Room Temperature Method for Increasing the Rate of DNA Reassociation by Many Thousandfold: The Phenol Emulsion Reassociation Technique†

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ABSTRACT: A phenol aqueous emulsion allows the reassociation of DNA at temperatures from 6 to 68 °C. This phenol emulsion reassociation technique (PERT) also promotes the very rapid reassociation of DNA. *E. coli* and human DNAs at a concentration of 4 μg/mL reacted at room temperature with the PERT reassociate many thousand times faster than under the standard conditions of 0.18 M Na^+, 60 °C. Solutions of DNA ranging in concentration from 6×10^{-5} to 6400 μg/mL have been successfully reassociated using the emulsion method. The greatest rate increases are seen at low DNA concentration. The half-time of reassociation does not decrease proportionally with an increase in DNA concentration when using the PERT. At 6400 μg/mL the phenol emulsion rate of reassociation is only about 10 times faster than under the standard aqueous reference conditions of 0.12 M phosphate buffer (PB), 60 °C. The rate of DNA reassociation observed with the emulsion technique is at least dependent on: (a) the presence of an emulsion; (b) the type and concentration of ion present; (c) an appropriate temperature of incubation; (d) the proper pH; (e) the rate and manner of agitating the emulsion; (f) the amount of phenol present; (g) the fragment size of the DNA; (h) the complexity of the DNA; (i) the concentration of DNA. The presence of salt is necessary for the emulsion reassociation, and the effect of a variety of salts ($NaHPO_4$, NaCl, $NaClO_4$, NaSCN, CsCl, RbCl, KCl, LiCl, and others) has been examined. As a general rule the more chaotropic the anion or cation, the faster the DNA reassociation in the emulsion system. DNA will reassociate over a wide range of pH (5-9) and phenol concentration (5-95%). Optimum emulsion reassociation rates are obtained by vigorous shaking of the emulsion. At high DNA concentrations the rate depends on the violence of shaking. The fragment size of the DNA used in the emulsion reaction does not greatly influence the rate of reassociation. DNAs from viruses, bacteria, and mammals ranging in G + C content from 30 to 70% have successfully and rapidly reassociated with the PERT. The criterion of those PERT mixtures examined is comparable to about 56 °C, 0.21 M NaCl in the aqueous system. RNA:RNA and RNA:DNA reactions also occur in the emulsion system. However, the greatest rate increase observed as yet is only 50-100 times the reference aqueous rate.

Nucleic acid reassociation has proved to be a powerful tool for analyzing the genetic material of a wide variety of organisms, from virus to man. The various applications of the technique are too numerous to list. An understanding of the rate of nucleic acid reassociation (Britten and Kohne, 1968; Wetmur and Davidson, 1968), the development of quick, efficient nucleic acid reassociation assays (Kohne and Britten, 1971; Britten et al., 1974) and the elucidation of the nucleotide sequence composition of the mammalian genome (Britten and Kohne, 1968) have greatly increased the versatility of this tool for genetic analysis. While nucleic acid reassociation has been used to answer many important questions, a major limitation on the feasibility of using this technique in many significant biological experiments is the basic rate of reassociation seen for the standard one-phase aqueous systems. We have discovered a technique which increases the rate of reassociation of DNA many thousandfold. The technique also allows reassociation to occur at or below room temperature. Most laboratories working with nucleic acids already possess the reagents and equipment needed for this technique which is simple and inexpensive.

Phenol is a common laboratory chemical which is frequently used to purify DNA and RNA. When phenol and water are mixed in the right proportions and shaken, an emulsion forms. When the shaking stops, the emulsion breaks and the two phases separate. Addition of single-strand DNA and salt to the water phase, and then shaking the mixture at room temperature results in the extremely rapid formation of double-stranded DNA. This two-phase system, water and phenol, is the basis for a new, rapid nucleic acid reassociation technique. We have named this method the phenol emulsion reassociation technique (PERT).[1]

Materials and Methods

DNA Preparations. DNAs used in these experiments were purified by the urea-phosphate-hydroxylapatite technique (Britten et al., 1974; Meinke et al., 1974) or by the standard phenol-chloroform technique. Iodinated DNA was prepared by a modification of the Commerford technique (Commerford, 1971). No nonradioactive carrier iodine was used and mercaptoethanol was utilized to terminate the iodination reaction

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[1] Abbreviations used: PERT, phenol emulsion reassociation technique; Tris, tris(hydroxymethyl)aminomethane; PB, phosphate buffers; Na-$DodSO_4$, sodium dodecyl sulfate; poly(a), poly(adenylic acid); poly(U), poly(uridylic acid); EDTA, ethylenediaminetetraacetic acid.

(Tareba and McCarthy, 1973). In order to prevent reassociation, the iodination mixture contained 0.01 M or less of monovalent cation. All iodinated preparations were examined for DNase lability. A small amount of each iodinated DNA preparation was also mixed with unlabeled DNA and assayed for its ability to reassociate with the PERT. Specific activities as high as 200×10^6 cpm/μg of DNA were attained by iodination. These iodinated DNAs, with one exception, reassociated to greater than 90% in the phenol system and reacted well for at least 2 weeks. [^{3}H]DNA was isolated from tissue culture cells or bacteria grown in the presence of [^{3}H]thymidine. Unless otherwise noted, the DNAs were sonicated before use. The procedure used for sonication produced fragments about 6-8 S (alkaline sucrose) in size. All of the DNAs used for this report reassociated to greater than 90% with one noted exception. The zero-time binding or binding of denatured DNA to hydroxylapatite varied from 1 to 6% for the different radioactive DNAs.

Phenol Reaction Mixture. The rapid reassociation is apparently dependent on the presence of an emulsion. Addition of just enough phenol to cause formation of two phases results in the rapid reassociation of DNA. The emulsions which promote rapid reassociation are unstable and will eventually break unless continually agitated. A practical, inexpensive shaker can be made out of a regular test tube vibrator such as the Vortex Genie mixer (Scientific Products, Evanston, Ill.). A styrofoam platform can easily be attached to the shaker in order to shake multiple samples simultaneously. All of the reaction mixtures reported here were shaken with the Vortex shaker unless otherwise noted. The shaker was run at $\frac{1}{2}$ to $\frac{3}{4}$ maximum speed.

Different aqueous solutions are used in this report in concert with phenol. The composition of these solutions is described in the text. Perhaps the most versatile aqueous phase consisted of 2 M sodium thiocyanate (NaSCN). The salt solutions were generally buffered to pH 6-8.5 with either Tris-HCl or phosphate buffers. Unless otherwise noted, the phenol:aqueous reaction mixtures consisted of 0.9 mL of aqueous phase plus a designated amount of phenol. The percent phenol present is calculated on a volume/volume basis. The phenol used was freshly distilled and diluted to 90% phenol for storage. Freshly prepared 90% phenol and 90% phenol kept at room temperature for 1-2 months gave similar results. Phosphate buffers (PB) used were mixtures of equimolar quantities of Na_2HPO_4 and NaH_2PO_4 (pH 6.8).

Assays for Reassociation. The DNA in an emulsion can be assayed for the extent of double strandness by rapidly and completely diluting an aliquot (usually 0.01-0.10 mL) of the emulsion directly into 2-4 mL of 0.14 M PB, 0.2% sodium dodecyl sulfate, and passing this solution over hydroxylapatite to separate double-stranded molecules from single-stranded molecules (Kohne and Britten, 1971). At low concentration the phenol does not affect this fractionation.

Different batches of hydroxylapatite respond differently to the presence of phenol. Each new batch should be examined for its ability to bind double-stranded DNA in the presence of phenol. When diluting the emulsion sample into the column buffer, transient areas of high phenol concentration at lower salt concentration are formed. These conditions may cause the dissociation of certain classes of double-stranded DNA. Mismatched reassociated repeat DNA fractions from higher organisms, mismatched interspecies hybrid DNA molecules or DNAs with very low G + C content would be most affected by such denaturing conditions. Both low and high G + C DNAs reassociate to greater than 90% using the methods described herein. If a situation occurs where a DNA does not appear to reassociate completely in the phenol system, try raising the salt concentration of the initial dilution buffer. A further dilution to 0.14 M PB is then necessary. In measuring the reassociation of sea urchin DNA with the PERT, emulsion aliquots diluted into 0.14 M PB and then frozen gave variable results (R. Britten, personal communication). Different DNAs may respond differently. If freezing of samples is necessary, conditions should be found so that double-stranded DNA is stable through the freeze-thaw cycle.

Most of the phenol can be removed from the reaction mix by shaking with an equal volume of chloroform and recovering the aqueous phase. The aqueous phase can then be diluted to 0.14 M PB, 0.2% $NaDodSO_4$, and passed over hydroxylapatite. Using the NaSCN aqueous solution is advantageous since the DNA can be ethanol precipitated out of the emulsion by the addition of 2 volumes of cold 95% ethanol. The precipitate is then collected by centrifugation and redissolved in 0.01 M NaCl. This is then adjusted to the proper salt conditions for S1 or hydroxylpatite assay.

The DNA in the emulsion can also be assayed by S1 nuclease digestion. In this case the aqueous phase should not contain phosphate buffer. For example, a PERT mixture consisting of 1.25 M $NaClO_4$, 8% phenol supports DNA reassociation and can be assayed by S1 nuclease. One-tenth milliliter of this reaction mix was added to 0.9 mL of 0.15 M NaCl, 0.05 M NaAc, pH 4.5, 0.003 M $ZnCl_2$. Twenty micrograms of RNA carrier and 1 unit of S1 nuclease are then added; the solution was incubated for 2 h at 37 °C and then assayed for acid precipitability.

Thermal Stability Measurements. The thermal stability of double-stranded DNA adsorbed to hydroxylapatite was determined as described by Kohne and Britten (1971). The thermal stability of double-strand DNA in the presence of phenol was done as described in Figure 3b.

Presentation and Comparison of Kinetic Data. PERT DNA reassociation kinetic data are presented in terms of the log C_0t method (Britten and Kohne, 1968) which is widely used to present aqueous reassociation kinetic data. This involves plotting the percent reassociation vs. the log of the product of the initial single-stranded DNA concentration (C_0) times the time (t) of reaction. The major advantage of this presentation method is the ability to depict the entire reassociation kinetic curve in one plot. The C_0t at one-half reassociation of the DNA ($C_0t_{1/2}$) is used to compare the rates of DNA reassociation from different experiments. $C_0t_{1/2}$ is equal to the reciprocal of the second-order rate constant (k) (Britten and Kohne, 1968). When obtained with the PERT, the data points from reassociation reactions done at different DNA concentrations *do not* all fall on the same reassociation kinetic curve since the $C_0t_{1/2}$ (or k) of the reassociation reaction changes with the DNA concentration (see the section on the effect of DNA concentration on the emulsion system reassociation rate).

Method Used for Determining the $C_0t_{1/2}$ of Reassociation. Two methods were used to determine $C_0t_{1/2}$ values. Method I: In those cases where many time points were measured (Figure 1), the $C_0t_{1/2}$ of reassociation was obtained by inspection of the graphed data. Method II: In these cases two different time points from each of two separate but identical reaction mixtures were obtained. It was assumed that the reaction followed second-order reaction kinetics. The second-order equation

$$\frac{c}{c_0} = \frac{1}{1 + kC_0t}$$

was then used to calculate the rate constant k (Britten and Kohne, 1968). The $C_0t_{1/2}$ is then equal to $1/k$. The $C_0t_{1/2}$

TABLE I: Characteristics of PERT Reassociated DNA.[a]

	Native DNA	PERT reassociated DNA	Aqueous reassociated DNA	Single-strand DNA incubated at RT
% Hyperchromicity exhibited from 60 to 100 °C	24.7	24.2	21.7	3–4
T_m	92.2	92.5	93	

[a] Three separate but identical DNA solutions were prepared. Each solution contained 344 μg of sonicated single-strand DNA per mL of 0.48 M PB. (a) One sample was incubated at 70 °C for 14 h. (b) Five milliliters of 90% phenol was added to a second 1-mL sample of DNA solution. This mixture was vigorously agitated at room temperature for 5 h. The phenol was then removed by chloroform extraction and the aqueous phase recovered. (c) A third solution was incubated at room temperature for 14 h. Each of the DNA solutions was passed through agarose (A-0.5m Bio-Rad) in order to change the salt to 0.11 M NaCl. A_{260} melting profiles were then determined for each sample using a Gilford recording spectrophotometer. Percent hyperchromicity is defined as the change in A_{260} from 60–100 °C divided by the A_{260} at 100 °C.

values presented in the various tables are the average of the $C_0t_{1/2}$'s calculated from multiple kinetic points.

Results

PERT-Mediated Formation of Helical Double-Stranded DNA. The data presented in Table I demonstrate that a phenol emulsion promotes the formation of stable helical double-stranded DNA from single-stranded DNA. Three identical solutions containing 0.48 M PB and single-stranded *E. coli* DNA were prepared. One tube was incubated for 14 h at 60 °C to a C_0t at which the DNA was completely reassociated. Another tube was incubated at room temperature for 14 h. Phenol was added to the third tube and the mixture was shaken for 5 h at room temperature. Table I provides the experimental details and summarizes the results of this experiment. The single-stranded DNA incubated at room temperature in the absence of phenol did not reassociate. Its thermal denaturation profile was broad and the extent of hyperchromicity low (3–4%). The single-stranded DNA shaken with phenol and that incubated at 60 °C showed both the sharp melting profile and high hyperchromicity characteristic of extensively double-stranded DNA. The hyperchromicity of the PERT DNA (24.2%) was very close to that of native DNA. The hyperchromicity of the DNA incubated at 60 °C was lower (21.7%) but typical of aqueous reassociated *E. coli* DNA (Kohne and Britten, 1971). Essentially complete reassociation of the emulsion reacted DNA occurred since the extent of hyperchromicity of the sonicated, emulsion reassociated DNA (24.2%) is close, if not identical, to that of unsonicated native *E. coli* DNA (24.7%). These data demonstrate that the emulsion system allows the formation of helical double-stranded DNA molecules from single-stranded molecules and furthermore does so at room temperature (about 22 °C) where DNA is normally incapable of reassociating in 0.48 M PB.

E. coli DNA Reassociation Kinetics: PERT vs. Standard Aqueous Method. The rapidity of *E. coli* DNA reassociation with the PERT is illustrated in Figure 1. The reassociation of ^{125}I-labeled *E. coli* DNA was measured by the PERT and the standard aqueous technique. The emulsion reaction mixture consisted of *E. coli* DNA (0.5 μg/mL) in 0.48 M PB and 9% phenol. The aqueous reaction consisted of 0.48 M PB and *E. coli* DNA (400 mg/mL). The emulsion was shaken on a

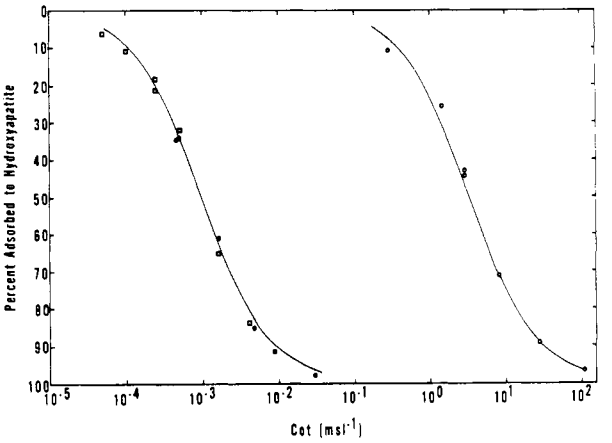

FIGURE 1: Reassociation kinetics of ^{125}I-labeled *E. coli* DNA done with the standard aqueous system (right-hand curve) and the PERT (left-hand curve). The aqueous system *E. coli* DNA (400 μg/mL) was incubated in 0.48 M PB at 68 °C (○). Two separate PERT mixes were used. Each contained 0.5 μg/mL of *E. coli* DNA, 0.9 mL of 0.48 M PB, and 0.1 mL of 90% phenol. PERT reaction mixes were continuously shaken by a Vortex mixer at room temperature (22–24 °C). Samples from separate PERT reaction vials are designated as □ and ●. Aliquots (0.05 mL) were taken from the incubation vials, diluted directly into 3 mL of 0.14 M PB, 0.2% $NaDodSO_4$, and passed over hydroxylapatite equilibrated to 60 °C, 0.14 M PB, 0.2% $NaDodSO_4$. After heating the ^{125}I-labeled DNA to render it single-stranded and then passing it immediately over hydroxylapatite as above, 4% of the radioactivity adsorbs to the hydroxylapatite. At the end of the reassociation reaction, 95% of the radioactivity adsorbed to hydroxylapatite. Each raw data point was first corrected for the amount of single-strand binding to hydroxylapatite (−4%), and the resulting number was normalized to 91% maximum reaction. The normalized points were then plotted. The aqueous system kinetic curve has been adjusted to the reference aqueous system condition of 0.12 M PB, 60 °C. The solid line curves shown are ideal second-order reaction curves.

Vortex mixer at room temperature while the aqueous reaction solution was incubated at 70 °C. The extent of reassociation was the same (see the legend of Figure 1) for both the PERT and aqueous reactions. The kinetics of the reassociation were determined as described in the legend. The form of the PERT kinetic curve is very similar to that of the aqueous reaction curve and closely approximates the shape of a second-order reaction. The $C_0t_{1/2}$ of reassociation for the *E. coli* DNA in 60 °C, 0.12 M PB, the reference incubation condition, was 3.5 mole seconds per liter (mol s L^{-1}). The PERT reassociated DNA had a $C_0t_{1/2}$ of reassociation of 9×10^{-4} mol s L^{-1}. Thus at 0.5 μg/mL in 0.48 M PB, 9% phenol, *E. coli* DNA reassociates about 4000 times faster than the same concentration of *E. coli* DNA reacted in the standard reference condition of 0.12 M PB, 60 °C. The open squares and closed circles represent different PERT reactions run on different days and help demonstrate that reproducible reassociation kinetic profiles can be obtained with this technique. Six different PERT reassociation kinetic reactions were done under the same conditions described in Figure 1 (0.5 μg/mL *E. coli* DNA, 0.48 M PB, 9% phenol, shaken at room temperature on a Vortex mixer) using just one preparation of sonicated ^{125}I-labeled *E. coli* DNA. The observed $C_0t_{1/2}$'s for the different reactions were 1×10^{-3}, 9×10^{-4}, 7.6×10^{-4}, 1×10^{-3}, 9×10^{-4}, 8×10^{-4} mol s L^{-1}.

Factors Affecting the Rate of DNA Reassociation with PERT. The rate of DNA reassociation observed with the PERT is at least dependent on: (a) the presence of an emulsion; (b) the violence of agitation of the emulsion; (c) an appropriate temperature of incubation; (d) the concentration of DNA in the reaction mixture; (e) the amount of phenol present; (f) the proper pH; (g) the type and concentration of ion; (h) the

TABLE II: Reassociation Rate vs. Degree of Emulsion.[a]

	% phenol	Calcd *E. coli* $C_0t_{1/2}$ (mol s L^{-1})	A_{500}
(A) 0.48 M PB	0		0.000
	0.9		0.002
	1.8		0.004
	2.6		0.006
	3.0		0.008
	3.5		0.009
	3.9	>1	0.012
	4.3		0.036
	4.7	0.07	
		0.1	0.075
	5.1	1×10^{-4}	
		10×10^{-4}	0.3-0.8
	5.6	0.8×10^{-4}	
		1.7×10^{-4}	
	6.3	3.2×10^{-4}	
		3.5×10^{-4}	
	6.9	7×10^{-4}	
		6.3×10^{-4}	
	9	1.6×10^{-3}	
		1.5×10^{-3}	
	15	1.8×10^{-3}	
		1.7×10^{-3}	
	20-21	1.5×10^{-3}	
		1.9×10^{-3}	
(B) 1.25 M $NaClO_4$	0		0.000
	0.9		0.003
	1.8		0.005
	2.6		0.007
	3.5		0.009
	4.3		0.025
	4.7		0.041
	5.1		0.150
	5.5		~1.0
	5.6	9.1×10^{-5}	
	6.9	2.4×10^{-4}	
	9	3.8×10^{-4}	
	15	1.13×10^{-3}	

[a] Each reaction mix consisted of 1 mL of (A) 0.48 M PB or (B) 1 mL of 1.25 M $NaClO_4$, 0.1 M PB, and an appropriate amount of phenol. ^{3}H-labeled *E. coli* DNA was present at a concentration of 0.5 μg/mL of aqueous solution. For kinetic assays all samples were agitated with a Vortex mixer at room temperature. The formation of the emulsion was monitored by mixing an appropriate amount of phenol with 1 mL of aqueous solution, shaking the mixture vigorously on a Vortex mixer and then quickly measuring the A_{500} of the mixture in a Gilford spectrophotometer.

fragment size of the DNA; (i) the complexity of the DNA. These different factors are discussed in detail in the following text.

Presence of an Emulsion and Rapid Reassociation. An emulsion is apparently necessary to obtain the rapid reassociation of DNA in this new system. Table II summarizes data which suggest a correlation between the appearance of an emulsion (as measured by light scattering) and the rapid reassociation of *E. coli* DNA. Phenol is soluble to about 4-4.5% (vol/vol) in 0.48 M PB. *E. coli* DNA begins reassociating rapidly in the phenol:aqueous system at between 4 and 5% phenol.

This suggests that the emulsion is necessary for rapid reassociation. At phenol concentrations where the emulsion is just forming, the rate of reassociation is not reproducible. At 5.1% phenol, for example, duplicate reaction mixtures gave $C_0t_{1/2}$ values ranging from 1.5 to 10×10^{-4} mol s L^{-1}. At higher phenol concentrations duplicate reaction mixtures gave much more reproducible $C_0t_{1/2}$ values although the *E. coli* DNA reassociates more slowly than at lower phenol concentrations. Practically, the optimum concentration of phenol to use in the PERT is the lowest concentration of phenol which will give rapid reassociation and reproducible results.

Agitation of the Emulsion and the Rate of Reaction. Agitation of the emulsion is necessary to promote reassociation with the PERT. Different types of agitation, such as a back and forth shaking, a swirling motion and placing the tube in a sonicator, are effective. The violence of the agitation needed is dependent on the concentration of DNA in the emulsion mixture. A 1.25 M $NaClO_4$, 7% phenol mix containing 0.5 μg/mL of radioactive *E. coli* DNA was briefly hand shaken for 1-2 s and then agitated no more. This emulsion is stable for quite some time without further agitation. An identical mix was agitated continually with a Vortex mixer. The Vortex mixer shakes at a rate of about 2000 oscillations per min. A third identical mix was continuously agitated by hand shaking at a rate of about 50 shakes per min. The $C_0t_{1/2}$ of reassociation of the *E. coli* DNA in each sample was determined. The two samples which were continuously agitated had essentially the same $C_0t_{1/2}$ of reassociation (8×10^{-5} mol s L^{-1}), while the sample which was not continuously agitated was about 6 times slower ($C_0t_{1/2} = 5 \times 10^{-4}$ mol s L^{-1}). Three different types of mechanical shakers, the Vortex Genie (Scientific Products, Evanston, Ill.), the Wig L Bug (Van Waters and Rogers, Los Angeles) (a shaker for mixing adjuvant with antigen), and a Buchler sucrose gradient mixing device gave essentially the same $C_0t_{1/2}$ for reassociation of low concentrations of *E. coli* DNA. At higher DNA concentrations different shaking methods give different rates of reassociation. Table VIII shows that in the PERT reaction of *E. coli* DNA at a concentration of 440 μg/mL, the rate depends on the manner of shaking the reaction mix. The more violent agitation of the Wig L Bug shaker increased the rate by a factor of 7. Extremely violent shaking methods have not as yet been tried for high DNA concentrations. It is possible that more violent shaking will increase the high DNA concentration emulsion reassociation rate.

Conditions of shaking will be a major variable in intercomparing PERT reassociation kinetics done in different laboratories where different methods of agitation are used. Most of the problems with the manner of agitation occur at higher DNA concentrations. The volume of the reaction mixture can also have an influence on the emulsion reassociation rate when using higher DNA concentrations. It is conceivable that the shape of the reaction vial may also have an influence on the emulsion rate. All of the reactions reported here were done in cylindrical 2.5 dram screwcapped vials with Teflon-lined caps.

PERT Reassociation Rate vs. pH and Temperature. The emulsion system supports rapid reassociation over a pH range of 5-9.2 (Table IIIA). The rate changes by a factor of about 2 over this range. Reassociation does not occur at pH 3.9. Double-stranded DNA added to the pH 3.9 mixture (Table IIIA) dissociates into single-stranded DNA.

The PERT promotes rapid reassociation of *E. coli* DNA over a temperature range of 6 to 68 °C. Table IIIB shows the effect of temperature on the $C_0t_{1/2}$ of reassociation of *E. coli* DNA over a range of 6 to 56 °C. Rates at temperatures below 6 °C could not be measured since the phenol solidified at a temperature slightly lower than 6 °C. The rate of reassociation

TABLE III.[a]

A. Effect of pH		B. Effect of temperature	
pH	$C_0t_{1/2}$ mol s L^{-1}	T (°C)	$C_0t_{1/2}$ (mol s L^{-1})
3.9		6	18×10^{-3}
5.0	1×10^{-3}	15	4.4×10^{-3}
6.8	1×10^{-3}	22	1.1×10^{-3}
9.2	5.5×10^{-4}	36	0.73×10^{-3}
		46	0.36×10^{-3}
		56	0.29×10^{-3}

[a] (A) Reaction mixes consisted of 0.9 mL of aqueous phase containing 0.5 μg of ^{125}I-labeled *E. coli* DNA and 0.1 mL of 90% phenol. The pH 9.2 mixture contained 0.96 M Na^+ and 0.48 M PO_4^{2-}; the pH 6.8 mixture 0.72 M Na^+ and 0.48 M PO_4^{2-}; the pH 5.0 mix 0.96 M Na^+ and 0.48 M PO_4^{2-}; the pH 3.9 mix 0.48 M Na^+ and 0.48 M PO_4^{2-}. All mixtures were agitated with a Vortex mixer at room temperature. The reactions were assayed as described in Materials and Methods. $C_0t_{1/2}$ determinations were done by method II described in Materials and Methods. (B) Reaction mixes consisted of 0.9 mL of 0.48 M PB containing 0.5 μg per mL of ^{125}I-labeled *E. coli* DNA plus 0.1 mL of 90% phenol. Each vial was placed in a water bath adjusted to the appropriate temperature and agitated with a Buchler sucrose gradient mixing device. The reactions were assayed as described in Materials and Methods. The *E. coli* DNA reacted to greater than 90% at all temperatures. $C_0t_{1/2}$ determinations were done by method II as described in Materials and Methods.

at 56 °C is about 3-4 times faster than at room temperature, while the rate at 6 °C is about 16 times slower than at room temperature. *E. coli* DNA reacted in 0.48 M PB, 9% phenol, reacts rapidly at 68 °C, but only about 10% of the DNA reassociates. The $C_0t_{1/2}$ of the reacting fraction was about 10^{-3} mol s L^{-1}. In the same PERT mixture about 50% of the DNA reassociates rapidly at 62 °C ($C_0t_{1/2}$ for the reacting fraction was about 5×10^{-4} mol s L^{-1}), and longer incubation results in little more reassociation. Figure 3a indicates that the T_m of *E. coli* DNA in 0.48 M PB, 9% phenol is about 70 °C. These data (Figure 3a and Table IIIB) show that the incubation temperature can be very close to the T_m of the DNA and rapid reassociation can still occur.

PERT Reassociation Rate vs. Concentration and Type of Anion and Cation. Table IV shows the effect of different sodium phosphate concentrations on the rate of PERT reassociation. The optimal rate is seen around 0.48 M PB. The reaction does not go to completion in either 0.14 M PB or 1.96 M PB. The rate changes by about twofold over the useful PB concentration range of 0.24 M to 0.48 M PB. Sodium sulfate, sodium chloride, sodium perchlorate, and sodium thiocyanate also promote rapid reassociation in the PERT. The rate of PERT *E. coli* DNA reassociation is close to optimum in 0.75 M sodium sulfate, 1 M sodium chloride, 1.25 M sodium perchlorate, and 2 M sodium thiocyanate. Table VA presents data comparing the effect of different anions on the $C_0t_{1/2}$ of *E. coli* DNA reassociated with the PERT. Thiocyanate promotes the most rapid reassociation in the phenol emulsion system, while sulfate is least effective. The order of anion effectiveness in promoting rapid reassociation in the emulsion system is $SCN > ClO_4 > Cl > PO_4 > SO_4$. The more "chaotropic" the ion the faster the *E. coli* DNA reassociation. Chaotropic agents act as hydrophobic bond breakers (Hamaguchi and Geiduschek, 1962). SCN^- is a much more effective chaotropic ion than Cl^-. The optimum salt concentrations were not determined for each different cation and the rates were compared at a 1 M concentration of each salt. The fastest rate was seen for lithium. Interestingly, cesium did not support DNA:DNA reassociation in the phenol emulsion system even though

TABLE IV: Effect of PB Concentrations.[a]

[PB] (M)	$C_0t_{1/2}$ (mol s L^{-1})
0.24	1.8×10^{-3}
0.48	9×10^{-4}
0.96	1.4×10^{-3}

[a] Reaction mixes consisted of 0.9 mL of aqueous phase containing 0.5 μg/mL of ^{125}I-labeled *E. coli* DNA and 0.1 mL of 90% phenol. The mixtures were shaken in a Vortex mixer at room temperature. Aliquots were assayed as described in Materials and Methods. $C_0t_{1/2}$ values were calculated as described in method II of Materials and Methods.

TABLE V: Ion Effects on the PERT Reassociation Rate.[a]

Salt	$C_0t_{1/2}$ (mol s L^{-1})
(A) Type of anion	
0.75 M Na_2SO_4	$3 \pm 1 \times 10^{-3}$
0.48 M PB	$1.4 \pm 0.18 \times 10^{-3}$
1 M NaCl	$7.8 \pm 1.5 \times 10^{-4}$
1 M NaAc	$3.6 \pm 0.4 \times 10^{-4}$
1 M NaI	$3.2 \pm 0.5 \times 10^{-4}$
1.25 M $NaClO_4$	$3.8 \pm 0.3 \times 10^{-4}$
1 M NaCHO	$2.7 \pm 0.3 \times 10^{-4}$
2 M NaSCN	$2.9 \pm 0.4 \times 10^{-4}$
1.5 M NaF_3Ac	$1.8 \pm 0.6 \times 10^{-4}$
(B) Type of cation	
1 M CsCl	No reassociation detected
1 M RbCl	$1.1 \pm 0.2 \times 10^{-3}$
1 M KCl	$9.4 \pm 1.1 \times 10^{-4}$
1 M NaCl	$7.8 \pm 1.5 \times 10^{-4}$
1 M NH_4Cl	$5.6 \pm 0.6 \times 10^{-4}$
1 M LiCl	$5.6 \pm 0.5 \times 10^{-4}$
(C) Different lithium salts	
1 M LiCl	$5.6 \pm 0.5 \times 10^{-4}$
1 M LiAc	$5.2 \pm 0.6 \times 10^{-4}$
1.25 M $LiClO_4$	$1.9 \pm 0.5 \times 10^{-4}$
2 M LiSCN	$2.2 \pm 0.1 \times 10^{-4}$

[a] Reaction mixes consisted of 0.9 mL of aqueous phase containing 0.5 μg/mL of ^{3}H-labeled *E. coli* DNA and 0.1 mL of 90% phenol. The mixtures were shaken at room temperature (22-24 °C) on a Vortex mixer. The salt concentrations used in A are close to optimum for that salt. In B and C the salt concentrations were not known to be the optimum concentrations. Reactions were assayed as described in Materials and Methods. $C_0t_{1/2}$ values were calculated according to method II presented in Materials and Methods.

reassociation of *E. coli* DNA does occur in CsCl under standard high temperature aqueous conditions. Again the rate in the emulsion system increases with the chaotropicity of the ion. Table VC presents the effect of different anions using lithium as the constant cation. The data of Table V suggest that in general the more chaotropic the ions involved, the faster the rate of reassociation in the phenol system. Trichloroacetate is a much more effective chaotrope than is thiocyanate. Unfortunately, phenol is essentially completely soluble in neutralized trichloroacetic acid solutions and an emulsion will not form. Other chaotropic compounds will be examined in an effort to further increase the rate of reassociation.

The Effect of DNA Fragment Size on Rate. In the standard aqueous reassociation system the rate of reassociation increases by the square root of an increase in the fragment size of DNA utilized in the reaction (Wetmur and Davidson, 1968). This is not the case for the phenol emulsion system. Varying the

TABLE VI: Effect of Piece Size on Reassociation Rate.[a]

Treatment of *E. coli* (DNA)	60 °C 0.12 M PB $C_0t_{1/2}$ (mol s L^{-1})	PERT $C_0t_{1/2}$ (mol s L^{-1})	Relative size of DNA pieces
(A) Unsonicated	1.18	1.1×10^{-3}	12.7
Moderate sonication	2.24	1.3×10^{-3}	3.5
Vigorous sonication	4.2	1.2×10^{-3}	1
(B) Unsonicated	0.9	1.6×10^{-3}	30.9
Vigorous sonication	5	0.7×10^{-3}	1

[a] Two different preparations of *E. coli* DNA (A and B) were examined. Aqueous reassociation mixtures were incubated at 68 °C in 0.48 M PB. The aqueous $C_0t_{1/2}$ s have been adjusted to 0.12 M PB, 60 °C. PERT mixtures consisted of 0.9 mL of 0.48 M PB and 0.1 mL of 90% phenol with DNA present at 0.5 and 4 μg per mL in A and B, respectively. The emulsion mixtures were shaken at room temperature on a Vortex mixer. Reassociation kinetics were measured as described in Materials and Methods. Each $C_0t_{1/2}$ value quoted above was determined by method II as described in Materials and Methods.

TABLE VII: Reassociation of Varying G + C DNAs with the PERT.[a]

	0.12 M PB, 60 °C		
DNA	G + C	Aqueous $C_0t_{1/2}$ (mol s L^{-1})	PERT $C_0t_{1/2}$ (mol s L^{-1})
Staph. aureus	32	1.7	$4.5 \pm 0.6 \times 10^{-4}$
B. subtilis	42	2.5	$2.3 \pm 0.4 \times 10^{-4}$
E. coli	50	3.5	$2.9 \pm 0.4 \times 10^{-4}$
Ps. aeruginosa	67	3.9	$3.3 \pm 0.9 \times 10^{-4}$

[a] All DNAs were reacted at a concentration of 0.5 μg/mL in a mixture consisting of 0.9 mL of 2 M NaSCN, plus 0.1 mL of 90% phenol. The samples were shaken at room temperature on a Vortex mixer. Reassociation was assayed as described in Materials and Methods. $C_0t_{1/2}$ values were calculated as described in method II of Materials and Methods.

TABLE VIII: Effect of DNA Concentration on PERT Reassociation Rate.[a]

E. coli DNA concn (μg/mL)	$C_0t_{1/2}$ (mol s L^{-1})
(A) 2 M NaSCN 12.9% phenol	
0.04	$1.5 \pm 0.3 \times 10^{-4}$
0.4	$3.7 \pm 0.4 \times 10^{-4}$
4.4	$7.9 \pm 0.7 \times 10^{-4}$
44	$1.6 \pm 0.2 \times 10^{-3}$
176	$3.8 \pm 0.2 \times 10^{-3}$
352	$1.3 \pm 0.4 \times 10^{-2}$
704	$2.7 \pm 1.5 \times 10^{-2}$
1600	$1.0 \pm 0.6 \times 10^{-1}$

E. coli DNA concn (μg/mL)	% phenol	Method of shaking	$C_0t_{1/2}$ (mol s L^{-1})
(B) 0.48 M PB			
0.4	9	Vortex	9×10^{-4}
4	9	Vortex	1.6×10^{-3}
44	9	Vortex	2.7×10^{-2}
440	9	Vortex	3
440	75	Vortex	0.74
440	9	Wig L Bug	1
440	75	Wig L Bug	0.1

Human DNA concn (μg/mL)	$C_0t_{1/2}$ nonrepeat DNA (mol s L^{-1})	Half-time of reaction of nonrepeat DNA (h)	Rate of increase relative to 0.12 M PB, 60 °C condition
(C) 2 M NaSCN 12.9% phenol			
40	0.97 ± 0.08	1.9	1445
200	2.8 ± 0.3	1.1	500
600	10.8 ± 1.9	1.4	130
1600	40.1 ± 10.9	2	35
3200	90.5 ± 20	2.3	15.5
6400	161 ± 26	1.85	8.7

[a] (A) ^{3}H-labeled *E. coli* DNA was reacted at the concentration shown in a mixture consisting of 0.9 mL of 2 M NaSCN, 0.01 Tris, pH 8.3, and 0.15 mL of 90% phenol. The *E. coli* DNA used for these had a 60 °C, 0.12 M PB, $C_0t_{1/2}$ = 3.5 ms L^{-1}. (B) Reaction mixes consisted of 0.9 mL of 0.48 M PB containing ^{125}I-labeled *E. coli* DNA plus the appropriate amount of 90% phenol. (C) Reaction mixes consisted of 0.9 mL of $NaSCN_3$, 0.01 M Tris, pH 8.3, plus 0.15 mL of 90% phenol and varying concentrations of ^{3}H-labeled human DNA. The human DNA exhibited a 0.12 M PB, 60 °C, $C_0t_{1/2}$ = 1402 ms L^{-1}. Reassociation was assayed as described in Materials and Methods. Samples were shaken at room temperature on a Vortex mixer. $C_0t_{1/2}$ values were calculated by method II as described in Materials and Methods.

DNA fragment size over a 30-fold range has little effect on the reassociation rate when using the PERT (Table VI). The largest fragment size effect seen thus far is summarized in Table VIB. In the aqueous system unsonicated *E. coli* DNA reassociated 5.6 times faster than did the sonicated *E. coli* DNA. This indicates that the unsonicated DNA was 30.9 (i.e. $(5.6)^2$) times larger than the unsonicated DNA (Wetmur and Davidson, 1968). These same DNAs showed only a 2-fold difference in rate when reassociated in the emulsion system. The large DNA was sized by alkaline sucrose sedimentation after the phenol emulsion reaction. Very little degradation had occurred during the course of the reaction. Thus the effect of fragment size on reassociation rate is small when using the PERT.

G + C Content and PERT Reassociation. The phenol emulsion technique promotes the reassociation of DNAs with widely varying G + C content. Bacterial DNAs ranging from 32 to 67% G + C react rapidly and completely in the phenol emulsion (Table VII).

Several different mycoplasmal DNAs, *Mycoplasma hyorhinis* and *Acholeplasma laidlawii* (about 30% G + C), and vaccinia and cowpox viral DNAs (about 35% G + C) and herpes simplex viral DNA (about 70% G + C) also reacted well in 0.48 M PB, 7% phenol. Rat, mouse, and human DNAs (about 42% G + C) have reassociated well in the PB, $NaClO_4$, and NaSCN, phenol emulsion mixes.

Effect of DNA Concentration on the PERT Reassociation Rate. In the standard aqueous method for reassociating DNA the half-time of reassociation is proportional to the concentration of DNA used in the reaction mixture. A tenfold increase in DNA concentration results in a tenfold decrease in the time needed to attain one half reassociation. In the aqueous system the

$$C_0t_{1/2} = \left(\frac{1}{\text{rate constant } k}\right)$$

for any specific DNA is the same regardless of the concentration of DNA used for the aqueous reaction. This is not true for the phenol emulsion system. With the PERT the $C_0t_{1/2}$ for any particular DNA increases with an increase in DNA concentration. Table VIIIA illustrates this and shows the effect of DNA concentration on the rate of reassociation of *E. coli* DNA in 2 M NaSCN with the emulsion system. When reacted

TABLE IX: PERT Reassociation of a Small Quantity of ^{125}I-Labeled Human DNA.[a]

PERT reaction of the equivalent of one cell's DNA			68 °C, 0.48 M PB aqueous reaction of the equivalent of one cell's DNA		
Hours of reaction	C_0t	% adsorbed to hydroxylapatite	Hours of reaction	C_0t	% adsorbed to Hydroxylapatite
0		6.5			
1	7.5×10^{-7}	16.4	23	1.7×10^{-5}	6.4
4.1	3.1×10^{-6}	20.7	50	3.8×10^{-5}	5.4
24	1.8×10^{-5}	26.3	73	5.5×10^{-5}	6.0
70	5.3×10^{-5}	38			

[a] The reaction mix consisted of 0.1 mL of 1.25 M $NaClO_4$ containing 0.27 μg of *E. coli* nonradioactive DNA and 6×10^{-6} μg of unfractionated human ^{125}I-labeled DNA, plus 0.0085 mL of 90% phenol. This was placed in a 1-mL vial and shaken at room temperature with a Vortex shaker. Multiple vials were set up in order to obtain kinetic points. The ^{125}I-labeled DNA had a specific activity of 6.1×10^7 cpm/μg and, when mixed with unlabeled human DNA, reassociated to 87%. A control consisted of the same human ^{125}I-labeled DNA (at 6×10^{-5} μg/mL) incubated at 68 °C, 0.48 M PB. Samples were assayed as described in Materials and Methods. The ^{125}I-labeled DNA reassociated to 87% extent when reacted with the PERT at 3 μg/mL in 1.25 M $NaClO_4$, 0.1 M PB, 7% phenol.

in the phenol system, the *E. coli* DNA $C_0t_{1/2}$ changes from 1.5×10^{-4} mol s L^{-1} at low DNA concentration to 0.1 mol s L^{-1} at very high DNA concentration, an increase of about 10^3. Table VIIIB shows a similar study done with 0.48 M PB, 9% phenol. Again the $C_0t_{1/2}$ increases at higher DNA concentrations. As is seen in Table VIIIB, the $C_0t_{1/2}$ at high DNA concentration (440 μg/mL) can be decreased by adding more phenol or by shaking the emulsion more violently. The Wig L Bug shaker agitated the emulsion more vigorously than the Vortex. Increasing the phenol concentration and the violence of shaking decreased the high DNA concentration $C_0t_{1/2}$ by about 30-fold. Table VIIIC presents the effect of DNA concentration on the $C_0t_{1/2}$ of human DNA. Again high DNA concentration resulted in increased $C_0t_{1/2}$.

About the same *E. coli* DNA $C_0t_{1/2}$ is obtained when a small amount of radioactive *E. coli* DNA is mixed with 400 μg/mL of unlabeled single-stranded *E. coli* or a comparable amount of unlabeled single-stranded cow DNA. This and other similar experiments indicate that the rate inhibition effect at high DNA concentration in the phenol system is due to some general property of single-stranded DNA and not to some property of the specific homologous DNA. Added single-stranded RNA (ribosomal RNA, poly(A), poly(U), tRNA) and native double-stranded DNA are much less effective than single-stranded DNA in increasing the $C_0t_{1/2}$ of radioactive *E. coli* DNA. Indeed, under certain circumstances the addition of RNA or nonhomologous double-stranded DNA to the emulsion actually decreases the $C_0t_{1/2}$ of radioactive *E. coli* DNA (relative to a control which lacks RNA or double-stranded DNA). Expansion of studies of this type may provide clues to the mechanism of rate increase in the phenol system.

Reassociation of Mammalian DNA with the PERT. Rat, mouse, and human DNAs have been successfully reassociated using the PERT. Reassociation kinetic profiles of human DNA obtained with the emulsion system are shown in Figure 2. About 40% of the DNA has reassociated by a $C_0t_{1/2}$ of 10^{-3} mol s L^{-1} (Figure 2). This fraction represents the rapidly reassociating repeated DNA sequences which are present in all mammals (Britten and Kohne, 1968). This 40% of the DNA has a thermal melting profile characteristic of repeated DNA. Little reassociation occurs between 5×10^{-4} to 10^{-2} mol s L^{-1}. This plateau region is characteristic of the reassociation kinetic profiles of many mammalian DNAs. The nonrepeated DNA starts reassociating around $C_0t = 10^{-2}$ mol s L^{-1} and the drawn curves represent the reassociation of this DNA. The lines drawn through the data points represent ideal second-order reactions and show that at this DNA concentration these emulsion reactions are approximately second order in nature. In the $NaClO_4$ mixture the nonrepeated DNA $C_0t_{1/2}$ is 0.09 mol s L^{-1}, while in the PB mixture the nonrepeated DNA $C_0t_{1/2}$ is 0.2 mol s L^{-1}. This illustrates that mammalian DNA also reassociates faster in the presence of the more chaotropic salt, $NaClO_4$. With the $NaClO_4$ the one half-time of nonrepeated DNA reassociation is about 2 h. At the same concentration of human DNA (4 μg/mL) reacted under the standard reference conditions (0.12 M PB, 60 °C) the nonrepeated DNA would require about 50 000 h to reach one half-reassociation. Thus at this DNA concentration the human nonrepeated DNA reacts 25 000 times faster in the phenol emulsion than in the standard aqueous system. Cat, rat, and mouse nonrepeated DNAs reassociate at a similar rate in the emulsion method and show the typical biphasic reassociation kinetic curve characteristic of mammalian DNA (data not shown). Table IX presents the reassociation kinetics of human repeated DNA.

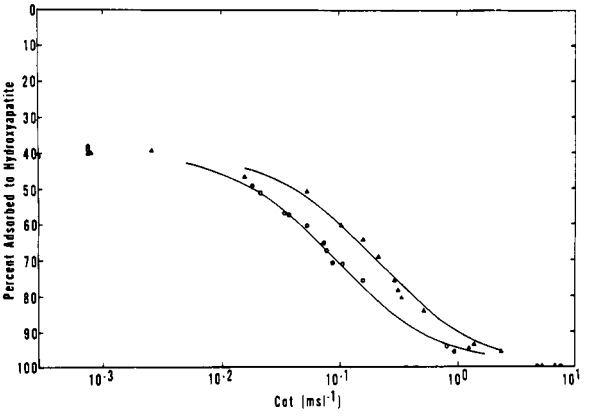

FIGURE 2: PERT room temperature reassociation kinetics of human ^{3}H-labeled DNA (A_{260} = 0.105) mixed with 1.25 M $NaClO_4$, 0.1 M PB, 7% phenol (●; ○); and 0.48 M PB, 7% phenol (▲; △). Each PERT condition was done in duplicate. The duplicates were run on different days. The reaction vials were shaken continuously by a Vortex mixer. Aliquots were removed at various times, diluted into 0.14 M PB, 0.2% $NaDodSO_4$, and assayed at 56 °C on hydroxylapatite. Upon heating the ^{3}H-labeled DNA to 100 °C in 0.14 M PB, 0.2% $NaDodSO_4$, to render it single stranded, and passing it over hydroxylapatite equilibrated to 56 °C, about 4% of the radioactivity adsorbed to the column. At the end of the reassociation reaction, about 93% of the radioactivity adsorbs to hydroxylapatite. Each data point has been normalized to 93% maximum reaction. The solid lines shown are ideal second-order reaction curves. The 60 °C, 0.12 M PB, $C_0t_{1/2}$ of the ^{3}H-labeled human nonrepeated DNA was 2400 mol s L^{-1}.

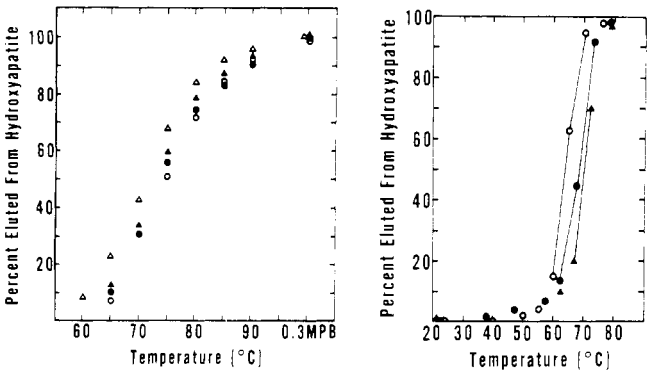

FIGURE 3: (Left, a) The thermal elution profiles of repeated human [^{3}H]DNA fractions obtained by PERT and standard aqueous incubations. Human [^{3}H]DNA was reacted to a C_0t of 19.0 mol s L^{-1} in 0.48 M PB, 68 °C (○); 3×10^{-4} mol s L^{-1} in 1.25 M $NaClO_4$, 0.1 M PB, 7% phenol at room temperature (●); 9×10^{-4} mol s L^{-1} in 0.48 M PB, 7% phenol at room temperature (▲). Aliquots of these reaction mixtures were diluted into 0.14 M PB, 0.2% $NaDodSO_4$, and passed over hydroxylapatite equilibrated to 56 °C, 0.14 M PB, 0.2% $NaDodSO_4$. A separate sample of [^{3}H]DNA was incubated in 0.48 M PB, 7% phenol, to a C_0t of 9×10^{-4} mol s L^{-1}. This reaction solution was diluted into 0.14 M PB, 0.2% $NaDodSO_4$, and passed over hydroxylapatite equilibrated to 51 °C, 0.14 M PB, 0.2% $NaDodSO_4$ (△). A thermal elution profile was then obtained for the [^{3}H]DNA which adsorbed to the hydroxylapatite. After the last temperature rise, the column was washed with 0.3 M PB to recover any DNA still adsorbed to the hydroxylapatite. (Right, b) The thermal stability of ^{125}I-labeled *E. coli* DNA in the presence of phenol. Double-stranded [^{125}I]DNA was added, at room temperature, to: 1.25 M $NaClO_4$, 0.1 M PB, 7% phenol (●); 1.25 M $NaClO_4$, 0.1 M PB, 9% phenol (○); 0.38 M Pb, 9% phenol (▲). The mixtures were incubated at increasing temperature. At each temperature an aliquot was removed and quickly diluted into 0.14 M PB, 0.2% $NaDodSO_4$. Each sample was then passed over hydroxylapatite at 60 °C in order to determine the amount of double-stranded DNA. No reassociation occurred during the dilution.

Human [^{125}I]DNA at a concentration of 0.5 μg/mL also reassociates well in the phenol emulsion. At this concentration, in a mixture of 1.25 M $NaClO_4$, 0.1 M PB, 7% phenol, the nonrepeated [^{125}I]DNA, $C_0t_{1/2}$ = 0.08 mol s L^{-1}, which corresponds to a half-time of reaction of about 16 h (data not shown). DNA is quite stable in the phenol emulsion and can be shaken for several hundred hours. Therefore it should be possible to attain at least one half reassociation of the nonrepeated mammalian DNA at concentrations as low as 0.05 μg/mL.

The $C_0t_{1/2}$ of nonrepeated human DNA increases with increasing DNA concentration (Table VIIIC). Over a concentration range of 40–6400 μg/mL the $C_0t_{1/2}$ increases from 0.97 mol s L^{-1} to 161 mol s L^{-1}. The half-time of reaction for all of these different concentrations was around 1–3 h. At 40 μg/mL the rate increase relative to the reference aqueous system is about 1400-fold, while at 6400 μg/mL the relative rate increase is about 9.7-fold. The data in Table VIIIB suggest that more violent shaking of the emulsion will increase the rate at higher concentrations. The conditions for the fastest possible rate increase for each DNA concentration have not been determined.

As discussed above, large rate increases have not been observed in the PERT when the DNA is reassociated at high DNA concentration. All of the human DNA concentrations checked thus far have had a half-time of reassociation of about 2 h (Table VIIIC). At high DNA concentrations, however, the observed order of the reassociation reaction no longer approximates second order. At 6400 μg/mL, for example, the apparent order of the PERT reassociation reaction is between first and second order. A practical benefit of this order of reaction, relative to second order, is a shorter time needed for the complete reaction of the DNA. All of the high DNA concentration (from 4 to 6400 μg/mL) PERT reactions are essentially complete in 20–24 h under PERT conditions.

Criterion of the Emulsion System. The "criterion" of a nucleic acid reassociation mixture determines the degree of complementarity which two single-stranded nucleic acid molecules must have in order to interact and form a stable double-stranded molecule. The criterion of the typical aqueous reassociation reaction is primarily a function of the temperature and cation concentration. In the phenol emulsion system the temperature, salt, and phenol all combine to determine the criterion. Figure 3a summarizes experiments which compare the criteria of two different PERT reaction mixes with the aqueous condition of 0.14 M PB, 56 °C. Human [^{3}H]DNA was reacted as described in the legend to Figure 3; (a) 0.48 M PB, 67 °C; (b) 1.25 M $NaClO_2$, 0.1 M PB, 7% phenol; and (c) 0.48 M PB, 7% phenol. Each of these reactions was incubated long enough to allow the repeated DNA to reassociate and the repeated DNA was then isolated via hydroxylapatite fractionation at 56 °C. The percentage of human [^{3}H]DNA reacting rapidly as repeated DNA was 41% in the standard aqueous system and 38% and 37%, respectively, in the $NaClO_4$ and PB mixes. A hydroxylapatite thermal elution profile was then obtained for each sample (Kohne and Britten, 1971). The thermal elution profiles of the three samples are quite similar (Figure 3a). A separate sample incubated in 0.48 M PB, 7% phenol for a time sufficient for the repeated DNA to react was fractionated on hydroxylapatite at 51 °C. Thirty-four percent of this DNA was recovered as repeated DNA, and its thermal stability profile was very similar to the other repeated DNA fractions (Figure 3a). These data indicate that the criteria of the room temperature 0.48 M PB, and 1.25 M $NaClO_4$, phenol mixes are comparable to that of 56 °C, 0.14 M PB in the standard aqueous system.

Figure 3b shows the effect of phenol on the melting temperature of *E. coli* DNA. As has been reported by other investigators, the presence of the phenol lowers the T_m of the DNA considerably (Leng et al., 1974). *E. coli* DNA in 0.48 M PB in the absence of phenol would have a T_m of around 97 °C. In 1.25 M $NaClO_4$ the T_m would be about 90 °C. Thus the presence of the phenol lowers the T_m by about 25–30 °C in both cases. In the emulsion system run at room temperature, the reassociation reaction is occurring roughly 45–50 °C below the T_m of the DNA in that system.

Complexity and PERT Reassociation Rate. In the aqueous reassociation system the rate of DNA reassociation is inversely proportional to the complexity of genome size of the DNA (Britten and Kohne, 1968; Wetmur and Davidson, 1968). Complexity can be defined as the number of nucleotides in the DNA of a haploid cell. An increase in complexity means a proportional decrease in reassociation rate. Thus far with the PERT the general trend is the same. In the PERT the smaller complexity DNAs reassociate faster than the larger complexity DNAs. The human genome size is 700 times larger than that of *E. coli*. Thus, in total human DNA the nonrepeated DNA $C_0t_{1/2}$ should be and is about 700 times larger than that of *E. coli* (Britten and Kohne, 1968) when reacted in the standard aqueous system. At a concentration of 44 and 1600 μg/mL in 2 M NaSCN, 12.9% phenol, *E. coli* DNA reassociates with $C_0t_{1/2}$'s of 1.6×10^{-3} and 0.1 mol s L^{-1} (Table VIII). At concentrations of 40 and 1600 μg/mL of human DNA in the same emulsion systems the $C_0t_{1/2}$'s of reassociation for the nonrepeated DNAs are 0.97 and 40 mol s L^{-1}, respectively. At the low concentration, the human DNA $C_0t_{1/2}$ is 606 times larger (0.97/0.0016) than that for *E. coli*. At the high DNA concentration the human nonrepeated DNA $C_0t_{1/2}$ is 400

times larger than that of *E. coli*. When compared with *E. coli* DNA under specific conditions, the PERT reassociation rates of vaccinia (about 2×10^8 daltons), herpes simplex virus DNA (about 10^8 daltons genome size), and mycoplasma DNA (about 10^9 daltons genome size) are within a factor of 2–3 of the rates expected from genome size differences. The precise relationships between different complexities and their PERT reassociation rates have not yet been determined. The PERT can, however, be used to get an estimate of the complexity of DNA accurate to within 2–3-fold.

RNA:DNA and RNA:RNA Reaction with PERT. RNA:RNA and RNA:DNA reassociation does occur in the phenol emulsion at room temperature. However, the rate increase (relative to standard aqueous conditions) is much smaller than seen for the DNA:DNA reassociation. The maximum rate increase seen thus far for both RNA:DNA and RNA:RNA PERT reactions has been 50–100 times that seen for the standard aqueous reaction in 0.12 M PB, 60 °C. The emulsion mixtures for these experiments consisted of 0.5 μg/mL nucleic acid in a mixture of 0.9 mL of a 2–2.5 M NaCl aqueous phase plus 0.075–0.1 mL of 90% phenol. The reaction mixes were shaken with a Vortex mixer. The RNA:RNA reactions were done by reacting minus strand VSV (vesicular stomatitis virus) virion RNA with VSV [^{3}H]cRNA plus strand synthesized by the VSV polymerase (Breindl and Holland, 1976). The RNA:DNA reactions were done by reacting minus strand VSV virion RNA with plus strand [^{3}H]DNA synthesized via reverse transcriptase. It would be very useful to obtain large rate increases for the RNA:RNA and RNA:DNA reactions in the phenol system. We are currently searching for conditions which will provide this.

Possible Problems and Useful Suggestions When Using the PERT. Following is a discussion of possible problems which may arise during use of the PERT and, where available, the solutions. Many of the observations are qualitative and are not well documented in a quantitative sense. Also included are useful procedures which may be helpful under certain circumstances.

(a) When sampling the emulsion, the aliquot should be diluted into a salt solution at least the equivalent of 0.12 M PB (0.18 M Na^+). Dilution into lower salt concentrations results in the denaturation of certain double-stranded molecules. After dilution, the samples should not be frozen, as the freezing and thawing cycle also results in the denaturation of certain classes of double-stranded DNA. This probably occurs due to concentration of the phenol and DNA at ice–liquid interfaces during the freezing and thawing.

(b) Enough hydroxylapatite should be used in order to bind the double-stranded DNA in the presence of phenol. Different lots of hydroxylapatite often vary in their DNA binding capacities and each lot should be checked. To minimize the amount of phenol present during the assay, it is best to use the smallest aliquot possible. Alternatively the phenol can be removed by extracting the emulsion with chloroform and recovering the aqueous phase. This can then be diluted as desired. The DNA can also be precipitated out of the emulsion by simply adding 2.5 volumes of 95% ethanol and cooling to −20 °C until precipitation occurs. If the concentration of DNA is too low for precipitation, add single-stranded carrier DNA from a far distant species or tRNA (tRNA does not adsorb to hydroxylapatite under the normal assay conditions) before adding the ethanol. Do not ethanol precipitate nucleic acids in the presence of phosphate buffer. An excellent aqueous phase for ethanol precipitation is 2 M NaSCN.

(c) Do not use citrate as a chelator in the reassociation mixture. Small amounts of citrate prevent DNA from binding to the hydroxylapatite. Around 10^{-4} M EDTA is usually acceptable.

(d) If you plan to use S1 exonuclease for the reassociation assay, do not use phosphate buffer as the aqueous phase. Phosphate interferes with the S1 assay. 1.25 M NaCl serves nicely in this case. The S1 enzyme works well in the presence of a small amount of phenol.

(e) Many compounds (e.g., ethanol, chloroform, and benzene) will inhibit the emulsion reassociation. The DNAs should be purified and free of extraneous solvents before using with the PERT. Glycogen at several milligrams per milliliter does not affect the emulsion reaction.

(f) Continuous agitation is necessary for optimum rate of reassociation. The effect of different shaking modes has not been well studied. Inadequate agitation of the emulsion usually results in irreproducible kinetics and/or incomplete reassociation of the DNA. One solution is to obtain a shaker which shakes adequately. Another is to cover the bottom of the reaction vial with small (1 mm diameter) glass beads. The shake rate is then adjusted so that the beads actively move through the emulsion. These problems will more likely be experienced with high concentrations of DNA.

(g) At very low concentrations (roughly less than 0.1 μg/mL) single-stranded DNA tends to adsorb to glass and plastic surfaces. The presence of phenol seems to enhance this adsorption. Addition of heterologous single-stranded carrier DNA alleviates this problem. It must be kept in mind that single-stranded DNA reduces the emulsion rate of reassociation and any controls should also contain the carrier DNA. In general, it is best to use as little carrier DNA as possible. Addition of 0.01–0.05%, final concentration, of $NaDodSO_4$ also helps but is somewhat less effective. $NaDodSO_4$ at these low concentrations does not affect the emulsion rate of reassociation. At higher concentration, however, the presence of $NaDodSO_4$ slows the reaction. Treating glassware with silicon compounds to reduce nonspecific adsorption of nucleic acids is not recommended. The silicon interacts with the DNA and phenol in some way so that DNA is lost during the reaction.

(h) When assembling the reaction mixture, the aqueous components should be mixed first and the phenol added last. This is particularly important when reacting a low concentration of radioactive DNA in the presence of a large amount of nonradioactive DNA. If the radioactive DNA is added to a preformed emulsion containing the nonradioactive single-stranded DNA, little inhibition of the radioactive DNA reassociation rate is observed (relative to an emulsion which contains only radioactive DNA). If the radioactive DNA is mixed with the nonradioactive DNA before adding the phenol, the radioactive DNA reaction is greatly inhibited by the presence of the single-stranded DNA. The present interpretation of this phenomenon is that the radioactive DNA is somehow excluded from the nonradioactive DNA emulsion system and forms a separate or partially separate reaction system in the same vial. If it is necessary to add radioactive DNA after the nonradioactive DNA emulsion has been formed, the mixture can be heated to the point where the emulsion disappears (the phenol dissolves) and then cooled to room temperature. The phenol comes out of solution at the lower temperature and the emulsion reforms.

(i) DNA can be denatured in the presence of phenol by heating the emulsion to a temperature above the melting temperature of the DNA (see Figure 3b). To start the reassociation, the mixture is then rapidly cooled to room temperature and shaken. Bacterial and mammalian DNAs denatured in this fashion reassociate rapidly and completely.

(j) When reacted with the PERT, sonicated bacterial DNAs

routinely reassociate to 95–98%, while the maximum extent of reassociation seen for sonicated mammalian DNAs is 92–95%. The 5–8% nonreassociated DNA proved to be smaller than the reassociated DNA. This and other data indicate that the more homogeneous in size a DNA, the greater the extent of reassociation. Thus, when it is necessary to react radioactive DNA with nonradioactive DNA, care should be taken that the two DNAs are the same size.

(k) It is clear that with the PERT the lower complexity DNAs react faster than high complexity DNAs. The precise relationship between complexity and PERT rate has not been determined as yet. If the PERT technique is to be used to determine genome size, the proper calibrations must be done. This will involve obtaining several DNAs of known genome sizes and comparing their PERT rates of reassociation. The comparisons should be done under identical conditions of temperature, DNA fragment size, phenol concentration, DNA concentration, pH, salt concentration, volume of reaction mixture, conditions of shaking and, if possible, G + C content.

(l) It is very useful to have a stock of radioactive bacterial DNA in the laboratory for trouble-shooting purposes. *E. coli* DNA is well characterized and is a good choice. When unspecified troubles arise in reassociation experiments, the rate and extent of reassociation of the bacterial DNA can quickly be checked.

Current Uses of the PERT. The emulsion system has already been successfully used in a variety of research projects. A short description of these projects is included here in order to illustrate some of the possible uses of the PERT.

(a) Radioactive DNAs are frequently produced from single-stranded RNAs via the reverse transcriptase of C-type viruses. Often only a small amount of these DNAs can be produced, and in many cases complementary DNA sequences are made and are present in the final product. This radioactive DNA is often used to detect the presence of viral-like nucleotide sequences in cell DNA. It is thus advantageous to remove the complementary DNA strands in order to minimize radioactive DNA self-reassociation. The PERT is useful for removing the complementary sequences from the radioactive DNA product. The radioactive DNA is reacted in the phenol system and then passed over hydroxylapatite to separate the single- and double-stranded DNAs. We have PERT reassociated radioactive DNA synthesized from feline leukemia, Rouse sarcoma, Molony leukemia and Rauscher C-type viruses to obtain single-strand DNA which contains no complementary DNA sequences.

(b) We have also used the PERT to characterize the reassociation behavior of small samples of different DNAs after labeling the DNAs with ^{125}I to very high specific activity. The DNA can be damaged during iodination and it is advantageous to be able to quickly characterize the newly labeled DNA.

(c) Male human cells possess a Y chromosome which contains male specific nonrepeated nucleotide sequences. The PERT has been used to separate radioactive Y specific DNA sequences from the rest of the radioactive male DNA (K. Smith and S. Boyer, personal communication).

(d) Very small amounts (<0.1 μg) of circular DNA isolated from *Drosophila* embryos were radioactively labeled by an in vitro method and characterized by reassociation kinetic analysis using the PERT (S. Stanfield, personal communication). These data provided an estimate of the complexity of the DNA sequences comprising the circular DNA.

(e) Mareks disease herpes virus labeled with radioactive DNA precursors is difficult to grow in large amounts. Only small amounts of the radioactive DNA can be obtained and the DNA is sometimes contaminated with host cell DNA. The PERT has been used to quickly characterize this DNA (W. Campbell, personal communication).

(f) The PERT has also been used to detect the presence of small amounts of C-type viral RNA in the media of tissue culture cells producing these viruses (E. Scolnick, personal communication). The RNA from the cell media is isolated and then reacted with radioactive viral DNA in the emulsion system using conditions similar to those described in the text.

(g) As described earlier, the PERT has been used to detect the presence of herpes viral DNA in infected and transformed cells (D. T. Kingsbury, personal communication). Radioactive herpes I and II and cytomegalo virus DNA probes were reacted with an excess of cell DNA in the phenol system.

Scope of Use of the PERT for Nucleic Acid Reassociation Studies. The emulsion reassociation system will be useful in virtually every phase of DNA:DNA reassociation. Following is a list of many of the present uses of DNA:DNA reassociation and a short discussion of how the PERT will apply to each particular case. In many of these cases the PERT has already been used successfully.

Straight DNA Reassociation Kinetic Analysis. The data of Figures 1 and 2 and Tables VII, VIII, and IX show that a variety of DNAs reassociate successfully in the emulsion system. Bacterial (Figure 1, Tables V, VII) and viral DNAs reassociate reproducibly and well. Nonrepeated DNA (Figure 2) as well as repeated DNA (Figure 2, Table IX) reassociate well and can be analyzed with the emulsion system. Rat, mouse, and cat DNAs have also reassociated readily with the PERT, giving reassociation kinetic profiles similar to the human profile seen in Figure 2. The emulsion system has also been used to reassociate *Drosophila* DNA (S. Stanfield, personal communication). A small amount of sonicated radioactive human, *E. coli*, *Staphylococcus aureus*, or herpes simplex DNAs mixed with a large amount of sonicated human DNA and reacted in the emulsion system, reassociated completely (data not shown). Bacterial and viral DNA can then reassociate in the presence of a large amount of heterologous DNA. The radioactive human DNA was present at such a low concentration that little reassociation could occur between radioactive nonrepeated complementary strands during the course of the PERT reaction. The radioactive nonrepeated strands could react only with an unlabeled complementary strand. This demonstrated that radioactive and nonradioactive DNAs sonicated separately and mixed to reassociate together in the emulsion system.

The data of Tables VIII and IX show various PERT reassociation reactions which vary in concentration by a factor of 10^8. Earlier it was mentioned that the nonrepeated fraction of human DNA was one-half reassociated at 14 h when reacted in the emulsion system at a concentration of 0.5 μg/mL. This DNA reassociated to greater than 90% in 100 h. This is essentially complete reaction. Five tenth of a microgram of human DNA is equivalent to about 10^5 diploid cells. It would be a simple matter to modify the system so that the essentially complete reassociation of DNA from 10^4 diploid cells could be measured.

The ability to do reassociation kinetic profiles using very small amounts of DNA is a very useful aspect of the PERT. In many tissue culture systems fewer cells will need to be grown for nucleic acid hybridization analysis. In many instances valuable animals need not be sacrificed in order to get sufficient DNA to do complete reassociation kinetics since a tissue biopsy will suffice. The PERT will be particularly useful in the botanical and insect areas where large amounts of certain DNAs can be obtained only at great expense. Thus, the PERT will be

TABLE X: Nucleotide Sequence Homology Study.[a]

Type of reaction	Total ^{125}I *E. coli* DNA C_0t (mol s L^{-1})	total unlabeled *Salmonella* DNA C_0t (mol s L^{-1})	Hours reacted	Max self-reaction of ^{125}I DNA	% of ^{125}I *E. coli* DNA absorbed to hydroxylapatite
(a) PERT	1.7×10^{-7}	7×10^{-3}	0.1	<1%	25.9
1.25 M $NaClO_4$	3.5×10^{-7}	1.5×10^{-2}	0.2	<1%	33.5
0.1 M PB	5.1×10^{-7}	2.2×10^{-2}	0.3	<1%	38.8
9.8% phenol	7×10^{-7}	2.9×10^{-2}	0.4	<1%	42.3
(b) same as a	1.7×10^{-7}	7×10^{-3}	0.1	<1%	33.2
	6×10^{-7}	2.6×10^{-2}	0.35	<1%	46.5
	6×10^{-7}	2.6×10^{-2}	0.35	<1%	44.7
	1×10^{-6}	4.2×10^{-2}	0.6	~1%	50
(c) 0.48 M PB, 70 °C	6.8×10^{-6}	30.8	4	<1%	40.3

[a] PERT reaction mixes contained 6 μg/mL of *Salmonella* DNA plus 1.6×10^{-4} μg/mL of ^{125}I-labeled *E. coli* DNA. The 0.48 M PB aqueous reaction contained 600 μg/mL of unlabeled *Salmonella* DNA plus 1.6×10^{-4} μg/mL of ^{125}I-labeled *E. coli* DNA. The ^{125}I-labeled *E. coli* DNA reassociated to 97% when reacted with excess unlabeled *E. coli* DNA. One percent of heat denatured single-strand ^{125}I-labeled *E. coli* DNA bound to hydroxylapatite when passed over a 60 °C column in 0.14 M PB, 0.2% $NaDodSO_4$.

very useful in situations where only small tissue samples can be obtained from patients, experimental organisms, or tissue culture. Methods for the rapid, complete isolation of DNA from small amounts of tissue (Britten et al., 1974) and for in vitro labeling of DNA with radioactive iodine (Commerford, 1971; Tareba and McCarthy, 1973) make it possible to exploit the full potential of the PERT.

Sensitivity of Detection of DNA by DNA Reassociation with the PERT. The potential sensitivity of DNA detection by reassociation depends largely on the complexity of the DNA, the specific radioactivity which can be attained for the DNA, and the basic rate of DNA reassociation. The smaller the genome size, the less DNA it takes to get reassociation. Thus a greater sensitivity of detection can be attained with a less complex DNA. It is possible to routinely produce DNAs with a specific radioactivity in the range of 10^7 to 10^8 cpm/μg. It is relatively easy then to detect the presence of 6×10^{-6} μg (360 cpm) of DNA with a specific radioactivity of 6×10^7 cpm/μg. This is an amount of DNA equivalent to that present in one diploid cell. The bulk of the mammalian repeated DNA has a complexity of roughly 10^7 daltons. Under standard aqueous conditions giving close to maximum rates of reassociation (0.48 M PB, 66 °C), 6×10^{-6} μg of radioactive human DNA in 0.1 mL would require about 10^4 h to reach one-half reassociation of repeated DNA. Radioactive human DNA, reacted in the emulsion system at the same concentration of 6×10^{-6} μg per 0.1 mL, would require roughly 2 h for one half-reaction of the repeat fraction. This was calculated by using the rate factor increase seen in Figure 2 and corrected to the 0.48 M PB, 66 °C aqueous condition. The corrected rate increase value is about 4500. Table IX shows the results of the experiment described above. The repeated DNA half-time of reassociation is 2–4 h, close to the predicted time for reaction. The same concentration of radioactive human DNA in 1 mL of 0.48 M PB showed little reaction of the repeat DNA even after 73 h of incubation.

The above observation gives an indication of the greatly increased sensitivity of DNA detection by reassociation made possible by the PERT. It also demonstrates that very small amounts of DNA will react rapidly in the phenol system. The experiment in Table IX detects by reassociation of the repeated DNA an amount of DNA equivalent to that contained in one diploid cell. In this case the specific radioactivity of the DNA (6×10^7 cpm/μg) limits the sensitivity of the detection. Since the half-time of reassociation was only about 3–4 h, a tenfold greater sensitivity could easily be attained with higher specific radioactivity DNA. The number of *E. coli* bacteria or SV-40 viruses equivalent to 6×10^{-6} μg of DNA is about 1400 and 2×10^6, respectively. These numbers represent the number of these organisms which could be detected by DNA reassociation, assuming the DNA has an appropriate specific radioactivity.

The ultimate stability of DNA in the phenol reaction is unknown. The phenol emulsion will prevent the growth of microorganisms and destroy any enzyme activity. By taking precautions to prevent oxidation of the phenol, it may be possible to shake DNA for thousands of hours at room temperature. If this is so, it will greatly increase the potential sensitivity of detection by reassociation.

DNA Relationship Studies. Table IX presents the PERT kinetics of reaction of human repeated DNA and Figure 3a illustrates the thermal stability profile of reassociated human repeated DNA. It is clear that imperfectly complementary DNA molecules can reassociate in the emulsion system to form stable double-strand DNA molecules. These molecules have a thermal stability lower than expected for perfectly base pair matched molecules. This observation shows that the PERT can be used to measure the nucleotide sequence similarities of DNAs from different species.

DNA relationship studies between ^{125}I-labeled *E. coli* DNA and unlabeled *Salmonella typhimurium* DNA are present in Table X. The fraction of *E. coli* DNA which reassociates with *Salmonella typhimurium* DNA is about the same in the phenol emulsion system (42–50%) as in the aqueous reaction system (40–50%). Further, the T_ms of the *E. coli*:*Salmonella typhimurium* hybrids formed in the aqueous (T_m = 78.8 °C) and emulsion system (T_m = 79.6 °C) are similar. Both the aqueous and PERT reactions were driven to a C_0t 30–60 times the $C_0t_{1/2}$ of the *Salmonella* DNA, which at this point will be 90–95% reacted.

The study of nucleotide sequence relationships between species is an area where it often has been difficult, if not impossible, to obtain sufficient quantities of DNA for analysis. In the standard aqueous system, large amounts of DNA (tenths of milligrams) are usually needed to observe the reassociation of the nonrepeated DNA of most higher organisms. The PERT is combination with in vitro radioactive labeling of nucleic acids will greatly expand the number of species which can be ana-

lyzed for nucleotide sequence homologies. This will be particularly important in the case of plants and insects.

Detection of Viral Sequences in Cell DNA. The PERT will be very helpful in detecting the presence of viral-like nucleotide sequences in cell DNA. When mixed together and reacted in the emulsion system, radioactive viral DNA reassociates with unlabeled viral-like sequences present in cell DNA. Reverse transcriptase synthesized Moloney virus [^{3}H]DNA reacts well with Moloney virus transformed cell DNA (data not shown). Radioactive DNAs from herpes simplex I and II and cytomegalo virus reacts completely with DNA from cells infected with the respective virus (personal communication, Dr. Kingsbury).

The emulsion system will greatly increase the potential sensitivity of viral detection and will greatly expand the number of instances where detection of viruses in cells by reassociation is possible. The feasibility and sensitivity of a viral detection test depends upon: (a) the genome size of the virus; (b) the amount of cell DNA available; (c) the specific activity of the viral DNA; (d) the basic rate of reassociation of DNA in the system being used; (e) the amount of time which the DNA will remain intact under the reassociation incubation conditions. The increased rate of reassociation obtainable with the emulsion system will allow a great increase in the sensitivity of detection of viral sequences. Even DNA sequences present in the cell DNA at a frequency much less than one copy per cell can be made to reassociate in a reasonable time before the DNA breaks down. Much less DNA is needed to perform a routine viral detection when the PERT is used in combination with the very high specific radioactivity viral DNAs which can be produced. With this combination certain large viruses (herpes simplex I and II, cytomegalo, Epstein Barr, Pox viruses) should be detected at a sensitivity of 0.5 copy per cell using only 2–3 μg of cell DNA. This amount of DNA is the equivalent of 2–3 $\times 10^5$ cells or roughly 2–4 mg of tissue.

The data of Figure 2 indicate that reproducible mammalian DNA kinetic reassociation curves can be obtained with the PERT. This makes it feasible to utilize the emulsion system to determine the number of viral DNA copies per cell by reassociation kinetic measurements, particularly when an internal standard of radioactive (labeled with a different isotope than present in the radioactive viral DNA), nonrepeated cell DNA is also included in the reaction mix. This marks the rate of reassociation of cellular DNA sequences which are present at a frequency of one copy per haploid cell. The reassociation rate of the radioactive viral DNA can be referred to the nonrepeat DNA rate and the copies per cell of the viral DNA calculated.

Discussion

The phenol emulsion has a twofold effect on the reassociation of single-stranded DNA. The emulsion allows DNA reassociation to occur at or below room temperature while at the same time very rapid reassociation is promoted. Phenol is very effective at lowering the thermal stability (T_m) of double-strand DNA (Levine et al., 1963; Massie and Zimm, 1965; Leng et al., 1974). Under standard aqueous conditions the optimum rate of reassociation occurs about 20–30 °C below the T_m of the double-stranded DNA (Wetmur and Davidson, 1968). At 50 °C below the T_m reassociation occurs very slowly and at lower temperatures is completely inhibited. This inhibition is presumably due to the increased intrastrand secondary structure which occurs at lower temperatures. In the emulsion system the phenol provides conditions under which the DNA can reassociate. A simple explanation for this would be that due to the denaturing properties of the phenol, certain areas in the emulsion are 20–30 °C below the T_m of the DNA and the DNA can and does reassociate in these areas.

The conditions in the emulsion mixture also permit the very rapid reassociation of the DNA. The most plausible explanation for this effect is that single-stranded DNA is somehow concentrated by its interaction with the emulsion. The single-stranded DNA may be concentrated at the phenol:aqueous interface or by forming aggregates or semiprecipitates elsewhere in the two-phase system. The rapid rate of reassociation would then be at least partially due to the high DNA concentration. The "volume exclusion principle" (Lerman, 1973) is a possible mechanism for the increased reassociation rate seen in the phenol emulsion. Wetmur (1975) describes conditions for increasing the reassociation of DNA by about 70 times (relative to 0.18 M Na^+, 60 °C) using this principle. The rate increase is produced by adding neutral or anionic dextran polymers to the DNA solution. These polymers make the effective solvent volume available to the single-stranded DNA much smaller and thus increases the effective DNA concentration, which results in an increase in reassociation rate. Lerman (1973) has described a similar system which results in very high local concentrations (230 mg/mL) of double-stranded DNA. This volume exclusion principle will probably be important in explaining the large increases in reassociation rates seen with the PERT.

Below is the current "best guess" description of the events occurring during the PERT reassociation. Conditions at certain places in the phenol reaction mixture are such that the single-stranded DNA forms a semiprecipitate or aggregate resulting in localized regions of very high DNA concentration. In other regions of the reaction mixture the DNA is soluble, and when the aggregated single-stranded DNA enters these regions, the DNA in the aggregate becomes solubilized. At a time when the DNA is highly concentrated the conditions are such that DNA reassociation can and does occur. The DNA must undergo many aggregation:dispersion cycles before all of the single-stranded DNA is reassociated. It seems likely that the observed rate of reassociation seen in any specific phenol emulsion system is limited by the rate of the aggregation:dispersion process possible in that system. We believe that the highly chaotropic ions allow a faster aggregation:dispersion process and thus increase the rate of reassociation. These agents tend to disrupt hydrophobic bonds, and their primary effect may be to increase the capacity of the aqueous phase to disperse the aggregated single-stranded DNA. Chaotropic ions are particularly effective in the PERT at high DNA concentrations (Table VIII), and a much smaller concentration effect on the PERT reassociation rate is seen when using NaSCN instead of PB. The concentration effect on the PERT reassociation rate is probably a consequence of a decreased capacity to disperse aggregated DNA at higher DNA concentrations. At high DNA concentrations the emulsion mixture is milky white as compared with the light gray appearance of the same emulsion mixture minus DNA. This suggests that much of the DNA is in an aggregated or precipitated state. Earlier it was mentioned that even at low DNA concentration, reassociation occurs faster when the reaction mixture is shaken continuously. A similar effect is seen at high DNA concentration (Table VIII, item B). We interpret this to mean that the constant intermixing of the phenol:aqueous phases is important for fast reassociation and that the agitation increases the rate of the aggregation:dispersion process.

It was also mentioned earlier that large amounts of double-stranded DNA or single-stranded RNA have little effect on the reassociation rate of single-stranded DNA in the emulsion system. The RNA:DNA reassociation reaction is also

much slower than the DNA:DNA reaction in the emulsion system. These effects are probably caused by the different precipitation characteristics of double-stranded DNA, RNA, and single-stranded DNA. In terms of the "best guess" model presented above, the single-stranded DNA probably precipitates under different conditions in the emulsion than the RNA or DS DNA and therefore precipitates in a different region of the emulsion than the RNA or double-stranded DNA.

The emulsion conditions may also increase the fraction of successful collisions between complementary DNA strands. A successful collision between two complementary DNA strands is one which results in a stable double-stranded DNA molecule. In the standard aqueous system not all collisions between complementary strands result in a stable double-stranded product. In the phenol emulsion the fraction of successful collisions between complementary strands may be greater than in the aqueous system.

Table IX presents the reassociation kinetics of human repeated DNA using an amount of DNA (6×10^{-6} μg in 0.1 mL) equivalent to that present in one human diploid cell. This experiment provides an indication of the reassociation sensitivity made possible by the PERT. In addition, it provides an experimental base for considering the reassociation of DNA within a single cell or nucleus. The concentration of DNA in an average mammalian cell is, very roughly, 1 mg/mL. The concentration of single-stranded DNA in a cell is much lower since very little of the cell DNA is single-stranded at any one time. The presence in the cell of a system which has reassociation enhancing properties similar to the phenol emulsion system would provide an opportunity for any single-stranded DNA to reassociate at physiological temperatures. Such reassociation could be instrumental in promoting rearrangement of nuclear or cytoplasmic DNA sequences within a cell, as well as the integration of foreign genetic information (virus or otherwise) into cellular DNA or vice versa. It is interesting to speculate further that a variety of organic–salt systems may be present in the cell which promote the concentration of a wide range of biologically important molecules. DNA polymerase, for example, needs the proper concentration of nucleotide triphosphates in order to function properly. The right organic–salt system might act as a device to concentrate the nucleotide substrate in a particular region of the cell. Such a region with its high concentration of substrate might form the classical precursor "pools" involved in intracellular biosynthetic reactions. Such concentrating systems could also have been important for accelerating prebiotic synthesis reactions.

The ultimate limit of rate enhancement by the emulsion technique is unknown. It seems unlikely that these initial studies have resulted in the optimum possible rate enhancement. Understanding the basic mechanism of rate enhancement is necessary to design the optimum system. As part of this study the reasons for the inhibiting effect of high DNA concentrations must be determined in the hope that an emulsion system can be designed which gives large rate increases at high DNA concentration. It will also be important to study the RNA:DNA and RNA:RNA reassociation in the phenol system in order to obtain larger rate increases for these reactions.

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