Inhibition of Deoxyribonucleic Acid Polymerases from Human Cells and from Simian Sarcoma Virus by Pyran

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Pyrans are co-polymers of divinyl ether and maleic anhydride. Four pyrans of various molecular weights more potently inhibited terminal deoxyribonucleotidyltransferase (EC 2.7.7.31) from a human cell line of acute lymphoblastic leukaemia origin (Molt-4) than they did DNA polymerases α , β and γ from these cells and DNA polymerase from simian sarcoma virus. For example, the concentrations of one pyran required for 50% inhibition of terminal deoxynucleotidyltransferase, DNA polymerases α , β and γ and viral DNA polymerase were 0.9, 110, 125, 35 and 47 $\mu\text{g/ml}$ respectively. Quantitatively similar results were obtained with the other pyrans. Inhibition of these enzymes by pyran was dependent on the concentrations of both the bivalent cation and template/primer or initiator in assay mixtures, but not on the concentrations of the substrate (deoxyribonucleoside 5'-triphosphate), enzyme, or bovine serum albumin. These results suggested that pyran inhibited these enzymes by complexing bivalent cations, which caused a decreased affinity of template/primer or initiator for each enzyme and a decrease in enzyme activity.

Pyran, a 1:2 co-polymer of divinyl ether and maleic anhydride, can be prepared to different molecular weights and is polyanionic in aqueous solution (Breslow *et al.*, 1973). It was reported that pyran preferentially inhibited DNA polymerase (EC 2.7.7.7) of RNA tumour viruses compared with cellular DNA polymerases and that pyran interacted with the viral polymerase by binding at a site other than the catalytic centre (Papas *et al.*, 1974). However, this mode of action by pyran has been challenged (Fiel *et al.*, 1976a). These investigators reported that pyran inhibited viral DNA polymerase by complexing with bivalent cations that were essential for enzyme activity. Furthermore, they proposed that the apparent selective inhibition by pyran of viral DNA polymerase compared with cellular DNA polymerases reported by Papas *et al.* (1974) could be due to differences in assay conditions which can influence the formation of pyran-bivalent-cation complexes (e.g. type and concentration of bivalent cation). Because of this controversy about the mode of action of pyran and because selective inhibitors of viral and mammalian cellular DNA polymerases are important for the characterization of these enzymes, we investigated the effects of four different pyrans on the activities of DNA polymerase from simian sarcoma virus and DNA polymerases α , β and γ .

Abbreviations used: terminal transferase, terminal deoxyribonucleotidyltransferase (EC 2.7.7.31); $\bar{M}_w$, weight-average molecular weight; $\bar{M}_n$, number-average molecular weight.

[the nomenclature used for eukaryotic DNA polymerases (DNA polymerase α , β and γ) has been explained in Weissbach *et al.* (1975)] and terminal transferase from Molt-4 cells. Two of the cellular enzymes, DNA polymerase γ and terminal transferase, were not previously examined for inhibition by any pyran and two of the pyrans were not previously tested for inhibition of any DNA polymerase. We found that under standard assay conditions, terminal transferase was more sensitive to inhibition by pyran than were the other enzymes. In addition, inhibition of all enzymes examined was dependent not only on the bivalent-cation concentration of assay mixtures but also on the template/primer or initiator concentration. Furthermore, an improved interpretation of past data on the binding of pyran to viral DNA polymerase is presented.

Materials and Methods

Pyrans were generously given by Dr. D. S. Breslow, Hercules Inc., Wilmington, DE, U.S.A. The four compounds donated were designated by the manufacturer as X19910-4, X18720-71, X146-85-2 and XA124-177 and had intrinsic viscosities of 0.215, 0.70, 0.76 and 1.65 (ml/g) respectively (D. S. Breslow, personal communication), indicating their increasing $\bar{M}_w$. Molecular weights of the methyl esters of compounds XA124-177 and X146-85-2 as determined by gel-permeation chromatography were 32000 and 22500 respectively (Breslow *et al.*, 1973). However,

the $\bar{M}_w/\bar{M}_n$ ratios for compounds X124-177 and XA146-85-2 were 7.2 and 3.7 respectively, demonstrating that these compounds have broad molecular-weight distributions.

Molt-4 cells are of acute lymphoblastic leukaemia origin (Srivastava, 1974). Terminal transferase and DNA polymerases α , β and γ were prepared from these cells by our published procedures (Srivastava, 1974; DiCiccio & Srivastava, 1976). DNA polymerase from simian sarcoma virus was prepared as described by Abrell & Gallo (1973). The specific activities of these enzymes were similar to those reported previously (DiCiccio & Srivastava, 1976; Abrell & Gallo, 1973) and their properties have been described (Srivastava, 1974; DiCiccio & Srivastava, 1977; Abrell & Gallo, 1973). One unit of enzyme activity is that which polymerizes 1 nmol of ^3H -labelled nucleoside monophosphate/h.

Enzymes were assayed in a final volume of 0.2 ml as follows: (a) for DNA polymerases α or β , 10 μmol of Tris/HCl (pH 8.3), 1.2 μmol of magnesium acetate, 4.0 μmol of dithiothreitol, 0.16 μmol each of dATP, dGTP and dCTP, 0.01 μmol of [^3H]dTTP (144 e.p.m./pmol) (Schwartz-Mann, Orangeburg, NY, U.S.A.), 30 μg of activated (Srivastava, 1974) calf thymus DNA (Worthington Biochemicals, Freehold, NJ, U.S.A.) and enzyme; (b) for DNA polymerase γ , 10 μmol of Tris/HCl (pH 7.5), 0.1 μmol of MnCl_2 , 20 μmol of KCl, 0.6 μmol of dithiothreitol, 8 μg of bovine serum albumin, 0.001 μmol of [^3H]dTTP (1036 c.p.m./pmol), 5.6 μg of poly(A)·(dT) $_{12-18}$ (Schwartz-Mann) and enzyme; (c) for terminal transferase, 10 μmol of Tris/HCl (pH 7.5), 0.1 μmol of MnCl_2 , 20 μmol of KCl, 0.6 μmol of dithiothreitol, 8 μg of bovine serum albumin, 0.01 μmol of [^3H]dGTP (115 c.p.m./pmol), 2.4 μg of (dA) $_{12-18}$ (Collaborative Research Inc., Waltham, MA, U.S.A.) and enzyme; (d) for DNA polymerase from simian sarcoma virus, same as for DNA polymerase γ except that 2.4 μg of poly(A)·(dT) $_{12-18}$ and 0.04 μmol of [^3H]dTTP (412 c.p.m./pmol) were used. The addition sequence for assay mixtures was template/primer or initiator, pyran (if added), the other components of the reaction mixture, and finally enzyme. Reaction mixtures were incubated at 37°C (30°C for DNA polymerase γ) for 30 min (20 min for terminal transferase), after which 50 μg of yeast RNA and 1 ml of 20% (w/v) trichloroacetic acid containing 3% (w/v) sodium pyrophosphate were added. The precipitates were collected on nitrocellulose membrane filters, washed with 5% trichloroacetic acid, dried, and counted for radioactivity by using a toluene-based scintillation fluid (Srivastava, 1974). All enzyme activities were linear with respect to time and protein concentration.

Data describing the inhibition of DNA polymerases by pyran at various concentrations of substrate, template/primer or initiator were plotted by the method of Lineweaver & Burk (1934), as exempli-

fied in Fig. 1 for DNA polymerase from simian sarcoma virus. Kinetic constants derived from such plots are defined as follows: K_m^s is the apparent Michaelis constant for substrate (dGTP for terminal transferase or dTTP for viral polymerase and DNA polymerases α , β and γ) obtained when substrate concentration was varied at constant concentration of template/primer or initiator; K_m^t is the apparent Michaelis constant for template/primer (activated DNA for DNA polymerases α and β and poly(A)·(dT) $_{12-18}$ for DNA polymerase γ and viral DNA

Fig. 1. Inhibition of RNA-dependent DNA polymerase from simian sarcoma virus by pyran at various concentrations of template/primer or substrate

DNA polymerase from simian sarcoma virus (0.03 unit) was assayed in reaction mixtures containing: (a) the indicated concentrations of pyran XA124-177 ($\circ$, 0; $\bullet$, 20 $\mu\text{g}/\text{ml}$) and poly(A)·(dT) $_{12-18}$; or (b) the indicated concentrations of pyran XA124-177 ($\circ$, 0; $\bullet$, 100 $\mu\text{g}/\text{ml}$) and dTTP. Other components of reaction mixtures and incubation conditions are specified in the Materials and Methods section. Data were plotted as described by Lineweaver & Burk (1934).

polymerase) or initiator [(dA)₁₂₋₁₈ for terminal transferase] obtained when template/primer or initiator concentration was varied at constant concentration of substrate; and V is the velocity of nucleoside monophosphate incorporation when enzyme is saturated with substrate and template/primer or initiator. The values of K_m^s , K_m^i and V may or may not change in the presence of inhibitor (pyran). Therefore, when these values are given, the presence or absence of inhibitor is specified. Inhibition constants (K_i) for pyran were not calculated, because K_i is the dissociation constant of an enzyme-inhibitor complex, and evidence indicated that inhibition by pyran was caused by the complexing of bivalent cations essential for enzyme activity and not by interaction with enzyme.

Results

Four pyrans of different intrinsic viscosities inhibited terminal transferase and DNA polymerases α , β and γ from Molt-4 cells and DNA polymerase from simian sarcoma virus in a concentration-dependent manner (Table 1). The degree of inhibition of any one enzyme by the four pyrans was similar, demonstrating no correlation between intrinsic viscosity and degree of inhibition. Terminal transferase was more potently inhibited by all four pyrans than were the other enzymes. For example, the concentration of compound XA124-177 required to inhibit terminal transferase by 50% was 0.9 $\mu\text{g/ml}$, whereas the concentrations required to inhibit viral DNA polymerase and DNA polymerases α , β and γ

by 50% were 47, 110, 125 and 35 $\mu\text{g/ml}$ respectively. Inhibition of all enzymes by pyran (e.g. XA124-177) was reversed by dilution of this compound to non-inhibitory concentrations (Table 2). In addition, inhibition of all enzymes by pyran (e.g. XA124-177) was a linear function of time, and addition of this compound to an on-going uninhibited reaction resulted in immediate inhibition of enzyme activity (Fig. 2 is representative for all enzymes).

To determine the mode of inhibition of DNA polymerases by pyran, individual components of assay mixtures were varied. Addition of extra template/primer or initiator overcame inhibition of each enzyme by pyran, but addition of extra enzyme or bovine serum albumin had no substantial effect on inhibition (Table 3). Furthermore, enzymes assayed at bivalent-cation concentrations 10 times those required for optimal enzyme activity were resistant to inhibition by pyran (Table 3). These data show that

Table 1. *Inhibition of DNA polymerase activities from Molt-4 cells and simian sarcoma virus by pyrans*

Terminal transferase (0.12 unit), DNA polymerase α (0.11 unit), DNA polymerase β (0.15 unit), DNA polymerase γ (0.02 unit), and simian sarcoma virus DNA polymerase (0.03 unit) were tested for inhibition by pyrans (six different concentrations of each) under standard assay conditions. Controls without pyrans incorporated 40.3, 56.9, 74.3, 8.6 and 14.8 pmol of ³H-labelled nucleoside monophosphate respectively for each of the above enzyme assays. The pyrans are listed in order of increasing intrinsic viscosity, indicating their increasing $\bar{M}_w$.

Pyran	Terminal transferase	Concentration yielding 50% inhibition of each enzyme activity ($\mu\text{g/ml}$)			
		DNA polymerase			
		α	β	γ	Viral
X19910-4	0.8	120	155	52	79
X18720-7	0.8	150	160	60	55
X146-85-2	0.7	90	135	43	64
XA124-177	0.9	110	125	35	47

Table 2. *Effect of dilution on the inhibition of DNA polymerase activities from Molt-4 cells and simian sarcoma virus by pyran*

Duplicate standard reaction mixtures (controls) and duplicate experimental mixtures [standard reaction mixtures containing pyran (XA124-177)] were incubated at 37°C. After 1 min, one of the experimental and one of the control mixtures were diluted 10 times into complete reaction mixtures lacking enzyme and inhibitor, but containing bovine serum albumin (200 $\mu\text{g/ml}$). After 30 min at 37°C, the reaction mixtures were processed as described in the Materials and Methods section. Amounts of enzymes used in standard reaction mixtures were 0.11, 0.12, 0.02, 0.12 and 0.03 unit of DNA polymerases α , β and γ , terminal transferase and simian sarcoma virus DNA polymerase respectively. Experiments with DNA polymerase γ were done at 30°C. dNMP is deoxyribonucleoside monophosphate, which was dGMP for terminal transferase assays and dTMP for all other enzyme assays.

Expt. no.	Conditions	dNMP incorporated (pmol)	
		Undiluted	Diluted
1	Controls (polymerase α)	57	53
	Polymerase α + pyran (200 $\mu\text{g/ml}$)	15	49
2	Controls (polymerase β)	58	51
	Polymerase β + pyran (150 $\mu\text{g/ml}$)	33	50
3	Controls (polymerase γ)	10	8
	Polymerase γ + pyran (40 $\mu\text{g/ml}$)	4	8
4	Controls (terminal transferase)	39	35
	Terminal transferase + pyran (2 $\mu\text{g/ml}$)	13	30
5	Controls (viral polymerase)	14	12
	Viral polymerase + pyran (100 $\mu\text{g/ml}$)	4	11

Fig. 2. Time course of inhibition of DNA polymerase γ from Molt-4 cells by pyran

DNA polymerase γ (0.02 unit) was assayed by using standard reaction mixtures with or without pyran (XA124-177). ○, Enzyme activity without pyran; ●, enzyme activity with pyran (40 μ g/ml) added at zero time; △, enzyme activity with pyran (100 μ g/ml) added 10 min after the start of uninhibited reaction; ▲, enzyme activity with pyran (100 μ g/ml) added at zero time.

inhibition was dependent on the concentrations of both the bivalent cation and the template/primer or initiator in assay mixtures. For each enzyme, double-reciprocal plots were made of enzyme velocity versus substrate and template/primer or initiator concentration at different concentrations of pyran. These results are summarized in Table 4 from plots analogous to Fig. 1. For experiments in which the concentration of substrate was varied, the K_m^s remained essentially unchanged in either the presence or absence of pyran, but the V of each enzyme decreased with increasing concentrations of pyran. These results show that pyran had no effect on the affinity of each enzyme for substrate and that inhibition of each enzyme by pyran was independent of substrate concentration. However, for experiments in which the concentration of template/primer or initiator was varied, the K_m^t increased with increasing pyran concentrations, whereas the V of each enzyme remained virtually the same. These results demonstrate that pyran decreased the affinity of template/primer or initiator for each enzyme and that inhibition by pyran was dependent on the concentration of template/primer or initiator. This corroborates data presented in Table 3.

Discussion

The data presented demonstrated that inhibition of cellular and viral DNA polymerases by pyran was dependent on the concentrations of bivalent cation and template/primer or initiator in assay mixtures, but not on substrate or enzyme concentration. Previously, Fiel *et al.* (1976a) showed that inhibition of mouse leukaemia virus DNA polymerase and eukaryotic RNA polymerases I and II by pyran was dependent on bivalent-cation concentration. Although Papas *et al.* (1974) reported that inhibition of a viral DNA polymerase by pyran was dependent on enzyme concentration, their data in fact, like ours, show no such dependence when calculated properly. They reported that a complete reaction mixture incorporated 5300 c.p.m. of radioactivity into DNA under normal conditions, but that addition of pyran (100 μ g/ml) decreased incorporation to 700 c.p.m., resulting in $(700/5300) \times 100 = 13.2\%$ of control enzyme activity. Addition of 10 times the amount of enzyme used above to reaction mixtures containing pyran (100 μ g/ml) resulted in 5610 c.p.m. of radioactivity incorporated. This was interpreted by Papas *et al.* (1974) as a complete reversal of inhibition by excess enzyme, since the incorporation of radioactivity was restored to that of their first experiment. However, we dispute this interpretation, because the proper control for this last experiment is a reaction mixture containing 10 times the amount of enzyme in the absence of pyran. If we assume that the enzyme activity reported by Papas *et al.* (1974) was directly proportional to enzyme concentration, then a reaction mixture containing 10 times the amount of enzyme in the absence of pyran would incorporate approx. 53000 c.p.m. of radioactivity and $(5610/53000) \times 100 = 10.6\%$ of control enzyme activity. This is virtually the same degree of inhibition reported for experiments with 10-fold less enzyme and does not demonstrate a reversal of inhibition by excess enzyme. This result is comparable with ours for both viral and cellular DNA polymerases (Table 3). We cannot explain, however, why Papas *et al.* (1974) found inhibition by pyran to be independent of template/primer concentration. Nevertheless, our discovery of a dependence of enzyme inhibition by pyran on template/primer or initiator concentration is consistent with the concomitant dependence of inhibition on bivalent-cation concentration. Bivalent cations can bind to polynucleotides (Eichhorn & Shin, 1968; Vamvakopoulos *et al.*, 1977) and these interactions can alter their secondary structure. In one DNA polymerase system (Vamvakopoulos *et al.*, 1977), evidence indicated that a particular bivalent cation caused a specific change in the secondary structure of a template/primer which increased enzyme activity. Formation of such favourable secondary structure may be an important

Table 3. *Effect of various additions or substitutions to assay mixtures on inhibition of DNA polymerase activities from Molt-4 cells and simian sarcoma virus by pyran*

Standard assay mixtures (0.2 ml final volume) contained: (a) for DNA polymerase α or β , 30 μ g of activated DNA, either 0.11 (α) or 0.15 (β) unit of enzyme, no bovine serum albumin and 1.2 μ mol of Mg^{2+} ; (b) for DNA polymerase γ , 5.6 μ g of poly(A)·(dT)₁₂₋₁₈, 0.02 unit of enzyme, 8 μ g of bovine serum albumin and 0.1 μ mol of Mn^{2+} ; (c) for terminal transferase, 2.4 μ g of (dA)₁₂₋₁₈, 0.12 unit of enzyme, 8 μ g of bovine serum albumin and 0.1 μ mol of Mn^{2+} ; (d) for simian sarcoma virus DNA polymerase, 2.4 μ g of poly(A)·(dT)₁₂₋₁₈, 0.03 unit of enzyme, 8 μ g of bovine serum albumin and 0.1 μ mol of Mn^{2+} . Other components of assay mixtures are listed in the Materials and Methods section. When added, pyran (XA124-177) concentrations were 200 μ g/ml for DNA polymerase α and β assays, 40 μ g/ml for DNA polymerase γ assays, 2 μ g/ml for terminal transferase assays, and 100 μ g/ml for viral polymerase assays. dNMP is deoxyribonucleoside monophosphate, which was dGMP for terminal transferase assays and dTMP for all other enzyme assays.

Enzyme	Additions or substitutions to assay mixtures	dNMP incorporated (pmol)		Enzyme activity (% of control)
		Pyran absent (controls)	Pyran present	
Polymerase α	None	57	18	32
	200 μ g of activated DNA	59	50	85
	1.1 units of polymerase α	578	231	40
	50 μ g of bovine serum albumin	62	21	34
	12 μ mol of Mg^{2+}	12	12	100
Polymerase β	None	74	28	38
	200 μ g of activated DNA	79	70	89
	1.5 units of polymerase β	718	323	45
	50 μ g of bovine serum albumin	80	32	40
	12 μ mol of Mg^{2+}	43	47	110
Polymerase γ	None	9	4	44
	33.8 μ g of poly(A)·(dT) ₁₂₋₁₈	11	9	82
	0.2 unit of polymerase γ	96	47	49
	50 μ g of bovine serum albumin	10	5	50
	1.0 μ mol of Mn^{2+}	1	1	100
Terminal transferase	None	40	13	33
	22.4 μ g of (dA) ₁₂₋₁₈	46	41	89
	1.2 units of terminal transferase	381	156	41
	50 μ g of bovine serum albumin	46	16	35
	1.0 μ mol of Mn^{2+}	33	23	70
Viral polymerase	None	16	5	31
	33.8 μ g of poly(A)·(dT) ₁₂₋₁₈	18	11	61
	67.6 μ g of poly(A)·(dT) ₁₂₋₁₈	18	14	78
	0.3 unit of the enzyme	141	45	32
	50 μ g of bovine serum albumin	18	6	33
	1.0 μ mol of Mn^{2+}	9	9	100

function of bivalent cations in DNA polymerase systems and would explain the specific bivalent-cation requirements of DNA polymerases for different template/primers. Pyran may prevent the formation of a favourable secondary structure of template/primer by complexing bivalent cations, resulting in a decreased affinity of the template/primer for enzyme and a decrease in enzyme activity. Such effects would obviously be dependent on the concentrations of both bivalent cation and template/primer in assay mixtures.

The more potent inhibition of DNA polymerase γ , viral DNA polymerase and terminal transferase by pyran compared with DNA polymerases α and β (Table 1) is consistent with the greater concentration of bivalent cations and template/primer used to assay DNA polymerases α and β (see the Materials

and Methods section) and with the greater ability of pyran to complex Mn^{2+} as compared with Mg^{2+} (Fiel *et al.*, 1976b). DNA polymerase γ and viral DNA polymerase reaction mixtures are virtually identical and these enzymes are inhibited to a similar degree by pyran (Table 1). However, terminal transferase, whose reaction mixture is the same as for DNA polymerase γ and viral DNA polymerase except for the substitutions of (dA)₁₂₋₁₈ for poly(A)·(dT)₁₂₋₁₈ and dGTP for dTTP, was more potently inhibited by pyran than were the other enzymes (Table 1). This indicates that other factors (e.g. type of template/primer or initiator) besides bivalent-cation requirement or concentration of template/primer or initiator may also influence the degree of inhibition by pyran.

Addition of pyran to cellular and viral DNA poly-

Table 4. *Summary of kinetic constants derived from the inhibition of DNA polymerases from Molt-4 cells and simian sarcoma virus by pyran at various concentrations of substrate, template/primer or initiator*

Kinetic constants (V , K_m^s and K_m^t) were defined in the Materials and Methods section. Kinetic constants describing the inhibition of simian sarcoma virus DNA polymerase by pyran (XA124-177) were obtained from Fig. 1. Similarly, kinetic constants for terminal transferase and DNA polymerases α , β and γ were obtained from plots analogous to Fig. 1. V has units of pmol of dTMP incorporated/30min per μg of protein for DNA polymerases α , β and γ and for simian sarcoma virus DNA polymerase, and pmol of dGMP incorporated/20min per μg of protein for terminal transferase. Concentrations of DNA and (dA)₁₂₋₁₈ were calculated on the basis of mol of nucleotide/litre.

Enzyme	Pyran	Variable component	V	K_m^s or K_m^t
Polymerase α	0	dTTP	182	6.3
	100	dTTP	125	6.7
	150	dTTP	77	6.5
	200	dTTP	50	6.3
Polymerase β	0	dTTP	63	2.6
	100	dTTP	32	2.3
	150	dTTP	29	2.5
Polymerase γ	0	dTTP	77	0.52
	10	dTTP	63	0.50
	20	dTTP	52	0.49
Terminal transferase	0	dGTP	118	3.9
	0.5	dGTP	75	4.0
	1.0	dGTP	40	4.1
	3.0	dGTP	25	4.0
Viral polymerase	0	dTTP	641	53
	100	dTTP	147	50
Polymerase α	0	DNA	200	115
	100	DNA	205	417
	150	DNA	227	556
	200	DNA	212	833
Polymerase β	0	DNA	69	42
	50	DNA	71	51
	100	DNA	65	57
Polymerase γ	0	poly(A)·(dT) ₁₂₋₁₈	72	17
	20	poly(A)·(dT) ₁₂₋₁₈	72	27
Terminal transferase	0	(dA) ₁₂₋₁₈	107	20
	1	(dA) ₁₂₋₁₈	91	30
	2	(dA) ₁₂₋₁₈	100	43
Viral polymerase	0	poly(A)·(dT) ₁₂₋₁₈	596	13
	20	poly(A)·(dT) ₁₂₋₁₈	610	25

merase reaction mixtures at either zero time or 10 min after the beginning of an uninhibited reaction caused immediate inhibition of DNA synthesis (Fig. 2 is representative). The lack of any delay in inhibition suggests that pyran prevents both the initiation of DNA synthesis and the elongation of DNA chains by DNA polymerases.

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