Molecular Specificity Associated with Alleviation of Actinomycin D Inhibition of Protein Synthesis

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(Received 5 April 1971)

The inhibition of protein synthesis by actinomycin D can be prevented or reversed by selected aromatic compounds. Durham & Keudell (1969) reported that *Pseudomonas fluorescens* NND produced an inducible enzyme, protocatechuate oxidoreductase (EC 1.13.1.3), in the presence of the antibiotic. The inducer, protocatechuate, prevented and reversed the inhibition by actinomycin D of growth and macromolecular syntheses in a synthetic succinate-salts medium. It has also been established that protocatechuate prevented or reversed the inhibition by actinomycin D of synthesis of an acyltransferase (amidase; acylamide aminohydrolase, EC 3.5.1.4) in this organism (Ferguson & Durham, 1970).

Actinomycin D has been reported to inhibit by binding to the guanine moiety of double-stranded helical DNA (Cerami, Reich, Ward & Goldberg, 1967), although the presence of guanine may not be a sufficient requisite (Wells, 1969; Hyman & Davidson, 1971). The prevention and reversal studies with protocatechuate emphasize the unique ability of microbial cells to grow or synthesize proteins in the presence of a DNA-binding component. Alleviation of the inhibition suggests that complexing of the antibiotic with cellular DNA is a reversible process, and it has been proposed that protocatechuate (3,4-dihydroxybenzoate) interacts with actinomycin D to negate the antimicrobial action (Durham & Keudell, 1969).

The present study establishes that selected aromatic compounds demonstrate the potential for reversal and that the specificity of the molecule is very important in determining the ability of the compound to complex or interact with the antibiotic.

Growth and enzyme-induction studies with *P. fluorescens* NND were conducted with a synthetic salts medium containing 0.2% sodium chloride, 0.2% ammonium chloride, 0.32% potassium dihydrogen phosphate, 0.42% dipotassium hydrogen phosphate and 0.2% sodium succinate. Acetamide (0.08M) was added as the inducer for the acyltransferase. The medium was adjusted to pH 7.0 and sterilized, and 0.1 ml of a sterile mineral salts solution (Durham, 1958) was added to each 100 ml of medium.

The medium containing the inducer was inoculated with succinate-grown cells to E_{540} 0.20

(18 mm light-path). The flasks contained 35 ml of succinate medium with acetamide, 25 μ g of actinomycin D/ml, the indicated test compound (0.8 mM) and sterile water to a total volume of 40 ml. The flasks were incubated at 37°C with constant shaking. Samples (1.0 ml) were taken at periodic intervals and frozen. Acyltransferase activity was assayed by thawing the samples and adding 0.25 ml to 0.75 ml of the substrate mixture containing equal volumes of 0.4M-acetamide, 2.0M-hydroxylamine hydrochloride (freshly neutralized) and 0.1M-tris-HCl buffer, pH 7.2. The mixture was incubated for 15 min at 37°C and the reaction terminated by addition of 2.0 ml of ferric chloride (6%, w/v, in 2% hydrochloric acid). The E_{540} was read in a Coleman Junior II spectrophotometer and the amount of acetohydroxamate formed during the reaction was calculated from a standard curve.

Compounds tested to ascertain their potential to prevent the inhibition of synthesis of acyltransferase by actinomycin D are listed in Table 1. The results established that equimolar concentrations of 3,4-dihydroxybenzoate and 2,3-dihydroxybenzoate were the most effective in preventing the inhibition of protein synthesis. These two compounds showed approximately equal potential for preventing the inhibition of acyltransferase by the antibiotic. The dihydroxyl structural moiety appears to be critical for interaction of the benzenoid and antibiotic molecules. In addition, the results indicate a requirement for the two hydroxyl groups to be on adjacent carbon atoms since 2,4- and 2,5-dihydroxybenzoate showed very little, if any, preventive potential. The 3-position on the benzene ring apparently has some importance, but 3,5-dihydroxybenzoate, in which the 3-position is occupied, showed little effect on actinomycin D inhibition. These results stress the importance of an occupied 3-position, coupled with the need for a hydroxyl group on one of the adjacent carbon atoms (either C-2 or C-4).

The presence of a single hydroxyl group at either C-2, C-3 or C-4 (*o*-, *m*-, *p*-hydroxybenzoate) was not effective, also emphasizing the need for reactive groups on adjacent carbon atoms. 3,4,5-Trihydroxybenzoate did not alleviate the action of actinomycin D. Although this molecule does have a hydroxyl group on the 3-position and hydroxyl

Table 1. *Prevention by selected compounds of inhibition by actinomycin D of acyltransferase synthesis*

Relative acyltransferase activities, as compared with the control value of 100%, are reported for the 6 h incubation and assay. The indicated compounds were tested at a concentration of 0.8 mM. The actinomycin D concentration was 25 µg/ml.

Additive	Acyltransferase activity (% of control)
None (control)	100
Actinomycin D	0
3,4-Dihydroxybenzoate (protocatechuate)	92
2,3-Dihydroxybenzoate	89
2,4-Dihydroxybenzoate	17
2,5-Dihydroxybenzoate	14
3,5-Dihydroxybenzoate	19
3,4,5-Trihydroxybenzoate	6
<i>o</i> -Hydroxybenzoate	6
<i>m</i> -Hydroxybenzoate	5
<i>p</i> -Hydroxybenzoate	10
<i>o</i> -Aminobenzoate (anthranilate)	19
<i>m</i> -Aminobenzoate	0
<i>p</i> -Aminobenzoate	4
Benzoate	7
4-Aminosalicylate	9
3,4-Dichlorobenzoate	6

groups on adjacent carbon atoms, the presence of three adjacent hydroxyl groups apparently gives a molecule with structural and conformational integrity that prevents an interaction with actinomycin.

Reactive groups such as hydroxyl groups apparently are a requisite for interaction between these molecules, since substitution at C-3 and C-4 (3,4-dichlorobenzoate) completely negated the potential to prevent the inhibition.

The presence of an amino group in the 2-position (anthranilate), 3-position (*m*-aminobenzoate) or 4-position (*p*-aminobenzoate) was not effective.

Growth studies revealed that the compounds capable of preventing the inhibition by actinomycin

D of acyltransferase also alleviated the inhibition of growth by the antibiotic in the synthetic salts medium.

These findings establish that selected aromatic compounds prevent the inhibition of protein synthesis and growth by actinomycin D. The steric specificity of the molecule and its conformational integrity are very important. Molecular models depict that the benzenoid compounds may layer or align themselves along the horizontal plane of the unreduced quinoidal ring structure of the antibiotic. It is suggested that a type of molecular complex may be formed between the compounds in which the π -orbitals overlap and are co-ordinated so that the two molecules interact. Although charge transfer has not been completely eliminated, it is questionable if a full electron transfer does exist between the molecules.

Complexing of the dihydroxybenzoates with actinomycin D prevents the antibiotic from interacting with cellular DNA. Since these compounds can also overcome an established inhibition, the actinomycin D-DNA complex would appear to be reversible and in a dynamic state. Thus, when the antibiotic is released, the benzenoid compounds interact with it and prevent any additional inhibition. The complex formed between the benzenoid compounds and actinomycin D would appear to have greater stability than the antibiotic-DNA complex.

The greater affinity of the dihydroxybenzoates for actinomycin would contribute to a shift in the equilibrium.

This investigation was supported by American Cancer Society Grant IN-91B.

Cerami, A., Reich, E., Ward, D. C. & Goldberg, I. H. (1967). *Proc. natn. Acad. Sci. U.S.A.* **57**, 1036.

Durham, N. N. (1958). *Can. J. Microbiol.* **4**, 141.

Durham, N. N. & Keudell, K. C. (1969). *Antimicrob. Agents Chemother.* p. 67.

Ferguson, D. V. & Durham, N. N. (1970). *Bact. Proc.* p. 53.

Hyman, R. W. & Davidson, N. (1971). *Biochim. biophys. Acta*, **228**, 38.

Wells, R. D. (1969). *Science, N.Y.*, **165**, 75.