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SHORT COMMUNICATIONS

Neomycin inhibition of DNA polymerase

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AMINOGLYCOSIDIC antibiotics, streptomycin, neomycin and kanamycin, have a high affinity to DNA.[1–4] It has been shown, for example, that the interaction of streptomycin with DNA (a) increases the thermal transition temperature,[5] (b) forms a salt[1] leading to precipitation[1,2] and (c) remains bound throughout purification.[4,5] Neomycin (Nm) inhibits DNA synthesis up to 30 per cent in *Chlorella in vivo*[6] although its mode of action is the inhibition of protein synthesis by the specific binding to the 30 S ribosome subunit.[7,8] However, in the presence of Nm, ribosomes curiously retain substantial activity to enable them to translate *in vitro* single-stranded[4,7,9] and f2 supercoiled DNA,[10] synthetic deoxypolynucleotides with repeating nucleotide sequences[11] and misread poly U.[3] Nm affects the interaction of aminoacyl-tRNA with ribosomes,[4,7,8,11] while the binding of DNA to ribosomes occurs in its absence.[7,10,11] The possibility exists that Nm might also function at the level of its interaction with DNA.[3,4,7] To test this hypothesis, we assayed the overall template efficiency of DNA-Nm complexes with DNA polymerase. In this communication we present evidence that Nm effectively inhibits DNA polymerase by specifically interacting with the DNA template.

DNA polymerase was a partially purified preparation from the cytoplasmic extracts of a baby hamster kidney cell line (BHK 21).[12] The peak of activity which eluted between 0·15–0·18 M NaCl from a DEAE-cellulose column was fractionated with ammonium sulfate. The precipitate of the 50–60 per cent (w/v) saturation was dialyzed against 10 mM tris–HCl, pH 7·9, 1 mM EDTA, 2 mM 2-mercaptoethanol containing 10% (v/v) glycerol and used throughout these experiments. The preparation exhibited a single, symmetric peak of activity on sucrose gradients corresponding to 7·2 S and was absolutely dependent on input DNA.[12] The 100 μl reaction mixture contained 10 mM Tris–HCl, pH 7·94 (at 37°), 8 mM $MgCl_2$, 2 mM dithiothreitol, 0·02 mM each dATP, dCTP and dGTP, 0·11 μM (^{3}H)TTP (sp. act. 28·1 Ci/mmole; The Radiochemical Centre, Amersham, England), DNA (see Figs. 1 and 2) and 3·9 μg protein. Following 5 min preincubation on ice with Nm and the other components, the reaction was initiated by adding enzyme and transfering the tubes to 37°. After 30 min the reaction was terminated by cooling and adding pyrophosphate (PPi) and trichloroacetic acid (TCA) to 0·04 M and 25% (v/v) respectively. Precipitates were collected on Whatman GF/C glass-fiber filters, washed with 1% TCA (v/v) TCA-10 mM PPi, acetone dried and radioactivity determined

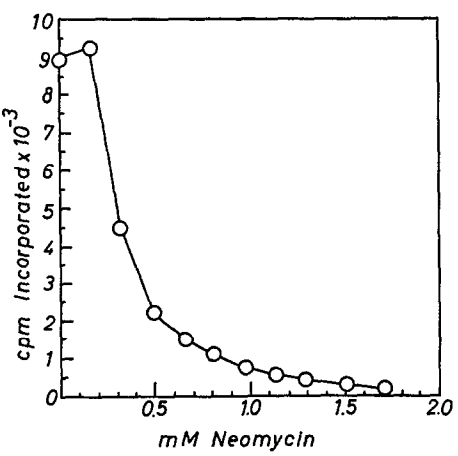

FIG. 1. Inhibition of DNA polymerase activity as a function of neomycin concentration. Reaction mixtures contained 2·5 μg activated DNA and were assayed as given in the text. Points are averages of duplicate samples. No visible turbidity was detected at any concentration.

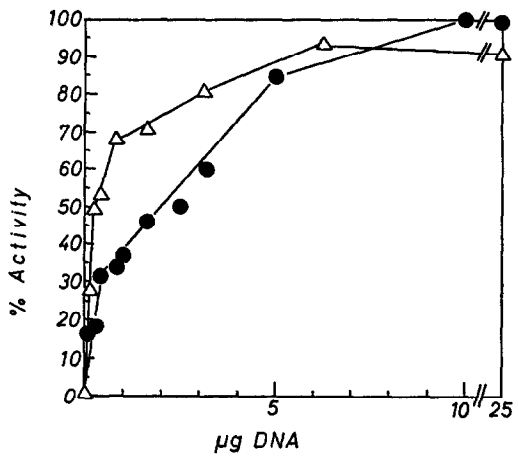

FIG. 2. Relative activity of DNA polymerase with varying concentrations of DNA template. The concentration of single-stranded (△) and activated DNA (●) varied 1000-fold from 0·1 to 100 μg at a constant level of 0·32 mM Nm. Percent activity of the Nm treated samples is relative to a non-treated control at each DNA concentration. Maximum control activity (100 per cent) for single-stranded and activated DNA was 3100 and 11,900 counts/min respectively. The points for activated DNA are taken from two experiments.

by liquid scintillation spectrophotometry. Salmon sperm DNA (Worthington) was either heat denatured (100°, 20 min; 34 per cent hypochromicity) or activated with DNase I according to Aposhian and Kornberg.[13] Reactions were carried out in duplicate.

All chemicals used were of reagent grade and obtained from commercial sources. Neomycin was the free base and a 162 mM stock solution was adjusted to pH 7·9 and made 10 mM in tris-HCl.

Neomycin asserted a strong inhibitory effect on the polymerization of nascent DNA chains (Fig. 1). At a concentration of 0·32 mM Nm, DNA polymerase activity was inhibited 50 per cent and reached complete cessation with a 10-fold increase in Nm concentration. At this high level, Nm was still 10 times more effective than kanamycin as an inhibitor of polymerase activity. The site of inhibition could involve competition with the substrate nucleoside triphosphates, complexing with the metal ion cofactor, binding to the enzyme or to the DNA template.

Although the neobiosamine portion of the Nm molecule could conceivably compete with the substrate deoxynucleoside triphosphates, excess dATP, dCTP and dGTP (0·56 mM) and/or 1·0 mM ATP failed to relieve inhibition. The level of Mg^{2+} present in the system (8 mM) would *a priori* eliminate the possibility of an inactive Nm–metal ion complex. Similarly, inhibition remained constant over a 16-fold increase in enzyme concentration (data not shown). Therefore it seemed reasonable to us that the mode of inhibition was related to the well documented binding of Nm to DNA.[3,4,7]

Inhibition of DNA polymerase activity was found to occur in an inverse relationship to the input DNA template (Fig. 2). At DNA levels as low as 0·1 μg, 0·32 mM Nm suppressed activity approximately 83 and 72 per cent on activated and single-stranded DNA, respectively. Even though the single-stranded DNA template elicited approximately 0·25 the activity compared with activated DNA, it was more effective than activated DNA at low template concentrations in reversing Nm inhibition (Fig. 2). This might reflect a greater Nm binding capacity by single-stranded DNA. In the presence of Nm, single-stranded DNA stimulates protein synthesis to a much greater extent than native DNA.[4] Large excess of DNA reversed Nm inhibition; above 5 μg DNA, Nm produced little or no effect (Fig. 2). Reversal presumably occurred by oversaturating the system with template, analogous to the effect of polyamino acid inhibition of Foot-and-Mouth Disease virus replicase.[14] All the Nm appears to be bound to DNA and the availability of Nm-free stretches of DNA (as well as DNA free of Nm) enabled the detection of fully active DNA polymerase.

Since Nm inhibits DNA polymerase activity *in vitro*, it suggests the possibility that it acts in a similar manner *in vivo*.[6] The concentration of Nm used *in vitro* was approximately 5 to 6-fold less than that required for a similar degree of DNA synthesis inhibition *in vivo*.[6] Alternatively, however, Nm might influence DNA biosynthesis indirectly through inhibition of protein synthesis:[8] deletion of a labile protein cofactor involved in DNA synthesis or, if DNA polymerase has a high turnover rate in

Chlorella, either reduce the amount of enzyme or increase the number of nonfunctioning enzyme molecules caused by misreading errors.[3,11]

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Biliary excretion of methadone by the rat: identification of a para-hydroxylated major metabolite

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THE EXTENSIVE biotransformation of methadone in both man and the rat has been shown to result in production of the *N*-demethylated cyclic metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP),[1,2] which is further *N*-demethylated to 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (EMDP).[2] Hydroxylated derivatives of EDDP and EMDP were found in human urine and rat urine and bile.[3] Baselt and Casarett[4] described the biliary secretion by the rat of a conjugated methadone metabolite, representing nearly one-third of an administered methadone dose, and assumed it to be a hydroxylated EDDP derivative. In the present study we identified this metabolite as conjugated *p*-hydroxy-EMDP.

MATERIALS AND METHODS

Male Sprague–Dawley rats (200–300 g) were used.

dl-Methadone hydrochloride was purchased from Merck Chemical Co. EDDP perchlorate and EMDP hydrochloride were gifts of Eli Lilly & Co. The *p*-methoxy analogue of methadone (*p*-OCH_3-methadone) was synthesized by the method of Shapiro[5] and was crystallized as its perchlorate salt (m.p. 163–164°).

Under ether anesthesia, the common bile ducts of the rats were cannulated using AWG-30 teflon tubing. Drugs were administered for metabolite collection by subcutaneous injection at a dose of 20 mg/kg and bile was collected for a period of 20 hr. For the excretion rate study, the drugs were administered by injection into the femoral vein at a dose of 1 mg/kg and bile collected for 4 hr.