical density at 260 mμ. Open circles: counts/min/fraction. Numbers refer to approximate S values calculated from the position of a marker (arrow) with a sedimentation coefficient of 17S.

posure of the splenic cells to unlabeled DHSM to reduce the amount of H^3-DHSM taken up into forms not removed by washing with cold PBS suggests that DHSM may be taken up into some sort of an intracellular pool without being firmly bound to specific sites. The binding of DHSM in a TCA-insoluble state was reversible because cells cultured in the presence of DHSM, but washed free of the drug before addition of H^3-DHSM, bound the same amount of the antibiotic in TCA-insoluble form as did cells cultured without DHSM.

Table I further demonstrates the reversibility of DHSM uptake and binding by showing that 2 centrifugations in MEM (10^{-3} M in Mg^{++}) or PBS + 10^{-3} M Mg^{++} removed nearly all the H^3-DHSM taken up and bound by the splenic cells. In contrast, centrifugation in PBS (no Mg^{++}) alone did not remove H^3-DHSM. This reversibility of DHSM binding is in accord with the observed reversibility of the effect of SM and related antibiotics on antibody synthesis in similar splenic cultures(7). In cell-free bacterial systems, Mg^{++} reverses a variety of SM effects including ribosomal synthesis of protein (16).

Fig. 1 shows that the integrity of splenic ribosomes, which have a sedimentation coefficient of 76S, is dependent upon Mg^{++} concentration. Lowering the Mg^{++} concentration caused the ribosomes to dissociate into 60S and 40S subunits. Fig. 1 also shows that H^3-DHSM actually penetrated intact splenic cells and was bound to the 40S ribosomal subunit. This subunit is comparable to the 30S subunit of the 70S *E. coli* ribosome which Cox *et al*(1) found is the subunit to which SM apparently binds. They demonstrated that the site of SM sensitivity is the 30S subunit by performing reaggregation experiments with 50S and 30S subunits from sensitive and resistant *E. coli* and testing for SM inhibition of polyuridylic acid-directed phenylalanine incorporation. The lower binding of H^3-DHSM to 40S subunits in the buffer A could have resulted from the effect of the higher Mg^{++} concentration on either the association of the ribosomal subunits or on the actual binding of the antibiotic to the 40S subunit.

Discussion. Mammalian cells are generally regarded as relatively impermeable to SM (17) because the antibiotic, at the concentration which penetrates intact splenic cells, is routinely incorporated without toxic effect into the culture media used for many different kinds of cells. However, SM does penetrate intact mammalian cells as evidenced by its ability to inhibit the multiplication of bacteria growing within cultured macrophages (18). In these experiments, packed cell volumes of 0.1 to 0.2 ml per ml suspension (2.4 to 5.2 × 10^7 cells) took up 1.7 to 3.6% of the total H^3-DHSM present in the medium. Therefore, as a rough approximation, the intracellular concentration of H^3-DHSM was only one-tenth that of the extracellular. The amount of H^3-DHSM bound to intact splenic cells was equivalent to 1 × 10^8 molecules per cell, which is of the order of magnitude of 1 molecule of DHSM per ribosome. Approximately 90% of the H^3-DHSM bound in TCA-insoluble form in intact cells was recovered in the ribosomal fractions.

These data demonstrate that H^3-DHSM, at a concentration of 200 μg/ml, was rapidly taken up by intact splenic cells and bound to the 40S ribosomal subunits. It has been suggested(8) that SM (and DHSM) exerts its effect on antibody synthesis to phage MS-2 in splenic cultures from immunized rabbits by either combining with the phages and altering their antigenic specificity or by combining with ribosomes and altering the reading of the messenger for antibody synthesis. These experiments show that DHSM, and presumably SM, binds to splenic ribosomes and therefore may be capable of altering antibody synthesis at the ribosomal level.

Summary. Tritiated dihydrostreptomycin (H^3-DHSM) was taken up by intact splenic cells cultured from rabbits immunized with bacteriophage MS-2 and was bound to the 40S ribosomal subunit of the 76S splenic ribosome. Splenic cells that had been maintained in the presence of 200 μg/ml unlabeled DHSM took up as much H^3-DHSM as cells not previously exposed to the antibiotic. However, only cells that had not been cultured in the presence of DHSM bound H^3-DHSM in cold trichloracetic acid-insoluble form. The binding of H^3-DHSM by splenic cells was reversible and dependent on the concentration of Mg^{++} in the suspending medium.

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Multiplicity Reactivation of Vaccinia Virus in the Cells of the Chorioallantoic Membrane.[*] (31416)

D. G. SHARP AND RUTH C. DUNLAP
Biophysics Laboratory, Bacteriology Department, School of Medicine, University of North Carolina, Chapel Hill

Multiplicity reactivation (MR) which involves the infection of a cell by the cooperative effort of 2 or more radiation-damaged virus particles, none of which is completely functional alone, has been demonstrated for vaccinia virus in several kinds of tissue culture cells(1,2) but not in the cells of an organized host. If tissue culture cells can find, among the damaged DNA or other parts of 2 or more such virus particles, the stimulus to produce normal virus progeny, one would expect to observe some such effect on the chorioallantoic membrane (CAM). The experimental difficulty encountered in previous attempts to demonstrate such effects was due in part to the exceedingly large and difficult-to-determine number of CAM cells that are available to the virus inoculum. This paper describes a means of observing and measuring the effect of MR without the need of determining or estimating the number of cells involved. We,

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