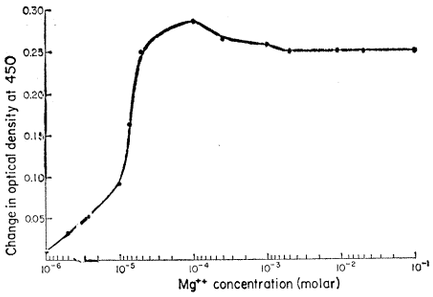

Fig. 6. The interaction of chromomycin with DNA; stoichiometry of the Mg^{++} requirement. The extent of chromomycin complex formation with DNA was measured by change in absorbance at 450 mμ as compared with a solution of chromomycin containing the appropriate concentration of Mg^{++}. All solutions contained chromomycin (100 mμmole/ml) and an excess of native calf-thymus DNA (850 mμmole/ml). The isosbestic point increases from 408 mμ to 421 mμ with increasing Mg^{++} concentration.

ificity of actinomycin binding is mediated by the amino group of guanine which is located in the minor groove of the DNA helix. That chromomycin fails to inhibit the template function of dIdC raises the possibility of the same being true for this antibiotic.

D. C. WARD
E. REICH
I. H. GOLDBERG

Department of Medicine, Harvard Medical School, and *Beth Israel Hospital, Boston, Massachusetts,* and *Rockefeller Institute, New York*

References and Notes

1. M. Chamberlin and P. Berg, *Proc. Nat. Acad. Sci. U.S.* **48**, 81 (1962).
2. C. F. Fox, W. S. Robinson, R. Haselkorn, S. B. Weiss, *J. Biol. Chem.* **239**, 186 (1964).
3. I. H. Goldberg, M. Rabinowitz, E. Reich, *Proc. Nat. Acad. Sci. U.S.* **48**, 2094 (1962).
4. R. Corbaz, L. Ettlinger, E. Gäumann, W. Keller-Schierlein, F. Kradilfer, L. Neipp, V. Prelog, P. Reusser, *Helv. Chim. Acta* **40**, 199 (1957).
5. H. E. Carter, C. P. Schaffner, D. Gottlieb, *Arch. Biochem. Biophys.* **53**, 282 (1954).
6. J. Berger, E. R. LaSala, W. E. Scott, B. R. Meltsner, L. H. Steinbach, S. Kaiser, S. Teitel, E. Mach, M. W. Goldberg, *Experientia* **13**, 434 (1957).
7. S. Ishihara, R. Utakara, M. Suzuki, Y. Okami, H. Umezawa, *J. Antibiotics (Tokyo), Ser. A.* **11**, 160 (1958).
8. T. Yoshida, K. Katagiri, S. Yolozawa, *ibid.* **14**, 330 (1961).
9. E. Reich, unpublished observations.
10. A. DiMarco, M. Gaetani, P. Orezzi, B. M. Scarpinato, R. Silverstrini, M. Soldati, T. Dasdia, L. Valentini, *Nature* **201**, 706 (1964).
11. A. Grein, C. Spalla, A. DiMarco, G. Canevazzi, *Giorn. Microbiology* **11**, 109 (1963); A. DiMarco, M. Gaetani, L. Dorigotti, M. Soldati, O. Bellini, *Tumori* **49**, 203 (1963); A. DiMarco, M. Soldati, A. Fioretti, T. Dasdia, *ibid.*, p. 235 (1963); L. Dorigotti, *ibid.* **50**, 117 (1964).
12. G. Hartmann, H. Goller, K. Koschel, W. Kersten, H. Kersten, *Biochem. Z.* **341**, 126 (1964).
13. Abreviations: dAT, deoxyadenylic-thymidylic copolymer; crab dAT, a deoxyadenylic-thymidylic copolymer containing 2 to 5 percent deoxyguanylic and deoxycytidylic acids, isolated from crabs of the genus *Cancer*; dAdT, deoxyadenylic-thymidylic homopolymer mixture; dGdC, deoxyguanylic-deoxycytidylic homopolymer mixture; dIdC, deoxyinosinic-deoxycytidylic homopolymer mixture; PolyA, polyadenylic acid; polyI, polyinosinic acid; polyU, polyuridylic acid; polyC, polycytidylic acid; polyUG, mixed polymer of uridylic and guanylic acids; AMP, adenosine monophosphate; GMP, guanosine monophosphate; GTP, guanosine triphosphate; CTP, cytidine triphosphate; TMV, tobacco mosaic virus.
14. The interaction of daunomycin with ribohomopolymers, nucleosides, and nucleotides was studied in 0.1*M* tris, *p*H 7.9. The concentration of reactants used was: daunomycin, 90 mμmole/ml; polyI, polyA, polyU, and polyC, 200 μg/ml; all nucleosides and nucleotides, 10 μmole/ml.
15. A. DiMarco, personal communication.
16. W. Kersten and H. Kersten, *Biochem. Z.* **341**, 174 (1965).
17. The concentration of daunomycin used in the enzymic reactions with DNA primers was below that required to precipitate DNA.
18. A. Peacocke and J. Skerrett, *Trans. Faraday Soc.* **52**, 261 (1956); L. Lerman, *J. Mol. Biol.* **3**, 18 (1961); I. H. Goldberg, E. Reich, M. Rabinowitz, *Nature* **199**, 44 (1963).
19. I. H. Goldberg and M. Rabinowitz, *Science* **136**, 315 (1962); J. Hurwitz, J. J. Furth, M. Malamy, M. Alexander, *Proc. Nat. Acad. Sci. U.S.* **48**, 1222 (1962); G. Hartmann, U. Coy, G. Kniese, *Z. Physiol. Chem.* **330**, 227 (1962).
20. E. Reich, *Science* **143**, 684 (1964).
21. L. F. Cavalieri and B. H. Rosenberg, *Progr. Nucleic Acid Res. Mol. Biol.* **2**, 1 (1963); E. Reich and I. H. Goldberg, *ibid.* **3**, 183 (1964); I. H. Goldberg and E. Reich, *Federation Proc.* **23**, 958 (1964).
22. L. D. Hamilton, W. Fuller, E. Reich, *Nature* **198**, 538 (1963).
23. B. A. Newton, *J. Gen. Microbiol.* **17**, 718 (1957); R. Tomchick and H. G. Mandel, *ibid.* **36**, 225 (1964).
24. W. H. Elliot, *Biochem. J.* **86**, 562 (1963); M. J. Waring, *Biochem. Biophys. Acta* **87**, 358 (1964).
25. W. Fuller and M. J. Waring, *Bunsenges. Ber. Physik. Chem.* **68**, 805 (1964).
26. The complex between ethidium and native DNA is relatively stable to heat, being disrupted only at temperatures in the range of the T_m of the DNA (80° to 90°C), whereas the complexes between ethidium and the ribohomopolymers were completely disrupted at approximately 40°C.
27. Similar results have been obtained by Dr. M. J. Waring (personal communication).
28. B. K. Bhuyan and C. G. Smith, *Proc. Nat. Acad. Sci. U.S.*, in press.
29. S. Tatsuoka, K. Nakazawa, A. Miyake, K. Kaziawara, Y. Aramaki, M. Shibata, K. Tanabe, Y. Hamada, H. Hitomi, M. Miyamoto, K. Mizuno, J. Watanabe, M. Ishidate, H. Yokotani, I. Ishkawa, *Gann* **49** (suppl.), 23 (1958).
30. K. V. Rao, W. P. Cullen, B. A. Sobin, *Antibiot. Chemotherapy* **12**, 182 (1962).
31. G. F. Gause, R. S. Uklolina, M. A. Sveshnikova, *Antibiotiki* **7** (3), 34 (1964).
32. G. F. Gause, *Advan. Chemotherapy,* in press.
33. G. Wakisaka, H. Uchino, T. Nakamura, H. Sotobayashi, S. Shirakawa, A. Adachi, M. Sakurai, *Nature* **198**, 385 (1963).
34. W. Kersten, *Biochim. Biophys. Acta* **47**, 610 (1961).
35. F. Arcamone, G. Franceschi, P. Orezzi, G. Cassinelli, W. Barbieri, R. Mondelli, *J. Amer. Chem. Soc.* **86**, 5334 (1964); F. Arcamone, G. Cassinelli, P. Orezzi, G. Franceschi, R. Mondelli, *ibid.*, p. 5335.
36. G. Morgan, L. P. Walls, C. H. Browing, R. Gulbranson, J. M. V. Robb, *J. Chem. Soc.* **1938**, 389 (1938).
37. W. Keller-Schierlein, M. L. Mihailovic, V. Prelog, *Helv. Chim. Acta* **42**, 305 (1959).
38. M. Miyamoto, K. Morita, Y. Kawamatsu, S. Noguchi, R. Marumoto, K. Tanaka, S. Tatsuoka, K. Nakanishi, Y. Nakadaira, N. S. Bhacca, *Tetrahedron Letters* **34**, 2355 (1964); M. Miyamoto, K. Morita, Y. Kawamatsu, M. Sasai, A. Nohara, K. Tanaka, S. Tatsuoka, K. Nakanishi, Y. Nakadaira, N. S. Bhacca, *ibid.*, p. 2367; M. Miyamoto, Y. Kawamatsu, M. Shinohara, K. Nakanishi, Y. Nakadaira, N. S. Bhacca, *ibid.*, p. 2371.
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Chondroitin Sulfate: Inhibition of Synthesis by Puromycin

Abstract. *Puromycin reversibly inhibits synthesis of chondroitin sulfate by vertebral chondrocytes from 10-day-old chick embryos. Treatment of these cells with puromycin for 5 hours does not affect viability or capacity to multiply on subsequent release from their matrix. It is suggested that synthesis of this polysaccharide is coupled with that of protein; there may be a feedback control in its synthesis.*

Our purpose is to present data indicating that synthesis of chondroitin sulfuric acid (CSA) is intimately associated with protein synthesis, since puromycin, an inhibitor of protein synthesis (*1*), also inhibits the uptake of sulfate and glucose into this polysaccharide. A similar observation has recently been reported on the uptake of S^{35}-sulfate by bone rudiments of chick embryo (*2*).

We used the cartilagenous vertebral columns from 10-day-old chick embryos, dissected free of connective tissues and perichondrium, which we shall refer to as "trunks" (*3*). In each series of experiments duplicate trunks of approximately equal volume were individually incubated at 37°C in 2 ml of Eagle's minimum essential medium (MEM) containing 10 percent of horse serum and both penicillin and streptomycin, each at 50 unit/ml (*4*). In experiments designed to demonstrate the reversibility of the inhibition induced by puromycin, trunks were transferred to a puromycin-free medium, either MEM or an enriched medium

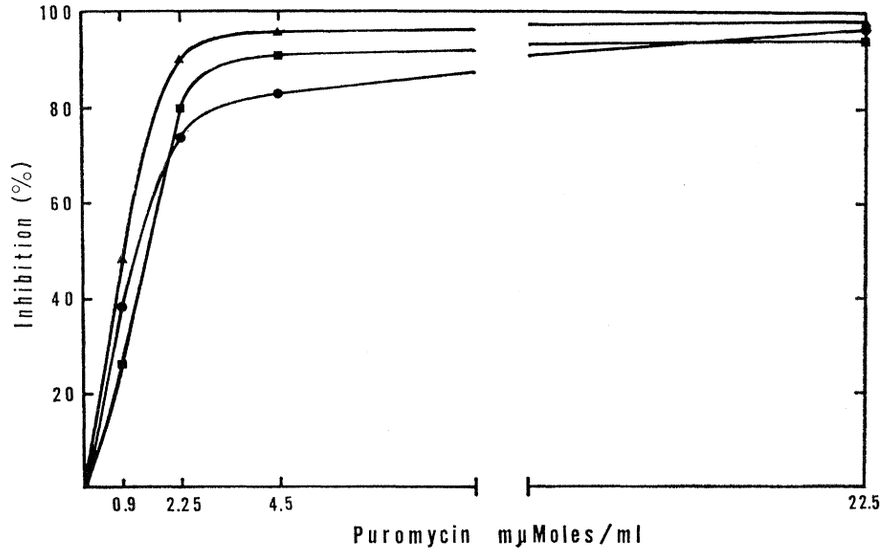

Fig. 1. Degree of inhibition of synthesis of protein and CSA at various concentrations of puromycin. After incubation for 30 minutes in the presence or absence of puromycin, trunks were removed to fresh medium before the desired substrate was added; 4½ hours later they were analyzed. Incorporation of valine into protein, ——●——; incorporation into CSA of sulfate, ——■——; and incorporation into CSA of glucose, ——▲——.

containing embryo extract (*3*). Because synthesis of DNA is negligible in this system (*5*), analysis is not complicated by problems of cell multiplication. In experiments designed to demonstrate the viability of cells after treatment with puromycin, chondrocytes were liberated from their matrix with trypsin and cultured (*3*).

Radioactive substrates and quantities employed per milliliter of culture medium were: C^{14}-L-valine, 0.1 μc; C^{14}-glucose, 1.0 μc; and S^{35}-sulfate, 5μc. Radioactivity was determined in a Packard liquid scintillation counter. The scintillation mixture used to count labeled protein consisted of 5 g of 2,5-diphenyloxazole and 0.3 g of dimethyl 1,4-di(5-phenyl-2-oxazolyl)benzene per liter of toluene; Bray's mixture was used to count labeled CSA (*6*).

For determination of incorporation of C^{14}-valine into protein, trunks were removed from the incubation medium at the desired times, rinsed briefly in cold balanced-salt solution (*3*), and individually homogenized in 0.4 ml of a solution of bovine serum albumin, 5 mg/ml. Then 5 ml of 5 percent trichloroacetic acid was added, and the denatured protein was further dispersed by homogenization. This suspension was cooled in ice for 30 minutes, placed in a water bath for 15 minutes at 90°C to hydrolyze aminoacyl-RNA, and again cooled in ice for 20 minutes; the insoluble protein was then plated with suction on 1.8-cm glass filter discs. The tubes were rinsed twice, each time with 5 ml of 2 percent trichloroacetic acid; the rinse solutions were filtered, and the solids were washed twice with 5 ml of 95 percent ethanol and twice with 5 ml of acetone and counted with 2 ml of the scintillation mixture.

For determination of incorporation of S^{35}-sulfate and C^{14}-glucose into CSA, the trunks were rinsed in the manner described and were individually homogenized with 2 ml of 0.2*M* tris buffer at *p*H 8.0. The suspension was placed in a boiling water bath for 3 minutes and then cooled; to it were added 0.08 ml of 0.04*M* $CaCl_2$, 0.08 ml of 95 percent ethanol, and 0.1 ml of pronase (grade B, Calbiochem), 4 mg/ml in 0.2*M* tris, *p*H 8.0, before incubation for 8 hours at 50°C (*7*).

The CSA labeled with sulfate was then isolated in this manner. To 1 ml of the pronase digest was added 0.2 ml of 2*M* tris (*p*H 8.0), 0.7 ml of water, 0.1 ml of CSA (10 mg/ml), and 1.0 ml of 1 percent cetylpyridinium chloride. After 5 minutes, samples were centrifuged at 12,000*g* for 15 minutes at room temperature. The supernatant fluid was removed and the precipitates were suspended in 1.0 ml of cold water and centrifuged again at 0°C. The precipitates were then dissolved in 1.0 ml of methanol, and 0.5 ml was used for determination of radioactivity with 15 ml of Bray's mixture.

In the case of CSA labeled with glucose, the procedure for its isolation and counting was the same as that described, except for an initial treatment with KOH to degrade RNA: 1 ml of 2*N* KOH was added to 1 ml of the pronase digest and the mixture was incubated for 2 hours at 30°C; 0.32 ml of 5*N* H_2SO_4 was then added to readjust the *p*H to 8.0. The CSA and cetylpyridinium chloride were then add-

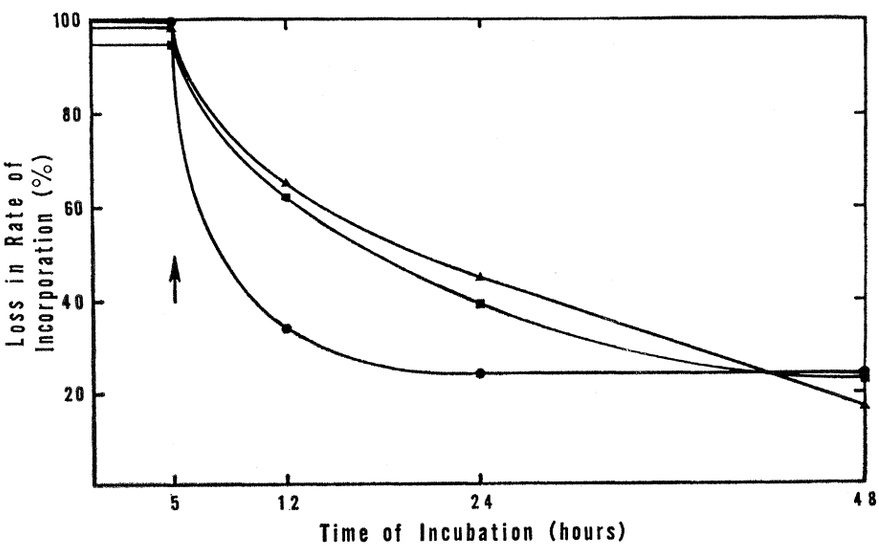

Fig. 2. Recovery of ability to incorporate valine into protein and sulfate and glucose into CSA. After incubation for 5 hours in MEM with and without puromycin (100 mμmole/ml), trunks were rinsed and transferred to 2 ml of enriched medium. For 2 hours before the times indicated, they were pulsed with respective radioactive substrates and then analyzed. After the 24th hour of incubation the remaining trunks were transferred to fresh medium. Symbols as in Fig. 1.

Table 1. Multiplication of cells released from trunks, either normal or treated with puromycin; eight trunks were used in each series.

Incubation period (hr)	Cells ($\times 10^6$) from trunks: Normal	Treated with puromycin
0	2.06	1.89
48	4.20	4.76
72	12.22	12.20

ed, as with the CSA labeled with sulfate.

Considerable variation in the degree of inhibition of incorporation of sulfate was observed for the lowest concentration of puromycin tested; the 26-percent inhibition (Fig. 1) represents the average of values from six experiments, which ranged from 13- to 46-percent inhibition. This fluctuation was not encountered with incorporation of either glucose or valine at this same concentration of puromycin, nor with incorporation of sulfate at the higher concentrations of puromycin tested; this discrepancy remains unexplained. The maximum degree of inhibition of synthesis of CSA requires about one-fifth the concentration of puromycin necessary for the complete inhibition of incorporation of valine.

The inhibition of synthesis of both protein and CSA is reversible (Fig. 2). When trunks are removed to puromycin-free medium, incorporation of valine into protein returns to a rate about 80 percent of normal, with gradual recovery of incorporation of sulfate and glucose into CSA.

The reversible nature of inhibition by puromycin has been observed in vivo (*8*), in cell-free systems (*9*), and in tissue slices (*10*). The release from the inhibition by puromycin, as shown by the return to the near-normal rate of incorporation of valine, does not in itself necessarily mean that these cells have survived this treatment. In order to demonstrate that cells in the trunks treated with puromycin could still be induced to synthesize DNA and all components necessary for cell multiplication, trunks were incubated in MEM in the presence and absence of puromycin; the same amount of tissue was obtained for each series so that the total number of viable cells could be estimated. After being incubated for 5 hours, they were transferred to puromycin-free MEM and incubated overnight. The next day they were treated with trypsin. The cells were then suspended, washed, and resuspended in 2 ml of medium, and 1 ml of the suspension was plated on clots and incubated for 48 and 72 hours, at which times the cells were recovered and counted. Almost the same number of cells (91 percent) were obtained (Table 1) from puromycin-treated trunks as from untreated trunks, and treated cells multiplied as well as normal cells.

Treatment of 10-day-old trunks with puromycin for 5 hours under the above conditions thus has no deleterious effect on the capacity of chondrocytes for cell division. However, treatment with puromycin beyond 5 hours under such conditions kills many cells: 70 percent are killed after a 6-hour exposure to puromycin.

Our results indicate that syntheses of CSA and protein are intimately associated. This idea is supported by the observation that actidione, another inhibitor of protein synthesis (*11*), inhibits synthesis of CSA (*12*). Since CSA is covalently linked to protein (*13*), it seems that its synthesis must be coupled to that of protein. This may also imply the existence of a feedback control of the synthesis of this polysaccharide whereby no protein-free CSA accumulates because it inhibits some step in its own synthesis. A recent report of a similar effect of puromycin on the synthesis of glycoprotein in rat liver is that inhibition of synthesis of protein led to the accumulation of uridine-diphospho-*N*-acetylglucosamine, which is an inhibitor of the first reaction in its own biosynthesis (*14*).

The long-term effect on CSA synthesis of a brief treatment of these cells with puromycin at a concentration that completely inhibits synthesis of protein is of some interest. The gradual return to normal synthesis of CSA, as determined by incorporation of sulfate and glucose, occurs only when the enriched medium is used; when trunks are instead transferred to MEM, a loss of 60 percent in the rate of incorporation of glucose or sulfate into CSA is still observed after 48 hours, whereas incorporation of valine returns to 70 percent of normal.

This failure to synthesize CSA may reflect a partial loss of some enzyme or enzymes in this pathway because of turnover during the inhibitory period, of accumulation of some inhibitor which affects synthesis of CSA more than protein synthesis, or of the loss of some cofactor necessary in this synthesis. It is significant that puromycin does not interfere with the release of radioleucine from labeled protein in liver slices (*12*), which fact is evidence for the turnover of protein (*15*); it has also been demonstrated that puromycin prevents neither the disappearance of tryptophan pyrrolase in rat liver when injected after full induction of this enzyme has been obtained (*16*), nor the turnover of rat liver amylase (*17*).

G. DE LA HABA
H. HOLTZER
Department of Anatomy, School of Medicine, University of Pennsylvania, Philadelphia

References and Notes

1. M. B. Yarmolinsky and G. L. de la Haba, *Proc. Nat. Acad. Sci. U.S.* **45**, 1721 (1959).
2. L. Adamson, S. Gleason, C. Anast, *Biochem. Biophys. Acta* **83**, 262 (1964).
3. H. Holtzer, J. Abbott, J. Lash, S. Holtzer, *Proc. Nat. Acad. Sci. U.S.* **46**, 1533 (1960).
4. H. Eagle, *Science* **130**, 432 (1959).
5. F. E. Stockdale, J. Abbott, S. Holtzer, H. Holtzer, *Develop. Biol.* **7**, 293 (1963).
6. G. A. Bray, *Anal. Biochem.* **1**, 279 (1960).
7. E. H. Eylar, *Biochem. Biophys. Res. Commun.* **8**, 195 (1962).
8. J. B. Flexner, L. B. Flexner, E. Stellar, G. de la Haba, R. B. Roberts, *J. Neurochem.* **9**, 595 (1962).
9. A. Morris, R. Arlinghaus, S. Favelukes, R. Schweet, *Biochemistry* **2**, 1084 (1963).
10. J. J. Ferguson, Jr., *J. Biol. Chem.* **238**, 2754 (1963).
11. H. L. Ennis and M. Lubin, *Science* **146**, 1474 (1964).
12. G. de la Haba, unpublished observations.
13. B. Anderson, P. Hoffman, K. Meyer, *Biochem. Biophys. Acta* **74**, 309 (1963).
14. S. Kornfeld, R. Kornfeld, E. F. Neufeld, P. J. O'Brien, *Proc. Nat. Acad. Sci. U.S.* **52**, 371 (1964).
15. M. V. Simpson, *J. Biol. Chem.* **201**, 143 (1953).
16. A. Nemeth, *ibid.* **237**, 3703 (1962).
17. R. L. McGeachin, B. A. Potter, A. C. Lindsey, *Arch. Biochem. Biophys.* **104**, 314 (1964).
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Right Horn Implantation in the Common Duiker

Abstract. *In the common duiker,* Sylvicapra grimmia, *implantation occurs only in the right uterine horn, but both ovaries ovulate.*

Reproductive tracts of 49 female common duikers (*Sylvicapra grimmia*) were collected in Bechuanaland and Rhodesia. In 39 of these tracts pregnancy was obvious and the single embryo was implanted in the right uterine horn. Thus the common duiker resembles three other African bovids in this