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Interaction Specificity of the Anthracyclines with Deoxyribonucleic Acid†

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ABSTRACT: The interaction specificity of salmon sperm DNA with various derivatives of daunorubicin has been studied. The results of binding, viscometric, ^1H nuclear magnetic resonance (NMR), flow dichroism, DNA template inhibition, rates of dissociation, and circular dichroism studies are found to be consistent with an intercalation mode of binding of the anthracycline ring as has been shown by other investigators. Moreover, it is observed that (i) strength of binding, (ii) the ease of dissociation of DNA–anthracycline complexes, and (iii) the degree of inhibition of the DNA-dependent RNA polymerase are dependent on the presence of the amino sugar moiety of daunoseamine. The results are consistent with specific H bonding of the amino group of the sugar moiety with DNA as has been suggested earlier by Pigram et al. (Pigram, W. J., Fuller, W., and Hamilton, L. D. (1972), *Nature (London), New Biol. 235,* 17). Peptide derivatives substituted at the amino sugar function of daunorubicin lower the affinity of the drug to DNA and presumably interfere with the "full insertion" of the anthracycline drugs between base pairs of DNA. The significance of these findings in relation to the biological efficacy of daunorubicin and related derivatives as antileukemic agents is discussed.

The interaction specificities of daunorubicin (a potent antileukemic drug) and various anthracycline derivatives with nucleic acids have been the subject of numerous studies in recent years (Henry, 1974; DiMarco and Arcamone, 1975; Zunino et al., 1974; Dalgleisch et al., 1974; Kersten et al., 1966; Waring, 1970; Pigram et al., 1972). It is now generally recognized that the biological activity of these drugs involves inhibition of the cellular RNA- and DNA-dependent replication and transcription processes (Meriwether and Bachur, 1972; Hartmann et al., 1964; Ward et al., 1965; Mizuno et al., 1975). Unfortunately, the unfavorable cytotoxic properties of daunorubicin on cardiac tissues have limited its usefulness as an effective chemotherapeutic agent in the treatment of cancer in humans (Smith, 1969; Raskin et al., 1973; Halazun et al., 1974; Mhatre et al., 1971; Herman et al., 1970; Bachur et al., 1974; Chalcroft et al., 1973; Cornu et al., 1974). For this reason, considerable interest is being focused on the synthesis of potentially nontoxic derivatives.

The present work deals with comparative studies of the interaction specificities of salmon sperm DNA with daunorubicin (**1**) and two types of substituted derivatives, i.e., (i) at the amino sugar, **2–5**, and (ii) at the 9-keto methyl moieties, **6** and **7** (Chart I).

Experimental Section

The syntheses of the peptide derivatives **2–4** were carried out by the mixed anhydride procedure of Anderson et al.

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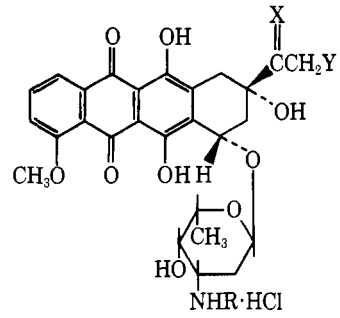
Chart I



Compound	X	Y	R	NSC
Daunorubicin (1)	O	H	H	82151
D-Me_2Gly (2)	O	H	$COCH_2N(CH_3)_2$	246104
D-Pr_2Gly (3)	O	H	$COCH_2N(C_3H_7)_2$	246105
D-Me_2GlyGly (4)	O	H	$COCH_2NHCOCH_2N(CH_3)_2$	246132
D-Gly (5)	O	H	$COCH_2NH_2$	256465
Adriamycin (6)	O	OH	H	
Rubidazone (7)	$NNHCOC_6H_5$	H	H	

(1967) utilizing daunorubicin·HCl (gift from Rhone-Poulenc) and *N,N*-dialkylglycine and *N,N*-dimethylglycylglycine. The nonmethylated glycyl derivative **5** was obtained by mild alkaline hydrolysis (pH 10.5 and room temperature for 12 h) from the corresponding *N*-trifluoroacetylglycyl adduct of daunorubicin. The authenticities of the synthesized derivatives, **2–5**, were checked by thin-layer chromatography, elemental analysis, ultraviolet, circular dichroism, and ^1H nuclear magnetic resonance (NMR) spectroscopy. Adriamycin and rubidazone were kindly supplied by Rhone-Poulenc, France.

Analyses were performed by Atlantic Microlab, Inc., Atlanta, Ga. ^1H NMR spectra were recorded on a Varian XL-100-15 spectrometer equipped with a Nicolet Technology Corporation FT accessory in $CDCl_3$ using tetramethylsilane (Me_4Si) as the internal standard. Absorption spectra were recorded on a Cary 15 spectrometer; circular dichroism spectra were recorded on a Jasco J-20 spectropolarimeter at 25 °C. Stop-flow kinetics were carried out using a Durrum-Gibson apparatus (Durrum Instrument Corp.) thermostated with a Lauda K-2/R constant temperature circulator. The kinetics of DNA-dependent *Escherichia coli* RNA polymerase reactions were followed by the incorporation [5-^3H]UMP into Cl_3CCOOH insoluble RNA according to the procedure of Preston et al. (1975). Radioactivity was counted on a LS-133 liquid scintillation counter (Beckman Instrument Co.). Flow dichroism measurements were carried out at 260 and 505 nm using the Cary 15 spectrometer with a Glan-Taylor calcite polarizing prism. The DNA solution was allowed to flow through a quartz capillary (0.415 mm radius) by means of a Sage syringe pump. Viscometric studies were performed on a low-shear Zimm viscometer (Beckman Instrument Co.).

Salmon sperm DNA was obtained from Worthington Corporation and found to be free of any detectable protein contaminants (<0.1%) as determined by the fluorescamine assay technique (Gabbay et al., 1973b). Stock solutions of the polymer were made in either 10 mM 2-(*N*-morpholino)-ethanesulfonic acid buffer (Mes)[1] (pH 6.2, 5 mM Na^+) or in 2 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) buffer (Pipes) (pH 7.8, 1 mM Na^+) and stored at 4 °C. The stock solutions were removed and diluted in the various buffers as indicated in the tables and figures. *E. coli* RNA polymerase, the nucleotide triphosphates, ATP, GTP, and CTP were obtained from Sigma Chemicals. [5-^3H]UTP (53.3 Ci/mol) was obtained from ICN.

[1] Abbreviations used are: Mes, 2-(*N*-morpholino)ethanesulfonic acid buffer; Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid) buffer.

Results

Ultraviolet, Fluorescence, and Circular Dichroism Studies. (a) Assignment of Transitions. Absorption and circular dichroism data on the anthracyclines **1–7** are given in Tables I and II, respectively. The CD spectra of the anthracyclines, **1–7**, indicate the presence of two electronic transitions above 300 nm, i.e., at 470 and 340 nm (Figure 1). The allowed low-energy transition (ϵ 11 500) at 470 nm is typical of the short axis transition of α-nitrogen or oxygen-substituted 9,10-anthraquinones, $L^1{}_b \leftarrow A$ (Sidman, 1956, and references therein). The disallowed higher energy transition ($\epsilon \leq 2000$) at 340 nm is typical of the long axis transition of unsubstituted and/or β-alkyl-substituted 9,10-anthraquinones, $L^1{}_a \leftarrow A$. Consistent with this assignment is the observation that the 340-nm long axis transition (sensitive to β substituent) shows larger optical activity than the low-energy short axis transition at 470 nm ($L^1{}_b \leftarrow A$). For example, the values of the dichroic strength, $(\Delta\epsilon_i)/\epsilon_i$ (where $\Delta\epsilon_i$ is the difference in the molar extinction coefficients for the left and right circular polarized light, and ϵ_i is the molar extinction coefficient of the ith transition) are found to be 1.5×10^{-3} and 1.2×10^{-4} for the long and short axis transitions, respectively. Since the asymmetry in the daunorubicin systems, **1–7**, resides at the β position of the 9,10-anthraquinone ring and the long axis transition is strongly affected by β substitution as compared to the short axis transition, the above assignments are reasonable.

(b) Effect on the Optical Properties of the Anthracyclines upon Binding to DNA. (i) Absorption Studies. Large hypochromic effects on the short and long axis transitions of the anthracyclines, **1–7**, are observed in the presence of excess salmon sperm DNA. The results are summarized in Table I for compounds **1–7**. It is noted that substitution at the amino group of the sugar moiety of daunorubicin (compounds **2–5**) lowers the hypochromic effect observed for the anthracycline electronic transitions (320–600 nm) upon binding to DNA. For example, the following order of decreasing hypochromism, H (where $H = 100(1 - f_b)/f_f$; f_b and f_f are the oscillator strengths of the anthracycline drugs in the presence and absence of DNA; see Table I), is observed: daunorubicin (**1**) > adriamycin (**6**) ≥ rubidazone (**7**) > D-Gly (**5**) > D-Me_2Gly (**2**) > D-Pr_2Gly (**3**) ≥ D-Me_2GlyGly (**4**). It should be noted that the absorption studies cited above were carried out under

Table I: Absorption and Hypochromicity (H) Data on the Anthracycline Drugs in the Presence and Absence of Salmon Sperm DNA in 10 mM Mes Buffer (pH 6.2 and 5 mM Na^+) at 25 °C.[a]

Compound	Free				DNA Complex		
	λ_{max}	ϵ_{max}	λ_{max}	ϵ_{max}	λ_{max}	ϵ_{max}	H^b
Daunorubicin (**1**)	477	11 500	495	11 200	506	6825	33
D-Me_2Gly (**2**)	477	11 500	495	11 500	505	7450	26
D-Pr_2Gly (**3**)	477	11 500	495	11 500	506	7670	24
D-Me_2GlyGly (**4**)	477	11 500	495	11 500	505	7470	23
D-Gly (**5**)	477	11 500	495	11 500	507	7280	28
Adriamycin (**6**)	476	11 500	495	11 500	505	6930	31
Rubidazone (**7**)	477	11 500	494	11 600	506	6950	30

[a] Absorption spectra were taken in 10-cm cells using 10 μM of the drug in the absence and presence of increasing concentrations of salmon sperm DNA (40–200 μM P/l.). The tabulated data are given in the presence of excess DNA P/l., i.e., 200 μM P/l. Under these conditions, the drugs are totally bound to DNA. [b] The hypochromicities (H) in the anthracycline electronic transitions (between 330 and 600 nm) were determined by direct measurements of the oscillator strengths, f, for the free (f_f) and DNA-bound drug (f_b) according to the following equation: $H = 100(1 - f_b/f_f)$.

Table II: Molar Ellipticity, $[\theta]_m$, of Daunorubicin and Its Derivatives in the Presence and Absence of Salmon Sperm DNA in 10 mM Mes Buffer (pH 6.2 and 5 mM Na^+) at 25 °C.[a]

Compound	Free				DNA Complex			
	λ^{P_1}	$[\theta]^{P_1} \times 10^{-3}$	λ^{P_2}	$[\theta]^{P_2} \times 10^{-3}$	λ^{P_1}	$[\theta]^{P_1} \times 10^{-3}$	λ^{P_2}	$[\theta]^{P_2} \times 10^{-3}$
Daunorubicin (**1**)	346	8.4	468	4.2	378	8.2	490	6.1
D-Me_2Gly (**2**)	341	8.2	466	5.9	357	6.3	490	4.6
D-Pr_2Gly (**3**)	343	9.0	465	6.7	360	7.7	490	5.2
D-Me_2GlyGly (**4**)	342	8.6	467	6.1	355	6.5	490	4.5
D-Gly (**5**)	346	8.9	474	6.3	370	8.0	500	6.3
Adriamycin (**6**)	345	6.7	470	3.1	381	6.7	492	4.4
Rubidazone (**7**)	346	7.5	473	3.7	378	7.5	499	6.6

[a] Circular dichroism spectra were taken in 10-cm cells using 10 μM of the drug in the presence and absence of salmon sperm DNA (200 μM P/l.). The drugs are totally bound to DNA under these conditions.

conditions of total binding to DNA and at a base pair to drug ratio of 10. (In all cases, the absorption spectra of the DNA bound anthracyclines reach a limiting value at a base pair to drug ratio greater than 3.0.)

(ii) Circular Dichroism Studies. A large red shift in the circular dichroism of the short and long axis transitions of the anthracyclines, **1–7**, is observed upon binding to DNA. For example, the peak at 470 nm (short axis transition) is red shifted by approximately 20–25 nm for compounds **1–7**. The extent of the red shift of the peak at 340 nm (long axis transitions) of **1–7** in the presence of DNA, however, is found to depend on the anthracycline structure, i.e., the value decreases as follows: daunorubicin (**1**) ≈ adriamycin (**6**) ≈ rubidazone (**7**) > D-Gly (**5**) > D-Me_2Gly (**2**) ≈ D-Pr_2Gly (**3**) ≈ D-Me_2GlyGly (**4**). Similarly, the molar ellipticity, $[\theta]$, for the long axis transition at 340 nm is observed to decrease in the presence of DNA to a greater extent for compounds **1, 6,** and **7** as compared to the peptide derivatives **2–5** (Table II). It should be noted that the circular dichroism studies were carried out under conditions of total binding to DNA and at a base pair to drug ratio of 10. In summary, the visible absorption and circular dichroism studies show that differences exist in the interaction specificities of the peptide derivatives **2–4** as compared to **1, 6,** and **7** with DNA.

Binding Studies and Distribution Coefficients. The binding of the anthracycline drugs to salmon sperm DNA was studied by three different methods, i.e., equilibrium dialysis, spectral titration, and solvent distribution techniques. The equilibrium dialysis method was found to be unsatisfactory due to the strong binding of the anthracyclines to the dialysis membrane. The results obtained with the other two techniques are described below.

(i) Spectral Titration Studies. The effect of increasing concentration of salmon sperm DNA (5–60 M) on the absorption of the anthracycline drugs, **1–7** (6.5–7.0 M), at 480 nm was studied in 10 mM Mes buffer (Na^+, 21 mM), pH 6.2, in 10-cm cells utilizing the Cary 15 spectrophotometer thermostated at 25 °C. The spectral titration data were analyzed by the Scatchard technique (Scatchard, 1949) according to the following equation:

$$\bar{n} = \bar{n}_{max} - (1/K_a)(\bar{n}/D_f)$$

where n = the number of moles of x bound per mole of DNA phosphate, $\bar{n}_{max}$ represents maximal binding, K_a is the association constant for the DNA–x complex, and D_f is the concentration of unbound x. A plot of $\bar{n}/D_f$ vs. n gives the values of $\bar{n}_{max}$ (x-axis intercept) and $\bar{n}_{max}K_a$ (y-axis intercept). The values of D_f and D_b were calculated from the spectrophotometric data in a manner similar to that described by Hyman and Davidson (1971) utilizing the determined values of ϵ_f^{480} and ϵ_b^{480}, the extinction coefficients of the free and DNA-bound anthracyclines. The binding isotherms for daunorubicin (**1**) and D-Gly (**5**) are shown in Figure 2 and the results are summarized in Table III. The apparent binding affinity, K_a, of the peptide derivatives of daunorubicin, i.e., D-Me_2Gly (**2**), D-Pr_2Gly (**3**), D-Me_2GlyGly (**4**), and D-Gly (**5**), to DNA is found to be lower by a factor of 2–3 as compared to daunorubicin and/or the C-9 substituted analogue, rubidazone.

(ii) Distribution Coefficients. It should be noted that the DNA binding studies utilizing the spectral titration technique

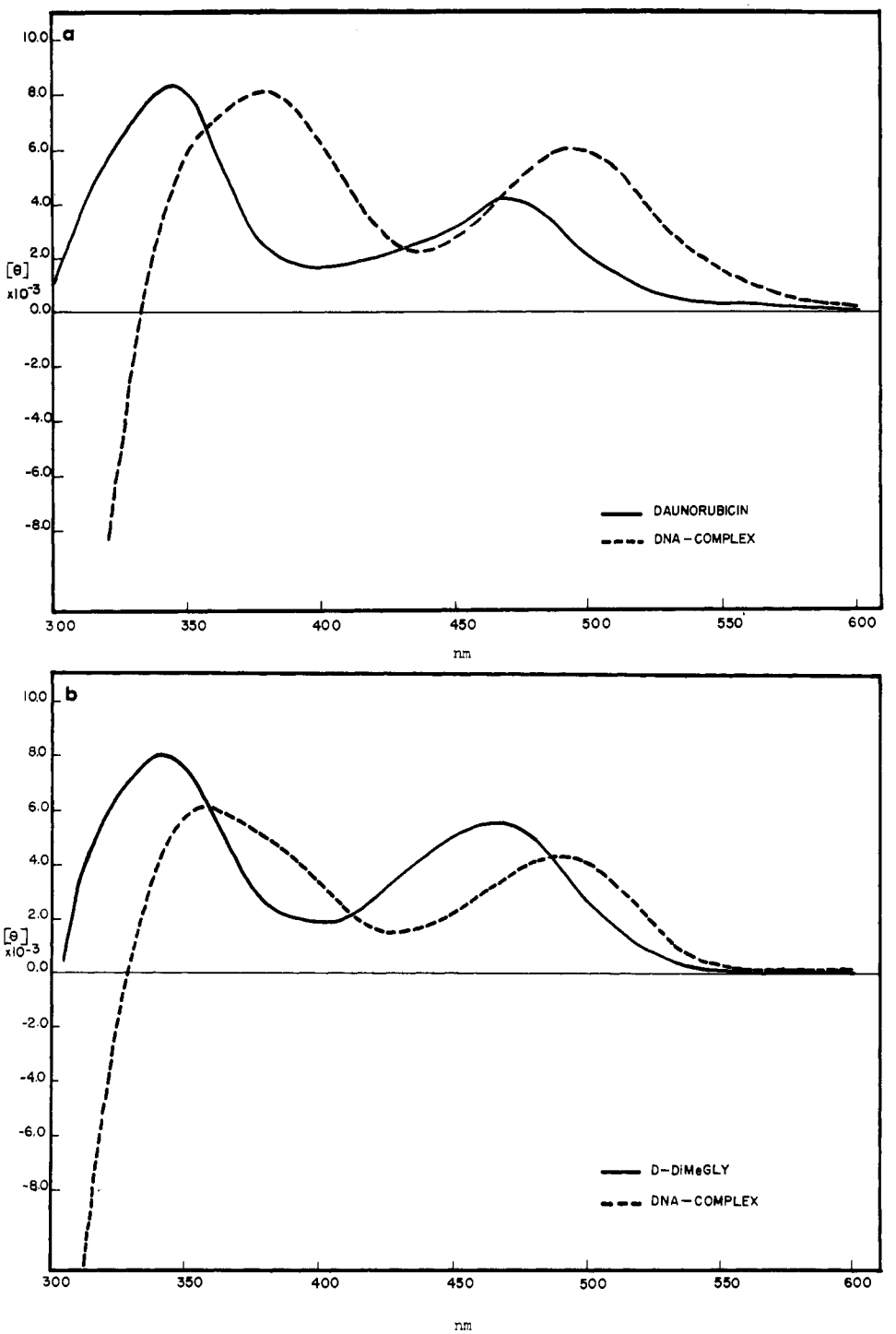


FIGURE 1: The circular dichroism spectra of daunorubicin (**1**) (a) and D-Me_2Gly (**2**) (b) in the presence and absence of excess salmon sperm DNA (see Table II for details).

described above are based on the assumption that the hypochromic change in the electronic transition (at 480 nm) of the anthracycline drugs, **1–7**, is independent of the nature of the DNA binding sites. In order to test the validity of such an assumption, the binding affinity of daunorubicin (**1**) and D-Gly (**5**) to DNA was also determined by a solvent distribution technique.

The distribution coefficients ($D = c_{CHCl_3}/c_{H_2O}$) of the anthracycline drugs in 10 mM Mes buffer (21 mM Na^+), pH 6.2, in water vs. chloroform at 25 °C were determined spectrophotometrically by measuring the concentration of the drug in each solvent (Table III). The values were found to be independent of the total concentration of the drug in the range from 2 to 20 μM. The DNA binding studies were carried out in duplicate using successively increasing concentrations of the anthracyclines (2–10 μM) in the presence of 0.1 mM P/l. of salmon sperm DNA. The concentration of the free drug in the chloroform layer was determined spectrophotometrically and the free (D_f) and DNA-bound (D_b) drug concentrations were calculated using the determined values of the distribution coefficients, D. The data were analyzed by the Scatchard technique (Figure 2) and the results are shown in Table III. It is noted that the determined values of K_a and $\bar{n}_{max}$ for daunorubicin (**1**) and D-Gly (**5**) utilizing the solvent distribution

Table III: Apparent Binding Affinity, K_a, of the Anthracycline Drugs to Salmon Sperm DNA and the Distribution Coefficient, D, of the Drugs in Chloroform/Water at 25 °C in 10 mM Mes Buffer (21 mM Na^+) (pH 6.2).

Compound	Absorption Method[a,b]		Distribution Method[b]		
	$K_a \times 10^{-6}$	$\bar{n}_{max}$	D	$K_a \times 10^{-6}$	$\bar{n}_{max}$
Daunorubicin (**1**)	6.8	0.17	8.16	9.3	0.17
D-Me_2Gly (**2**)	1.7	0.18	66.2		
D-Pr_2Gly (**3**)	1.7	0.16	>500		
D-Me_2GlyGly (**4**)	1.7	0.18	24.0		
D-Gly (**5**)	2.2	0.15	5.65	2.5	0.16
Rubidazone (**7**)	7.0	0.18	9.66		

[a] The absorption studies were carried out at 480 nm whereby the anthracycline drugs undergo a large hypochromic effect upon binding to DNA. [b] The values of K_a and $\bar{n}_{max}$ are found to be reproducible to ±20 and ±5% error, respectively.

Table IV: Effect of the Anthracycline Drugs on the Relative Specific Viscosities, η_{sp}(complex)/η_{sp}(DNA), of Native High Molecular Weight and Sonicated Low Molecular Weight DNA at Bound Drug (D_b) to DNA Phosphate (P_t) Values of 0.2 and 0.3.[a]

Compound	η_{sp}(complex)/η_{sp}(DNA)			
	Native DNA[b]		Sonicated DNA,[c] D_b/P_t = 0.3	
	D_b/P_t = 0.2	D_b/P_t = 0.3	200 μM P/l.	400 μM P/l.
Daunorubicin (**1**)	3.25 ± 0.05	3.59 ± 0.07	1.60 ± 0.02	2.24 ± 0.03
D-Me_2Gly (**2**)	1.78	2.03		
D-Pr_2Gly (**3**)	2.19	2.37		
D-Me_2GlyGly (**4**)	2.08	2.18	1.30	1.64
D-Gly (**5**)	2.46	2.82		
Adriamycin (**6**)	3.60	3.64		
Rubidazone (**7**)	3.02	3.22		

[a] The viscometric measurements were carried out at 37 °C in 10 mM Mes buffer (Na^+, 21 mM) (pH 6.2) using the low shear Zimm viscometer. The values of D_b/P_t are calculated from the binding isotherm data shown in Table III. [b] Native salmon sperm DNA was used at 167 μM P/l. [c] Sonicated low molecular weight salmon sperm DNA, obtained according to our previously described procedure (Gabbay et al., 1973b), and used at 200 and 400 μM P/l. at a D_b/P_t = 0.3. The viscometric measurements were carried out 37 °C in 10 mM Mes buffer (Na^+, 21 mM) (pH 6.2) using an Ubelohde viscometer.

method are in close agreement with the values obtained by the spectral titration method.

Viscometric Studies. The effect of increasing concentrations of the anthracyclines (**1–7**) on the relative specific viscosity (η_{sp}/η_{sp_0}, where η_{sp} and η_{sp_0} are the specific viscosities in the presence and absence of the drugs) of native salmon sperm DNA (mol wt ≃6 × 10^6) solution at 37 °C was studied. Viscometric measurements were carried out at near infinite dilution of the nucleic acid (0.167 mM P/l., i.e., 0.005% in DNA) in 10 mM Mes buffer (21 mM Na^+) using the low shear Zimm viscometer. In all cases, the solution viscosity is found to increase and finally to level off at a drug to DNA phosphate ratio of 0.25. The viscometric data at a bound drug (D_b) to DNA phosphate (P_t) values of 0.2 and 0.3 are summarized in Table IV. It is noted that complex formation with daunorubicin (and/or the C-9 analogues **6** and **7**) leads to a larger increase in the specific viscosity of DNA solution as compared to the *N*-peptide derivatives (**2–5**). Such an effect may be due to drug induced differences in the relative degree of (i) lengthening and stiffening of the DNA helix and/or (ii) an intramolecular aggregation effect (Muller and Crothers, 1968). In order to shed light on this problem, the effect of bound daunorubicin (**1**) and D-Me_2GlyGly (**4**) on the relative specific viscosity of rod-like sonicated DNA (mol wt ≤5 × 10^5) was studied and the results are given in Table IV. The viscometric measurements were carried out at near infinite dilution of the nucleic acid (0.2 and 0.4 mM P/l., i.e., 0.01 and 0.02% in DNA) and D_b/P_t ratio of 0.3 in 10 mM Mes buffer (pH 6.2)

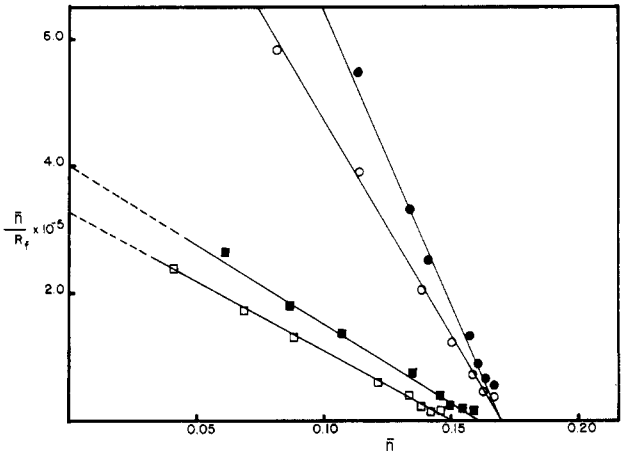


FIGURE 2: The Scatchard plot of the binding of daunorubicin (**1**) (○,●) and D-Gly (**5**) (□,■) to salmon sperm DNA using the spectral titration (○,□) and distribution (●,■) methods. For details see text and Table III.

(21 mM Na^+) using the Ubelohde viscometer thermostated at 37 °C. Results identical with those obtained with high molecular weight native DNA are observed, i.e., a greater increase in the relative specific viscosity of a solution containing DNA–**1** as compared to DNA–**4** complexes.

In addition, the intrinsic viscosities, [η], of native salmon sperm DNA and its complexes with daunorubicin (**1**) and D-Me_2Gly (**2**) were also determined at 37 °C using the Ube-

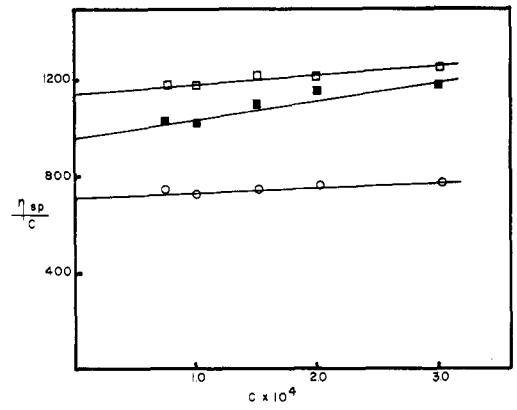


FIGURE 3: The intrinsic viscosity, $[\eta]$, of native salmon sperm DNA (○), DNA-D-Me_2Gly (■), and DNA–daunorubicin (□) complexes. The specific viscosities were determined at 37 °C using 10 mM Mes buffer (21 mM Na^+) (pH 6.2) in a Ubelohde viscometer at a DNA phosphate to drug ratio of 3. Under these conditions, the strong DNA binding sites are totally occupied by the drugs even at the lowest DNA concentration used, i.e., 75 μM P/l.

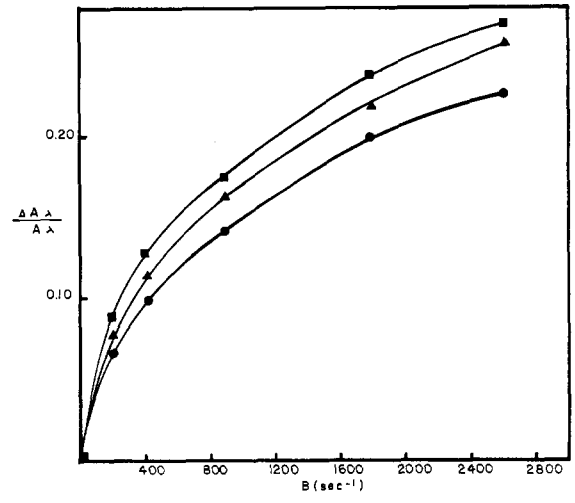


FIGURE 4: The effect of increasing shear rate, B, on the reduced dichroism of salmon sperm DNA solutions at λ 260 nm (●), in the presence of daunorubicin at λ 505 nm (■), and in the presence of D-Me_2GlyGly (**4**) at λ 505 nm (▲).

lohde viscometer. The results are shown in Figure 3. It is noted that the values of the intrinsic viscosity, $[\eta]$ (where $[\eta] = \eta_{sp}/c$ as $c \rightarrow 0$), for native DNA, DNA-**2**, and DNA-**1** are found to be 710, 960, and 1140 l./mol, respectively. Assuming the Kirkwood–Auer equation is valid for rod shaped particles (Kirkwood and Auer, 1951) (whereby the intrinsic viscosity is proportional to L^2, and L is the length of the rod) the above data indicate that the DNA helix is elongated by 16 and 27% in the presence of D-Me_2Gly (**2**) and daunorubicin (**1**), respectively.

The above data suggest that the differences in the enhanced relative specific viscosity of DNA solutions induced by the anthracyclines, **1–7** (see Table IV), are due to differences in the lengthening and stiffening of the helix (upon intercalation) rather than an intramolecular aggregation phenomenon. Such an effect could arise from a different extent of insertion of the anthracycline ring of daunorubicin (**1**) (and/or the C-9 analogues **6** and **7**), as compared to that of the N-peptide derivatives (**2–5**), between base pairs of DNA, i.e., "full insertion" leading to lengthening and stiffening vs. "partial insertion" leading to lengthening (and concomitant bending) of the helix.

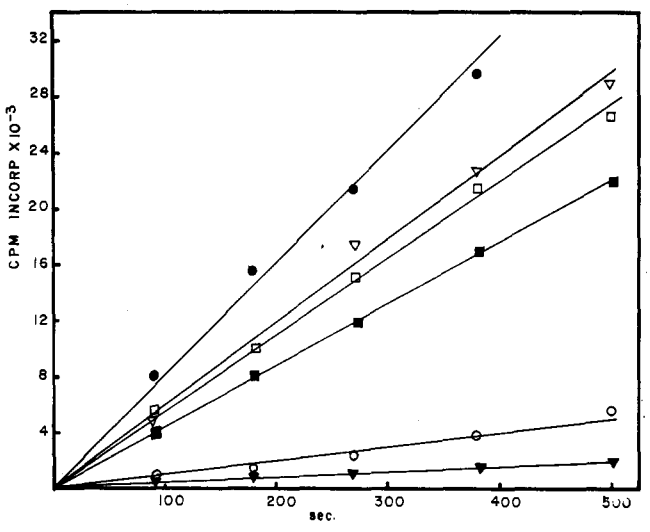


FIGURE 5: The effect of the anthracyclines **1–7** on the kinetics of incorporation of [5-^{3}H]UMP into RNA by the DNA-dependent *E. coli* RNA polymerase. Calf-thymus DNA (0.12 mM P/l.) and the drugs at 0.12 mM were used: daunorubicin, adriamycin (▼), rubidazone (○), D-Gly (■), D-Me_2Gly (□), D-Me_2GlyGly (▽), control (●).

Such effects have been previously observed for various types of intercalating agents (Gabbay et al., 1973c; Kapicak and Gabbay, 1975; Gabbay et al., 1976).

Flow Dichroism Studies. The results of the flow dichroism studies on solutions of salmon sperm DNA in the presence and absence of daunorubicin (**1**) (and/or the peptide derivative, D-Me_2GlyGly (**4**)) are shown in Figure 4. It is noted that the value of reduced dichroism, $\Delta A_\lambda/A_\lambda$ (where $\Delta A = (A_\perp - A_\parallel)_\lambda$, $A_\parallel$ and $A_\perp$ are the absorbances of light polarized parallel and perpendicular to the axis of flow, and A_λ is the absorbance of the nonflowing solution), increases with shear rate, B, for free DNA (measured at 260 nm), DNA-**1**, and DNA-**4** complexes (measured at 505 nm). The data indicate that the anthracycline ring of **1** and **4** is in a plane parallel to that of the base pairs of DNA and suggest a common mode of binding of the drugs.

Inhibition of DNA-Template Activity. The effect of the anthracyclines **1–7** on the DNA-dependent *E. coli* RNA polymerase is shown in Figure 5. It is noted that daunorubicin and the C-9 substituted derivatives, adriamycin (**6**) and rubidazone (**7**), inhibit the incorporation of [5-^{3}H]UMP into RNA to a greater extent than the corresponding *N*-acyl peptide analogues, **2**, **4**, and **5**. Moreover, the extent of the inhibition (as determined from the slope of the data shown in Figure 3) is found to decrease in the following order: adriamycin (**6**) (95%) ≃ daunorubicin (**1**) (95%) > rubidazone (**7**) (82%) > D-Gly (**5**) (45%) > D-Me_2Gly (**2**) (32%) > D-Me_2GlyGly (**4**) (26%).

Kinetics of Dissociation. The effect of increasing ionic strength on the dissociation of the DNA–drug complexes was studied by flow techniques utilizing sodium dodecyl sulfate according to previously published methods (Gabbay et al., 1973a; Muller and Crothers, 1968). The reactions are found to be first order to at least 2.5 half-lives with respect to the DNA–drug complexes. In addition, increasing the concentration of sodium dodecyl sulfate ($NaDodSO_4$) (0.1–1%) and/or the concentration of DNA (base pair/drug = 3–30) has a minimal effect on the observed first-order rate constant, i.e., the value of k remains constant to ±5%. The data are consistent with the following mechanism whereby the first-order rate constant, k, of the dissociation process is the rate-

Table V: Effect of Ionic Strength on the Dissociation of the Salmon Sperm DNA–Drug Complexes in 2 mM Pipes Buffer (pH 7.8) at 15 °C.[a]

Salmon Sperm DNA Complex	21 mM Na^+		51 mM Na^+		201 mM Na^+	
	k (s^{-1})	Rel Rate	k (s^{-1})	Rel Rate	k (s^{-1})	Rel Rate
Daunorubicin (**1**)	0.32	1.00	0.42	1.00	0.68	1.00
D-Me_2Gly (**2**)	4.32	13.5	6.10	14.5	9.74	14.3
D-Pr_2Gly (**3**)	4.95	15.5	10.7	27.1	>20	>25
D-Me_2GlyGly (**4**)	8.30	26.0	11.4	27.5	>20	>25
D-Gly (**5**)	2.78	8.70	2.95	7.00	3.52	5.18
Adriamycin (**6**)	0.29	0.91	0.41	0.98	0.55	0.81
Rubidazone (**7**)	0.44	1.38	0.56	1.33	0.86	1.26

[a] Kinetics of dissociation were measured at 480 nm by stop-flow techniques using 0.6% sodium dodecyl sulfate.

controlling step and the bimolecular sequestering process, k_2, is diffusion controlled:

$$\text{DNA–D} \xrightarrow{k} \text{DNA} + \text{D} \quad (1)$$

$$\text{NaDodSO}_4 + \text{D} \xrightarrow{k_2} \text{NaDodSO}_4\text{–D} \quad (2)$$

The results of the dissociation studies are given in Table V. The following are noted: (i) at any given ionic strength (e.g., 21, 51, and 201 mM Na^+), the relative rates of dissociation of the DNA–drug complexes are 5- to 30-fold greater for the peptide derivatives, **2–5**, as compared to daunorubicin (**1**) and/or the C-9 analogues, **6** and **7**, and (ii) the rates of dissociation of the anthracyclines (**1–7**) from the DNA complexes increase with increasing ionic strength.

Biological Activity. Preliminary screening by the Drug Research and Development Branch of the National Cancer Institute of the peptide derivatives, **2–5**, using 0.25–10 mg/kg daily doses in the treatment of the mouse P388 lymphocytic leukemia tumor indicates the following order of decreasing biological activity: adriamycin (**6**) ≥ daunorubicin (**1**) > rubidazone (**7**) > D-Me_2Gly (**2**) > D-Gly (**5**) > D-Me_2GlyGly (**4**) > D-Pr_2Gly (**3**) (zero activity).

Discussion

Considerable interest is being devoted in various laboratories to the synthesis of derivatives of daunorubicin in order to improve the chemotherapeutic index of the anthracycline drugs (Yamamoto et al., 1972; Henry, 1974; Arcamone et al., 1974, 1975; DiMarco and Arcamone, 1975; DiMarco et al., 1972; Bouchaudon and Jolles, 1971). Our preliminary approach has been centered on the modification of the amino group of the sugar moiety of daunorubicin and future efforts will consider the effect of modifications of the A and D ring substituents.

The interaction specificity of daunorubicin with DNA has been the subject of numerous studies (DiMarco and Arcamone, 1975; Kersten et al., 1966; Waring, 1970; Barthelemy-Clavey et al., 1973) and it is generally accepted that the anthracycline ring is inserted between base pairs of DNA via an intercalation mechanism (Lerman, 1961). The results of x-ray studies of DNA–daunorubicin fibers are consistent with the above interpretation and, in addition, model building studies of the DNA–**1** complex indicate that the ammonium group of the sugar moiety is involved in an electrostatic and H-bonding interaction with a neighboring phosphate group (Pigram et al., 1972). Such a DNA–daunorubicin complex is schematically illustrated in Figure 6a. The results obtained in the present studies, i.e., (i) the large hypochromism in the electronic transition of the DNA-bound drugs (Table I), (ii) the induced circular dichroism (Table II and Figure 1), (iii) the enhanced viscosity of DNA solution in the presence of the anthracyclines **1–7** (Table IV), and (iv) the flow dichroism data which indicate that the anthracycline ring is in a plane perpendicular to the helical axis of DNA (Figure 4), are entirely consistent with the intercalation mode of binding. It should be noted, however, that differences in the interaction specificities of **1–7** to salmon sperm DNA are observed. The data indicate that daunorubicin and the C-9 analogues (adriamycin (**6**) and rubidazone (**7**))

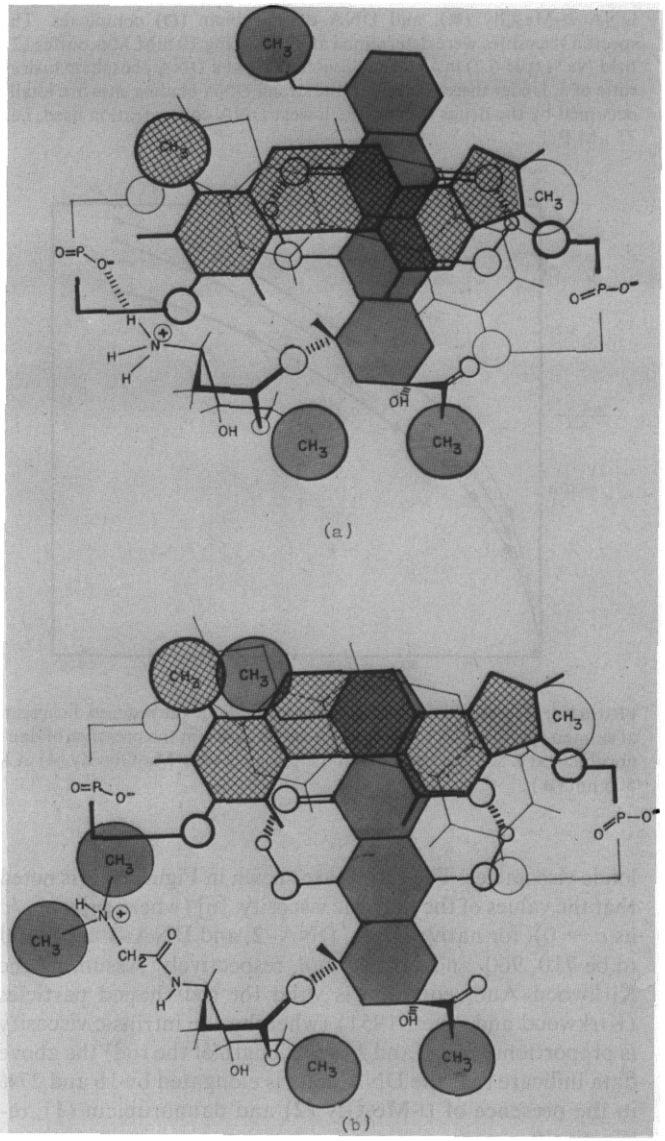


FIGURE 6: Schematic illustration of the DNA–daunorubicin (a) and DNA–D-Me_2Gly (b) complexes.

form more "intimate" complexes with DNA than the *N*-acyl peptide derivatives **2–5.** This conclusion is based on several lines of evidence, namely, (1) the observed lower hypochromism, *H* (known to arise from aromatic ring stacking interactions (Devoe and Tinoco, 1962)), in the electronic transitions of the peptide analogues, **2–5,** upon binding to DNA (Table I); (2) the observed lower bathochromic shift in λ^{peak} of the $L^1a \leftarrow A$ (long axis) transition in the CD spectra of **2–5** upon binding to DNA (Table II, Figure 1); (3) the observed lower binding affinity of **2–5** to DNA (Table III); (4) the faster rate of dissociation of the DNA-**2–5** complexes (Table V), and, finally, (5) the lower inhibition of the DNA-dependent *E. coli* polymerase by the peptide analogues **2–5** as compared to daunorubicin (**1**) and the C-9 analogues **6** and **7**.

At the molecular level, it is likely that the bulky tertiary ammonium cations, i.e., $-NH^+(CH_3)_2$ and $-NH^+(C_3H_7)_2$ of the amino acid side chains, in the derivatives **2–4** sterically hinder the full insertion of the anthracycline ring between DNA base pairs. This effect is schematically illustrated in Figure 6b. Consistent with the above interpretation is the observation that the glycyl derivative **5** (which contains a less bulky primary ammonium group, NH_3^+) exhibits a larger (i) hypochromism (*H*), (ii) induced CD, (iii) DNA binding, and (iv) a lower dissociation rate than the peptide analogues **2–4.** In addition, the data indicate that substitution at the amino sugar function (compounds **2–5**) strongly affects the DNA binding whereas substitution at the C-9 position shows minimal effects. The results are consistent with the specific electrostatic and H bonding of the amino sugar function with a neighboring DNA phosphate group as has been suggested earlier by Pigram et al. (1972).

The biological activity of daunorubicin is generally accepted to be due to the strong DNA (and/or RNA) binding which leads to the inhibition of the cellular replicative and transcription processes (Hartmann et al., 1964; Theologides et al., 1968; Ward et al., 1965; Meriwether and Bachur, 1972; Zunino et al., 1974). Therefore, in vitro assays of the (i) rate of dissociation of DNA complexes as well as (ii) the inhibition of DNA-dependent polymerases may provide a rapid estimate of the biological activity of daunorubicin and its derivatives. Moreover, important information is obtained at the molecular level which may be relevant to the proper design of chemotherapeutically more effective drugs. It is recognized, of course, that the in vivo situation is considerably more complicated due to problems of permeability, solubility, competitive binding to proteins and/or lipids, metabolic breakdown of the drugs, etc. Nonetheless, the in vitro studies on **1–7** indicate that these molecules (i) dissociate from the DNA complex in the order: adriamycin (**6**) = daunorubicin (**1**) < rubidazone (**7**) < D-Gly (**5**) < D-Me_2Gly (**2**) < D-Pr_2Gly (**3**) = D-Gly_2Gly (**4**) (Table V) and inhibit the DNA-dependent *E. coli* RNA polymerase in the inverse order (Figure 5). The above results (with one exception) are consistent with the relative in vivo biological activity on the mouse P388 lymphocytic tumor, i.e., **1** = **6** > **7** > **2** > **5** > **4** (**3** is biologically inactive). The one discrepancy, i.e., the glycyl derivative **5**, which shows a lower biological activity than D-Me_2Gly (**2**), could be due to differences in cellular permeability. In line with this interpretation is the observed tenfold higher distribution coefficient, *D* (and hence greater lipid solubility), of the D-Me_2Gly (**2**) as compared to **5** (Leo et al., 1971).

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Control of Ribonucleic Acid Synthesis in Eukaryotes. 2. The Effect of Protein Synthesis on the Activities of Nuclear and Total DNA-Dependent RNA Polymerase in Yeast†

Kurt J. Gross and A. Oscar Pogo*

ABSTRACT: A thermosensitive conditional yeast mutant (t_s-187) which suppresses protein synthesis at the nonpermissive temperature (36 °C) also suppresses RNA synthesis. The effect of temperature on the mutant is similar to the addition of cycloheximide—it inhibits the incorporation of labeled precursors into RNA in both whole cells and isolated nuclei. The effect of temperature is selective for the RNA polymerases bound to the nuclear template but not for the total RNA polymerases. Thus, the specific activities and total amounts of RNA polymerase species extracted and assayed with exogenous DNA template are similar in the t_s-187 cultured at 23 °C and at 36 °C. On the contrary, the nuclear polymerases, i.e., RNA synthesis in isolated nuclei, are dramatically inhibited in cells cultured at 36 °C. When amino acid starved t_s-187 cells are transferred to 36 °C, release from the inhibition of RNA synthesis is observed. As with the addition of cycloheximide, this relaxation is observed in cells but not in isolated nuclei. The parental strain, A364A, which responds by stimulating instead of inhibiting protein synthesis when the temperature is increased to 36 °C, also exhibits an inhibition in the incorporation of labeled precursor into RNA as well as reducing RNA synthesis in isolated nuclei. However, these are transitory inhibitions and afterward there is reinitiation of both processes. Reinitiation of RNA synthesis in isolated nuclei is similar to the relaxed phenomenon and it is called "nuclear relaxation". This relaxation can only be obtained if protein synthesis is not inhibited; however, cellular relaxation occurs in the absence of protein synthesis. The repression of the nuclear RNA polymerase activities which starvation and inhibition of protein synthesis produce appears to be due to a restriction in the nuclear DNA template. This notion is supported by the fact that a net diminution of these nuclear enzyme activities is observed in spheroplasts cultured under starving conditions. Studies of the four main ribonucleotide pools indicate that stringency and inhibition of protein synthesis (t_s-187 cultured at 36 °C) produce an increase in UTP and CTP pools. This is consistent with the concept that stringency and inhibition of protein synthesis affect the rate of utilization rather than the synthesis of these ribonucleotide residues. In the A364A and t_s-187 yeast strains, the conversion of uracil but not of uridine into the UTP and CTP is inhibited when there is inhibition of the nuclear RNA polymerases. This indicates that the uracil phosphoribosyltransferase but not the uridine-cytidine kinase is allosterically inhibited by UTP and CTP in yeast. The feedback inhibition in the metabolic pathway of the base explains why relaxation cannot be detected when uracil instead of uridine is used as the labeled RNA precursor.

Stringency has been defined as a gene-controlled process that exists in prokaryotes by which they can adapt to environmental changes of nutrients (Edlin and Broda, 1968). It operates by inhibiting the RNA synthetic machinery as well as the protein synthetic machinery in cells cultured in an amino acid deprived medium (Ryan and Borek, 1971). Although the coupling of both machineries had been originally observed in eukaryotes (Brachet, 1957), it is currently accepted that stringency is a genetic phenomenon restricted to prokaryotes (Edlin and Broda, 1968). Nevertheless, recent observations in ascites cells and yeast (Franze-Fernández and Pogo, 1971; Gross and Pogo, 1974) clearly indicate that stringency is a universal procedure for regulating RNA metabolism. This implies that any biological process which produces stimulation or inhibition in the eukaryotic RNA synthetic machinery has to exert its effect through the function of RC^{str} gene(s) (Tsukada and Lieberman, 1965; Pogo et al., 1966, 1967; Doly et al., 1965; Tata, 1966; Rubin and Cooper, 1965; Pogo, 1972).

The uncoupling of bacterial RNA and protein synthesis, known as relaxation, is the essential point of stringency since this phenomenon can be obtained by mutation (Stent and Brenner, 1961). The observation of phenotypic relaxation in yeast (Foury and Goffeau, 1973; Gross and Pogo, 1974) clearly established a further link in the basic mechanism by which

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