Effect of Reaction Conditions on the Reassociation of Divergent Deoxyribonucleic Acid Sequences†

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ABSTRACT: The effect of mispaired bases on the reassociation of divergent DNA sequences was studied under conditions which demanded different levels of specificity of base pair formation. Measurements were made of the rate of reassociation of both chemically modified DNA and DNA from related bacteria. Under the most commonly used conditions of renaturation, the effect of mispairing on the rate of reassociation appears to be minor. Incubation at 25° below the native T_m requires approximately a 15° T_m reduction to reduce the rate by a factor of two. However, under more stringent reaction conditions, the effect of mispairing becomes decidedly more pronounced, requiring only a 3.5° reduction in T_m at 10° below the T_{mn} to produce the same result. (T_m is the temperature required for 50% thermal denaturation and T_{mn} refers to the T_m of native DNA.) By determining the relative rates of reassociation of mispaired sequences, we have derived a mispairing coefficient (a) which can be used to predict renaturation rates for duplexes of varying degrees of homology under various criteria of stringency. The reduction in rate for a given duplex can be obtained by multiplying the ΔT_m by the mispairing coefficient (a), where a is obtained from the empirically derived relationship: $\log a = \log 0.27 - 0.0412(T_{mn} - T_i)$ (where T_i is the temperature of incubation). The practical connotations of this fact for experimental design are discussed. Two new methods for the analysis of homologous and heterologous reannealing reactions are described and discussed.

Analysis of the kinetics of DNA reassociation is a valuable tool for the study of genome size, structure, and organization as well as for the measurement of evolutionary relationships. In principle, both the degree of sequence repetition and the average length of the repeated segments can be established (Britten and Kohne, 1968). It has long been recognized that the size of the DNA fragments (Wetmur and Davidson, 1968), their G + C content, and their sequence complexity affect the rate of renaturation. However, the effect of mismatched base pairs has only recently received attention (Southern, 1971; Hutton and Wetmur, 1973b).

In order to determine the magnitude of this rate reduction, we have measured the kinetics of reassociation of alkali deaminated DNA and DNA from related bacteria. In the first case, mispairing results from the conversion of G·C pairs to G·U pairs (Marrian *et al.*, 1950; Ullman and McCarthy, 1973); in the latter mispairing is the result of natural evolutionary drift. Different conditions of stringency were obtained by changing the formamide concentration (McConaughy *et al.*, 1969) and the temperature of incubation. Reannealed chemically altered DNA or interspecific DNA duplexes exhibit a decreased thermal stability relative to native DNA. This results primarily from mispaired bases and is a linear function of the fraction of mismatched base pairs (Ullman and McCarthy, 1973). This experimental parameter is used to define the extent of mismatching and its effect on the rate of renaturation.

Materials and Methods

Preparation of DNA. DNA was isolated from *Bacillus subtilis* strains 746 and W23, *B. globigii*, and *B. licheniformis* (Chilton and McCarthy, 1969). Cells were harvested as they entered the stationary phase to minimize the number of fractional genomes present. DNA was prepared and purified by standard methods (Marmur, 1961), dissolved in 0.1 × SSC[1] at 1 mg/ml and sheared at 12,000 psi in a French pressure cell fitted with a steel ball bearing. This produced DNA fragments averaging 370 to 400 nucleotides in single-stranded length as measured by boundary sedimentation velocity in 0.1 M NaOH–0.9 M NaCl in a Beckman Model E analytical ultracentrifuge equipped with a ultraviolet (uv) monochromator. Molecular weights were calculated from the equivalent boundary sedimentation coefficient (Schachman, 1959) using the method of Studier (1965).

DNA Deamination. Cytosine residues were converted to uracil by alkali deamination of *B. subtilis* 746 DNA, thus permitting the formation of G·U base pairs in the renatured duplex (Ullman and McCarthy, 1973). DNA at 1.4 mg/ml in 1 × SSC was mixed with an equal volume of freshly prepared 2 M NaOH to give a final measured pH of 13.9. This solution was incubated at 70° and aliquots were removed at 30, 60, 90, 120, 180, and 240 min, added to equal volumes of ice cold 1 M KH_2PO_4 (pH 4.45), and dialyzed exhaustively against distilled water. Samples to be used as untreated DNA controls were incubated in 0.1 N NaOH at 60° for 15 min to hydrolyze all traces of RNA.

Reactions with Deaminated DNA. For all reannealing experiments, deaminated DNA was lyophilized and resuspended at approximately 0.5 mg/ml in 5 × SSC, containing either 48 or 70% (v/v) formamide, placed in 1-mm cuvets, and overlayed with light mineral oil to prevent evaporation. An untreated DNA control and two deaminated samples were renatured in parallel with a solvent blank and monitored by recording the absorbance at 260 nm using a Gilford spectrophotometer. The temperature of the cuvet chamber was first raised to 70° to denature the DNA and then cooled to the renaturation temperature. Renaturation was assumed to begin when the absorbance of the untreated control began to decrease. Since the DNA had already been denatured by the high pH of deamination, it was

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[1] Abbreviation used is: 1 × SSC, 0.15 M NaCl–0.015 M sodium citrate.

not possible to determine its concentration by measuring the absorbance of the native duplex at room temperature. Instead, concentrations were determined from the A_{260} at 70° assuming a 38% hyperchromic shift since samples from the same DNA preparation before deamination exhibited this same hyperchromicity during denaturation. The fraction reassociated was calculated from the decrease in absorbance compared to the theoretical maximum decrease. After the reaction was essentially complete at about 24 hr, the temperature of the cuvet chamber was gradually raised to 70° in order to measure the thermal stability of the hybrids. The T_m is defined as the temperature at which 50% of the material has melted as measured by the increase in hyperchromicity, and ΔT_m is defined as the difference between the T_m of renatured untreated DNA and that of renatured deaminated DNA.

Second-order rate constants for deaminated DNA were determined in two ways. Initially, results were plotted as the fraction reassociated *vs.* C_0t where C_0 is the concentration of denatured DNA in molarity of nucleotides and t is the time in seconds (Britten and Kohne, 1968). In this formulation, the second-order rate constant is equal to $1/C_0t_{1/2}$ where $t_{1/2}$ is the time necessary to reach half-maximum renaturation. Later, to avoid subjective errors in determining rate constants, a straight line function was derived, facilitating an objective determination of the best fit.

The equation for renaturation of homogeneous single-stranded DNA to form double-stranded DNA may be written

$$2C_s = C_d \tag{1}$$

where C_s and C_d refer to the concentrations of single- and double-stranded DNA, respectively. Since eq 1 does not provide for nonrenaturable DNA, C_s refers only to potentially renaturable DNA. The disappearance of single-stranded DNA may be written

$$\frac{-dC_s}{dt} = kC_s^2 \tag{2}$$

Integrating

$$\frac{1}{C_s} - \frac{1}{C_T} = kt \tag{3}$$

where C_T refers to the total concentration of *homologous* DNA and k is the second-order rate constant of renaturation. If all concentrations are expressed as moles of nucleotides per liter, then

$$C_T = C_s + C_d \tag{4}$$

Substituting for C_s and rearranging yield

$$\frac{C_d}{t} = kC_T(C_T - C_d) \tag{5}$$

For convenience, the equation can be divided by the square of total DNA concentration (C_0^2 in Britten's formulation (1968) noting that $C_T \neq C_0$)

$$\frac{C_d}{C_0}\frac{1}{C_0t} = \frac{kC_T}{C_0}\left[\frac{C_T}{C_0} - \frac{C_d}{C_0}\right] \tag{6}$$

Thus the equation is recast in terms of readily measurable parameters, *e.g.*, fraction reassociated and C_0t.

The equation can be used in this form by plotting the fraction reassociated over C_0t against the fraction reassociated with both axes starting at zero. With single copy DNA, such as *B. subtilis* DNA, the data yield a straight line. The slope is $k(C_T/C_0)$ and the abcissa intercept is (C_T/C_0), the maximum extent of reaction. Dividing the slope by the abcissa intercept permits second-order rate constants to be determined.

Interspecies Reactions. For experiments using DNA from different species of bacteria the procedure was quite different. A small amount of [^{3}H]thymidine-labeled DNA from *B. subtilis* 746 was mixed with a large excess (≥4000:1) of unlabeled DNA from *B. subtilis* W23, *B. globigii*, or *B. licheniformis* and the mixture was lyophilized and resuspended in 5 × SSC containing either 48 or 70% formamide. Samples were denatured at 90° for 10 min and allowed to renature at 37°. Aliquots (50 μl) were withdrawn at various times, diluted into 5 ml of ice-cold 0.14 M sodium phosphate buffer (pH 6.8), and applied to 1-ml hydroxylapatite (Bio-Rad) columns maintained at 60° in a water bath. The columns were washed with 5 ml of 0.14 M phosphate buffer to elute single-stranded DNA. Double-stranded DNA was eluted with 5 ml of 0.5 M phosphate buffer. The percentage of renatured DNA was determined from the fraction of trichloroacetic acid precipitable radioactivity recovered in each wash. To measure the thermal stability an aliquot was diluted tenfold with 0.14 M phosphate buffer and dialyzed extensively *vs.* 0.14 M phosphate buffer. The sample was then layered onto a 7-ml jacketed column of hydroxylapatite held at 60° with a Haake circulating water bath. The temperature was raised in 5° increments and the column allowed to equilibrate at each temperature. Single-stranded counts were eluted with a 5-ml wash of 0.14 M phosphate buffer which had previously been brought to the temperature of the column. To finally clear the column of any remaining counts a 100°, 0.5 M phosphate buffer wash was applied. The results are presented as the cumulative per cent counts eluted as a function of temperature. The T_m is the temperature at which 50% of the radioactivity is eluted from the column and ΔT_m refers to the difference between the T_m values of homologous and heterologous duplexes.

Relative rate constants for interspecies reactions were determined in the following manner. Interspecies reactions containing a small amount of radiolabeled DNA mixed with a large excess (≥4000:1) of unlabeled DNA were incubated for a time span which precluded any significant self-association of the labeled DNA. However, since the concentration of unlabeled DNA is constantly changing due to self-association, two equations are necessary to describe the formation of heteroduplexes

$$\frac{-dC_s}{dt} = kC_s^2 \tag{2}$$

$$\frac{-dC_s^*}{dt} = k^*C_sC_s^* \tag{7}$$

where C_s and C_s^* are the concentrations of unlabeled and labeled DNA remaining single stranded and k and k^* are the homologous and heterologus rate constants, respectively. The solution for the fraction of labeled DNA remaining single stranded is

$$\frac{C_s^*}{C_0^*} = \frac{1}{(1 + kC_0t)^{k^*/k}} \tag{8}$$

where C_0^* and C_0 are the total concentrations of labeled and unlabeled DNA, respectively. The fraction of labeled DNA (C^*) which cannot form stable heteroduplexes can be accounted for by subtracting the noncomplementary portion (N), inverting, and taking logs

$$\log\frac{(C_0^* - N)}{(C_s^* - N)} = \frac{k^*}{k}\log(1 + kC_0t) \tag{9}$$

Only when $k^*/k = 1$ does $1/C_0t_{1/2} = k^*$. Thus, theoretical curves for Figure 7 were calculated using eq 8 and the analysis of interspecies reactions shown in Figures 8A–D made use of eq 9 which is linear with respect to k^*/k.

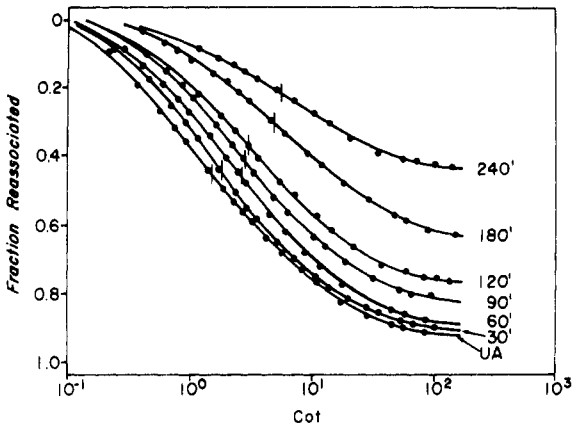

FIGURE 1: Rate of renaturation of alkali deaminated *B. subtilis* 746 DNA in 48% formamide. The DNA was sheared in a French pressure cell to a single-stranded molecular weight of about 125,000 and then deaminated for various times. Samples (0.5 mg/ml) were incubated in 48% formamide and 5 × SSC at 37° and renaturation was monitored by the change in absorbance at 260 nm on a Gilford recording spectrophotometer. Further details of the deamination and renaturation procedures are described under Materials and Methods. Renaturation kinetics are shown for DNA which was deaminated for 0 (UA), 30, 60, 90, 120, 180, and 240 min. Vertical bars refer to $C_0t_{1/2}$.

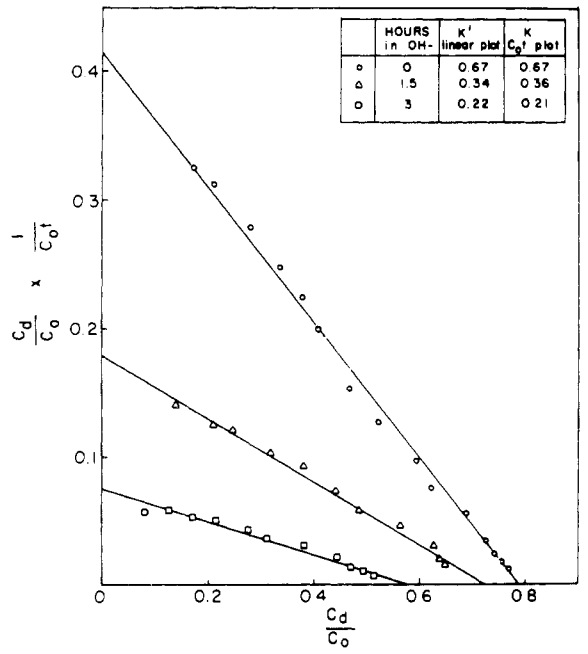

FIGURE 2: Examples of data from Figure 1 are replotted according to eq 6: (○) 0 min deaminated DNA; (△) 90 min deaminated; (□) 180 min deaminated.

Results

Renaturation of Deaminated DNA. Alkali deaminated DNA was annealed in 48% formamide at 28, 37, and 45°, and in 70% formamide at 28, 37, 40, and 44°. Data obtained in 48% formamide at 37° are shown in Figure 1. As indicated by the vertical bars, the time necessary to reach half-maximal reaction ($t_{1/2}$) increases as the time of deamination is increased; hence, the second-order rate constant is decreased as the time of deamination and presumably the amount of mismatching are increased. In Figure 2, the data from Figure 1 are plotted in linear form according to eq 6. This analysis confirmed the values of the second-order rate constants obtained from the semilog plots. The thermal stability of the renatured DNA was measured after each experiment. Melting profiles

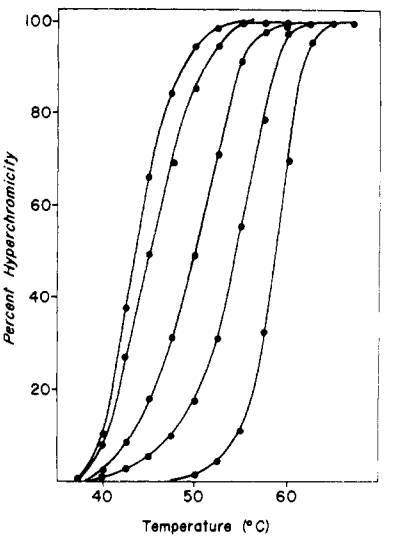

FIGURE 3: Thermal stability measurements. Duplexes formed in Figure 1 with untreated, 1-, 2-, 3-, and 4-hr deaminated DNA samples were heated slowly and the fractional increase in absorbance at 260 nm was measured as a function of temperature.

for renaturation experiments conducted at 37° are shown in Figure 3 and are typical of the data obtained. T_m values are summarized in Table I. In all cases T_m decreases as the time of deamination is increased.

The primary effect of alkali deamination is to remove the amino group from the 6 position of cytosine, converting deoxycytosine residues to deoxyuracil (Ullman and McCarthy, 1973). With increasing deamination an increasing proportion of the DNA is unable to form stable duplexes (Figures 1 and 2). We questioned whether the deamination was random or whether a particular region of the genome might be enriched in cytosine and hence be preferentially removed from the pool of renaturable DNA. Untreated [^{3}H]DNA from *B. subtilis* 746 was annealed with unlabeled *B. subtilis* 746 DNA which had been deaminated for 0, 30, and 120 min. If deamination were random, all the [^{3}H]DNA would form duplexes, while preferential loss of certain fractions of the genome would lead to incomplete reaction. In fact, the reaction of the two deaminated samples followed that of the untreated control exactly, leveling off at 95% reaction. Moreover, all three samples were equally resistant to later S1 nuclease digestion, thus verifying the absence of large unreacted tails in the deaminated samples.

Chain scission is a side reaction during alkali deamination (Marrian *et al.*, 1950; Hill and Fangman, 1973). Since the rate of renaturation varies as the square root of the molecular weight of the DNA (Wetmur and Davidson, 1968), any decrease in the rate of renaturation of the deaminated samples (Figures 1 and 2) may be attributable, at least in part, to molecular weight differences. Similarly, the T_m values (Figure 3) might also be affected since T_m varies inversely with the length of the polynucleotide chain (Hayes *et al.*, 1970). In order to determine the magnitude of the molecular weight effects on renaturation rates and T_m values, we measured size as a function of deamination time (Figure 4). For every 90 min of deamination, the single-stranded molecular weight was approximately halved. These data were then used to correct for molecular weight differences in the different deaminated samples.

Rate constants and values of ΔT_m obtained under various reaction conditions are compiled in Table I. When the normalized rate constants are plotted against T_m (Figure 5), a decline in the rate of reaction with increased mismatching is apparent. Data obtained in 48% formamide at 28, 37, and 45° are shown

TABLE I: Rate Constant and Thermal Stability Data for Reassociation of Deaminated DNAs.[a]

Reaction Condition	Temp (°C)	DNA Sample Deam. (hr)	ΔT_m (°C)	ΔT_m Corrected	$C_0t_{1/2}$	$(k_2/k_1)\cdot\sqrt{\text{mol wt(untreated)}}/\sqrt{\text{mol wt(deam)}}$
48% formamide, 5 × SSC	28	0	0	0	1.7	
		1	5	4.1	2.3	0.95
		3	15.5	11	4.3	0.89
		0	0	0	1.7	
		2	10.5	8.3	3.2	0.89
		4	19.5	12.9	6.3	0.79
	37	0	0	0	1.8	
		1	4.5	3.5	2.7	0.89
		3	13.5	8.8	5.9	0.70
		0	0	0	1.4	
		2	8.5	6.3	3.0	0.79
		4	15	8.3	5.5	0.74
		0	0	0	1.5	
		0.5	2.5	1.9	1.8	0.95
		1.5	7	5.6	2.8	0.81
		0	0	0	1.5	
		1.5	7	5.6	2.8	0.73
		3	13.5	8.6	4.8	0.82
	45	0	0	0	1.5	
		2	8.5	6.3	3.4	0.78
		4	12.5	5.6	7.5	0.68
		0	0	0		
		1	4	3.2	2.4	0.85
		3	9.5	5	5	0.70
		0	0	0	2.0	
		0.5	2.5	2	2.1	1.08
		1.5	8.5	7.3	3.9	0.79
70% formamide, 5 × SSC	28	0			4.0	
		1	4	3.2	6.4	0.82
		2	8.5	6.1	9.7	0.70
		0			4.3	
		3	10.5	5.8	10.0	0.57
		4	11.5	4.8	22.0	0.54
		0			4.3	
		0.5	1.5	1.2	5.4	0.91
		1.5	6	4.4	8.3	0.78
	37	0			4.4	
		1	3	2.2	8.0	0.72
		2	4.5	2.3	12.5	0.58
		0			4.7	
		0.5	1.5	1.2	6.6	0.81
		1.5	4	2.6	11.0	0.64
	40	0			4.8	
		1	2	1.2	8.0	0.79
		2	3	0.8	14.5	0.56
		0			5.0	
		0.5	1	0.7	7.3	0.78
		1.5	3	1.4	1.4	0.55
	44	0			4.8	
		1	1	0.2	10.5	0.6
		0			6.8	
		0.5	0.5	0.2	10.5	0.74
		1.5	1.5	0	22.0	0.46

[a] k_2 is the second-order rate constant for deaminated DNA. k_1 is the second-order rate constant for untreated control DNA. From studies of Hayes *et al.* (1970), the reduction in thermal stability due to length can be calculated from the relationship $\Delta T_m = 442/n$ where n is the length in nucleotides. Corrected ΔT_m values were obtained by subtracting the expected contribution due to length (obtained using the above relationship) from the observed ΔT_m values.

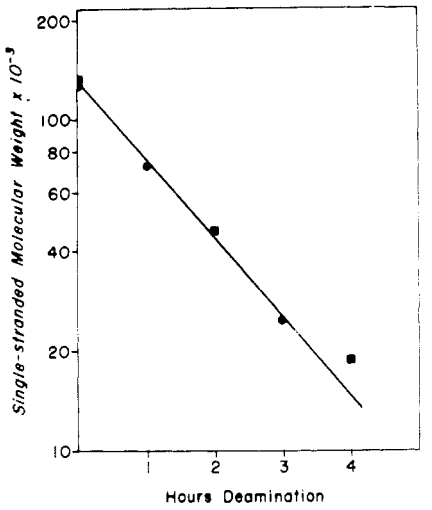

FIGURE 4: Effect of deamination on the molecular weight of DNA. The molecular weights of deaminated *B. subtilis* 746 DNA samples were determined by analytical centrifugation in alkali as described under Materials and Methods. The single-stranded molecular weight is plotted as a function of time of deamination. The results from two different DNA preparations deaminated for the indicated times are plotted together as ■ and ●.

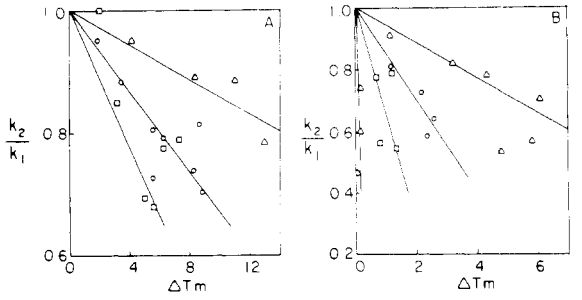

FIGURE 5: Effect of thermal stability on the rate of reaction. Deaminated DNA was allowed to reassociate at different temperatures in 5 × SSC containing either (A) 48% formamide or (B) 70% formamide. After complete reassociation the samples were melted and T_m values determined. Rate constants for deaminated samples (k_2) have been normalized to the rate constant of the untreated DNA (k_1) run in parallel. In A, renaturation in 48% formamide was at (△) 28°, (○) 37°, and (□) 45°. The native T_m in this solvent is 60°. In B, renaturation was in 70% formamide at (△) 28°, (○) 37°, (□) 40°, and (△) 44°. The native T_m in this solvent is 45°.

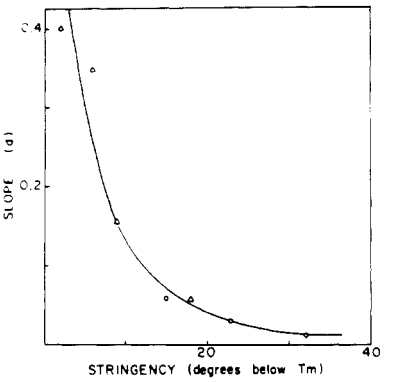

FIGURE 6: Effect of the relative degree of stringency upon the rate constant. The slopes from Figure 5 are plotted against the relative degree of stringency, expressed as the difference between the temperature of incubation and the T_m of native DNA: (○) slopes from Figure 5A; (△) slopes from Figure 5B.

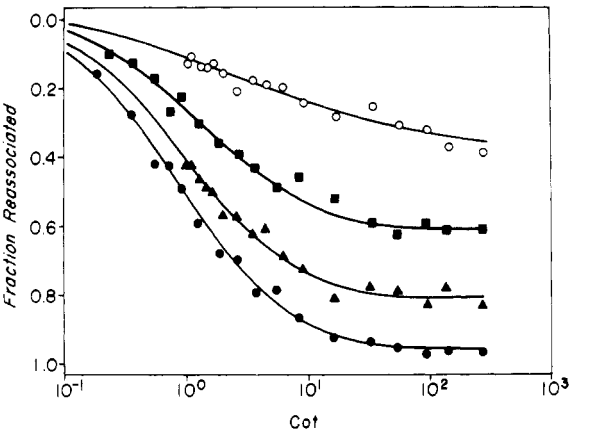

FIGURE 7: Interspecies DNA reactions. [^{3}H]DNA (10,000 cpm/μg) from *B. subtilis* 746 was added to excess unlabeled DNA from (●) *B. subtilis* 746, (▲) *B. subtilis* W23, (■) *B. globigii*, and (○) *B. licheniformis*, and sheared at 12,000 psi in a French pressure cell. The total DNA concentration was 1 mg/ml in 48% formamide and the ratio of unlabeled DNA to [^{3}H]DNA was 4000:1. Heteroduplex formation was measured by hydroxylapatite fractionation as described under Materials and Methods. Solid lines are theoretical curves generated from eq 8.

in Figure 5A. Figure 5B shows data obtained in 70% formamide at 28, 37, 40, and 44°. Mismatched base pairs cause a greater reduction in reaction rate under conditions of high stringency (*e.g.*, 70% formamide) than under the moderately stringent conditions of 48% formamide. For instance, it can be seen that in 48% formamide at 37° (25° below the T_{mn}) a 15° decrease in T_m reduces the rate by a factor of two, whereas in 70% formamide, a 3.5° reduction in T_m produces the same result. Compare data of Figures 5A and 5B at 37°. The lines in Figures 5A and 5B are described by the equation

$$k_2/k_1 = 1 - a\Delta T_m \qquad (10)$$

where a is the slope, reflecting the magnitude of the effect. For purposes of discussion, this will be called the mispairing coefficient. Values of the mispairing coefficient (a) are plotted in Figure 6 as a function of the stringency of the reaction, expressed as the difference between the temperature of incubation (T_i) and the T_m of the native DNA (T_{mn}). The resulting curve of Figure 6 is described approximately by the equation

$$\log a = \log 0.27 - 0.0412(T_{mn} - T_i) \qquad (11)$$

It is clear that the effect of mismatching on rate increases dramatically as the temperature of incubation approaches the T_m of the native DNA.

Interspecies Reactions. Although it is convenient to alter DNA by controlled chemical means, thus producing any desired degree of mismatching, this does not necessarily reflect the many types of alterations which occur during the normal course of evolution. This condition is best studied by monitoring heteroduplex formation between DNAs from related species. Moreover, in the case of interspecies reactions, the DNA may be sheared to uniform size, thus simplifying quantitative analysis by eliminating the necessity of molecular weight corrections. Therefore experiments were performed using DNA from related species of bacteria where the base sequences had diverged by natural evolutionary processes. *B. subtilis* 746, *B. subtilis* W23, *B. globigii*, and *B. licheniformis* are four species of bacteria sufficiently related to permit interspecific transformation (Chilton and McCarthy, 1969), yet whose DNA will form heteroduplexes of varying degrees of base sequence homology. [^{3}H]DNA from *B. subtilis* 746 was allowed to form heteroduplexes with unlabeled DNA from each of the four bacilli in 48% formamide (Figure 7) and 70%

TABLE II: Thermal Stability Data for Interspecies Heteroduplexes.[a]

Cross	Reaction Conditions % Formamide	ΔT_m (°C)
B. subtilis W23/*B. subtilis* 746	48	5
B. globigii/*B. subtilis* 746	48	12
B. licheniformis/*B. subtilis* 746	48	23
B. subtilis W23/*B. subtilis* 746	70	2

[a] All reactions were at 37° in 5 × SSC with varying amounts of formamide.

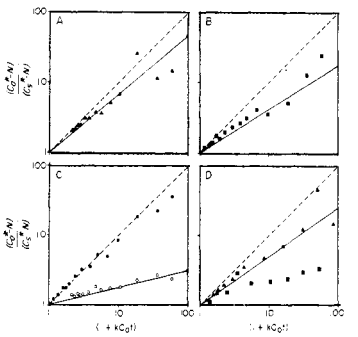

FIGURE 8: Data from interspecies reactions in 48 and 70% formamide are plotted according to eq 9 discussed under Materials and Methods.

formamide. Heteroduplexes formed at 37° in 48 and 70% formamide were assayed by the hydroxylapatite procedure described under Materials and Methods. Thermal dissociation profiles were obtained by successive elution at increasing temperatures from hydroxylapatite. T_m values are compiled in Table II. The solid lines of Figure 7 are theoretical curves generated from eq 7. Since these data were not sufficient to permit a meaningful determination of the best fit and hence, of the heterologous rate constant, a linear method of analysis was devised as described under Materials and Methods (eq 8). This method separates the two variables, *i.e.*, the maximum extent of reaction and the heterologous rate constant. Thus, the analysis is linear with respect to the ratio of the homologous and heterologous rate constants. The data from interspecies reactions in 48% formamide (Figure 7) and 70% formamide are analyzed by this method as shown in Figures 8A–D. Solid lines represent the relative renaturation rates predicted from the data obtained with deaminated DNA. These were calculated from eq 10 by measuring the ΔT_m of the heteroduplexes (Table II) and calculating the mispairing coefficient from eq 11. The predicted and observed behaviors are quite similar. A close correlation between these two independent measurements serves to establish the validity of the methods.

It is conceivable that the heterologous reaction, proceeding more slowly than the homologous reaction, may have terminated because the supply of unlabeled DNA had been depleted. To test this possibility, *B. subtilis* 746 DNA was allowed to react with a large excess of unlabeled *B. globigii* and *B. subtilis* W23 DNA. After apparent termination, a second excess of unlabeled single-stranded *B. subtilis* 746 DNA was added to each reaction mixture. No further reaction could be observed. Theoretical calculations using eq 8 corroborated this observation.

Discussion

We have measured the rate of reassociation using both chemically modified DNA and DNA from different species. We chose alkali rather than HNO_2 deamination since the latter produces extensive cross-linking perhaps resulting in the preferential loss of some DNA sequences. *B. subtilis* was chosen because it has a G + C content similar to most vertebrates and because measurements of the randomness of deamination demonstrated clearly that artifacts caused by regions of high G + C content were not present. Since chain scission is an unavoidable side reaction of chemical modification, molecular weights were determined and the appropriate corrections made. We have taken the relationship of Hayes *et al.* (1970) as the best estimate of the effect of molecular weight on thermal stability. However, because of the probable salt dependence and since our measurements were made at much higher ionic strength, their relationship probably represents an upper limit.

For the interspecies reactions, four species of bacilli were chosen which were known to be sufficiently related to permit interspecific transformation and hence could be expected to permit extensive heteroduplex formation. These experiments obviated the difficulties and criticisms pertinent to reactions using chemically modified DNA. The close correlation between the two methods indicates that chemically modified DNA is a reasonable model system for studying the effect of mismatching on rate of annealing.

The magnitude of the effect of mismatched base pairs on the rate of annealing is a contentious issue. Southern (1971), in an effort to reconcile conflicting estimates of the complexity of mouse satellite DNA, proposes a major effect. Sutton and McCallum (1971), who present experiments with mouse satellite DNA, have attempted to corroborate this prediction. Hutton and Wetmur (1973b) have recently proposed a theory which predicts a much smaller effect. Although the possibility of side reactions was not discussed, their data with HNO_2 deaminated and glyoxalated DNA indicate that the effect is relatively small. A small effect was also found by Bonner *et al.* (1973) who measured the rate of interspecies DNA renaturation. We conclude from our data that the effect of mismatching on rate of annealing is small under commonly used conditions of reassociation (*i.e.*, 25° below T_{mn}).

Although the effect of mismatching on the reaction rate is minor under commonly used conditions of renaturation, it is important to stress that the magnitude of the effect is quite dependent on the stringency of the reaction conditions. Not unexpectedly, a given degree of mismatching has a much greater effect on the rate of reaction in higher concentrations of formamide or at higher renaturation temperatures. However, this effect does have practical implications. For example, it is apparent that the cross-reaction of even closely related redundant sequences may be minimized or even totally precluded by appropriate choice of stringent reaction conditions. Similar issues often arise in cases of the hybridization of two messenger RNAs for proteins of similar amino acid sequences. The data of Figure 5 will be valuable in design and interpretation of experiments of this type.

In terms of the interpretation of the renaturation kinetics of redundant DNA, our results lead us to agree with Bonner *et al.* (1973) and Hutton and Wetmur (1973b), namely, a decrease in thermal stability of 10° reduces the renaturation rate by only a factor of two or less. The much larger effects described by Sutton and McCallum (1971) for renaturation or mismatched mouse satellite DNA may be attributable to the fact that when the repeat length is less than the fragment size, the amount of duplex formed per successful nucleation is indepen-

dent of the fragment size thus making the reaction rate proportional only to (mol wt)$^{-1/2}$ (Hutton and Wetmur, 1973a; Chilton, 1973). Therefore, the results obtained by Sutton should not be generalized to apply to all redundant DNA renaturation kinetics. Nevertheless, in some interspecies DNA renaturation experiments where very large degrees of mismatching occur, it is necessary to consider a sizable decrease in rate of heterologous reaction over the homologous before attempting to quantitate the absolute degree of homology.

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Role of Base Composition in the Electrophoresis of Heat-Treated Deoxyribonucleic Acid from HeLa and Mouse Cells in Composite Polyacrylamide Gels†

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ABSTRACT: Heat-treated DNA from HeLa and mouse cells could be electrophoretically resolved into two components representing the native and denatured fractions. In 1.7% polyacrylamide–0.5% agarose gels heat-denatured DNA migrated in a discrete band of significantly slower mobility than did native DNA. Spectrophotometric gel scanning allowed a quantitation of the per cent denaturation at various temperatures and thus permitted calculation of a T_m, which agreed well with that determined by the optically measured thermal transition profile method of Marmur and Doty (1962). In addition, fully heat-denatured mouse satellite DNA electrophoresed as two discrete bands, presumably as a result of compositional differences between the complementary strands. The role of base composition in the electrophoretic behavior of DNA was studied by labeling the cytosine or guanine bases of HeLa DNA with tritiated nucleoside precursors while simultaneously labeling the thymine bases with thymidine-^{14}C. The ratio of $^{3}H/^{14}C$ incorporation within the native HeLa DNA band indicated a linear gradient in GC content. These studies further demonstrated the ability of polyacrylamide gel electrophoresis to fractionate DNA molecules on the basis of their base composition.

In a recent series of experiments we have examined some of the electrophoretic characteristics of DNA in polyacrylamide gels (Zeiger *et al.*, 1971, 1972a; Dingman *et al.*, 1972). It was found that high molecular weight DNA from the mouse and crab could be separated by polyacrylamide gel electrophoresis into satellite and main band DNA species. The separation was possible because the dA-dT rich crab and mouse satellite DNAs had lower electrophoretic mobilities than their corresponding main band DNAs. Subsequent studies using microbial DNAs of differing GC content showed that DNAs with progressively greater GC content had correspondingly greater electrophoretic mobilities in polyacrylamide gels. Within the range of base compositions studied (25–67% mole per cent G + C) the relationship between base composition and electrophoretic mobility was linear. In the present study we examined the electrophoretic behavior of DNA, denatured to a varying extent, and found that the native and denatured fraction could be easily resolved. We again found that the electrophoretic mobility of high molecular weight DNA is dependent on base composition. We also observed that polyacrylamide gel electropho-

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