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Kinetic and Spectrophotometric Studies on the Renaturation of Deoxyribonucleic Acid

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The kinetics of the renaturation of *Escherichia coli* DNA in 0·4–1·0M-sodium chloride at temperatures from 60° to 90° have been studied. The extent of renaturation was a maximum at 65° to 75° and increased with ionic strength, and the rate constant increased with both ionic strength and temperature. The energy and entropy of activation of renaturation were calculated to be 6–7kcal.mole^{-1} and −40cal.deg.$^{-1}$mole^{-1} respectively. It has been shown that renaturation is a second-order process for 5hr. under most conditions. The results are consistent with a reaction in which the rate-controlling step is the diffusion together of two separated complementary DNA strands and the formation of a nucleus of base pairs between them. The kinetics of the renaturation of T7-phage DNA and *Bordetella pertussis* DNA have also been studied, and their rates of renaturation related quantitatively to the relative heterogeneity of the DNA samples. By analysis of the spectra of DNA at different stages during renaturation it was shown that initially the renatured DNA was rich in guanine–cytosine base pairs and non-random in base sequence, but that, as equilibrium was approached, the renatured DNA gradually resembled native DNA more closely. The rate constant for the renaturation of guanine–cytosine base pairs was slightly higher than for adenine–thymine base pairs.

It has been known for several years that denatured DNA can re-form into a complete double-helical structure, if it is maintained at a suitable temperature and salt concentration (Doty, Marmur, Eigner & Schildkraut, 1960; Marmur & Lane, 1960). This process is usually termed 'renaturation' and the extent of helical structure re-formed depends on the temperature and ionic strength of the solvent, and also on the source of the DNA: DNA from viruses or phage renatures more readily than that from bacteria or mycoplasma, which renatures more readily than DNA from plants or animals (Marmur, Schildkraut & Doty, 1962). The optimum temperature for renaturation is about 25° below the melting temperature, T_m, of the native DNA in the solvent employed (Marmur & Doty, 1961).

Although renaturation has found many applications in biological fields, the kinetics and mechanism of the renaturation process have not been fully clarified, since previous investigations have produced conflicting results (Cavalieri, Small & Sarkar, 1962; Subirana & Doty, 1966). Cavalieri *et al.* (1962) concluded that *Escherichia coli* DNA and *Diplococcus pneumoniae* DNA renatured by a process which was essentially first-order at 60°, deviating towards second-order at higher temperatures. From this they deduced that the reaction was probably unimolecular and also that there had been no previous strand separation on denaturation. Subirana & Doty (1966) inferred from their experiments with *Hemophilus influenzae* DNA, *E. coli* DNA and T4-phage DNA that renaturation was a second-order process under all conditions and that the process was bimolecular, indicating strand separation in the preceding denaturation. In both of these investigations the renaturation was followed by measuring the decrease with time of the extinction at 260mμ. The reasons for the discrepancies in the results reported by these two groups are not clear, but it may be significant that the methods of denaturation and of the analysis of results differed. Moreover, it is important in such kinetic experiments to be certain that the extinction when the renaturation is complete is known accurately, and it is not clear whether or not this was achieved.

The present investigation into the kinetics of

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renaturation was undertaken to determine the order of the process and if this, and the rate and extent of renaturation, varied with the conditions of renaturation, including the method by which the environment of the DNA was changed from one favouring denaturation to that favouring renaturation. It was decided to minimize the re-formation of non-specific hydrogen bonds, which occur on cooling heat-denatured DNA, by never allowing the temperature of the denatured DNA to fall below the temperature of renaturation. The quantitative relationships between the rate constant and temperature of renaturation, the viscosity of the solvent and the heterogeneity of the DNA sample have been examined and the energy and entropy of activation calculated. Particular care has been taken over the methods of kinetic analysis used, and the effect of errors in various parameters has been tested quantitatively.

It is already well established that renatured DNA is very similar to native DNA in many respects after renaturation is complete, but the structure of the DNA while renaturation is still in its early stages has not been closely analysed. Many of the methods available require the DNA to be shock-cooled in ice, thereby introducing another factor into the experiment and causing random hydrogen bonds to form. The technique of spectral analysis (Felsenfeld & Hirschman, 1965; Hirschman & Felsenfeld, 1966) has been applied to determine the base composition of the renatured DNA at various stages at the temperature of renaturation, without cooling the DNA at all; this technique also provides an indication of the randomness of re-formation of the helical structure with respect to the two kinds of base pairs.

EXPERIMENTAL

E. coli *DNA*. *E. coli* B was kindly provided by the Microbiological Research Establishment, Porton, Wilts., and had been grown as previously described (Nicholson & Peacocke, 1966). The DNA was isolated by the use of 4-aminosalicylate, sodium dodecyl sulphate and phenol (Kirby, 1964), and purified by two ethanol precipitations from a solution in 4% (w/v) sodium acetate. Before the final precipitation from a solution in 3M-NaCl by propan-2-ol, pancreatic ribonuclease [Sigma (London) Chemical Co. Ltd., London, S.W. 6] was added to destroy any remaining RNA. The DNA was redissolved in 1mM-NaCl solution and, after dialysis, was freeze-dried and stored in the solid state at 2° until needed. A 100g. sample of wet frozen *E. coli* yielded about 200mg. of DNA. Two preparations of DNA were made and designated as EC1 and EC2; the characteristics of these preparations and other samples of DNA are given in Table 1.

T7-phage DNA. *E. coli* B was cultured in a medium consisting of: Oxoid Tryptone (Oxoid Ltd., London S.E. 1), 10g.; Difco Yeast Extract Powder (Baird and Tatlock Ltd., Chadwell Heath, Essex), 5g.; NaCl, 10g.; M-glucose, 5ml.; 0·25M-$CaCl_2$, 10ml.; all made up to 1l. with water. The buffer for resuspension of phage consisted of: Na_2HPO_4, 7g.; KH_2PO_4, 3g.; NaCl, 5g.; 0·1M-$MgSO_4$, 10ml.; 0·01M-$CaCl_2$, 10ml.; all made up to 1l. with water.

A 1l. volume of growth medium in a 5l. flask was inoculated with 100ml. of a culture of *E. coli* B and incubated at 37° with vigorous swirling until the turbidity reached about unity (equivalent to about 1×10^8 cells/ml.) as measured by an EEL colorimeter (Evans Electroselenium Ltd., Halstead, Essex) with filter ND7.

Each litre of *E. coli* B was then inoculated with a fivefold excess of phage particles (a total of 5×10^{11}–$2{\cdot}5\times10^{12}$ particles/l. of bacterial suspension; the concentration of phage particles in the original phage suspension was estimated by plating). The solution was aerated by vigorous swirling until the bacteria were lysed, which took about 45min., and the turbidity of the suspension became constant at about 0·55. Chloroform was added to complete lysis.

The debris was spun off at 3000*g* for 1hr. and the phage particles were sedimented by centrifugation at 44300*g* for 1hr. The phage pellets were resuspended in a minimum amount of buffer and centrifuged at 4900*g* for 3–4hr. until a clear solution was obtained. Small quantities (1mg. each) of deoxyribonuclease and ribonuclease [Sigma (London) Chemical Co. Ltd.] were added and the suspension was incubated at 37° for 30min. to remove any remaining bacterial nucleic acids. By further centrifugation at 78400*g* for 1hr., phage pellets were obtained and these were washed several times in buffer, ready for the extraction of the DNA.

DNA was extracted from the phage pellet by essentially the same method as that given by Mandell & Hershey (1960). A 2l. volume of *E. coli* suspension yielded about

Table 1. *Characteristics of DNA samples*

Source of DNA	$\epsilon_{(P),259}$	R at 259mμ*	% of RNA (w/w)	% of protein (w/w)	$S^0_{20,w}$ (s)	$10^{-6}\times$ Mol.wt.
E. coli (EC1)	6600	1·40	1·5	1·2	30·2 ± 1·2	19·1†
E. coli (EC2)	6330	1·43	1·2	Trace	—	—
T7-phage	6710	1·39	—	—	33·0	23·7†
B. pertussis (DNA-5)‡	6710	1·36	0·5	2·1	27·8	17·4†

* Relative increase in extinction at 259mμ on heating to 100°.
† Molecular weight calculated from $S^0_{20,w}$ (Eigner & Doty, 1965; eqn. 3′).
‡ From Bacon *et al.* (1967).

10mg. of T7-phage DNA. The DNA was stored in 1mM-NaCl at −20° until needed.

Bordetella pertussis DNA. This was the same preparation (DNA-5) as isolated and described by Bacon, Overend, Lloyd & Peacocke (1967).

Analytical methods. Protein content was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), and RNA content by that of Ceriotti (1955), after separation of the DNA and RNA by alkaline hydrolysis (Schmidt & Thannhauser, 1945). Absolute DNA concentrations were measured by phosphorus analysis. For concentrations of 10–100μg. of P/ml. the method of Jones, Lee & Peacocke (1951) was used. More dilute solutions of DNA (1–10μg. of P/ml.) were digested with the same mixture as described by Jones *et al.* (1951), but with the reagents in two-thirds concentration, and the colour was developed by ammonium molybdate and ascorbic acid (Chen, Toribara & Warner, 1956). A fine reddish-brown precipitate which formed was centrifuged down at 2750*g*. This resulted in no loss of colour, provided that only a very small amount of selenium powder was added to the digestion mixture.

Apparatus. The renaturation of DNA was followed by extinction measurements with a Beckman–Gilford recording spectrophotometer. A Gilford 200 extinction meter and cell compartment (Hilger and Watts Ltd., London N.W. 1) was substituted for the photocells and cell compartment of a Beckman DU spectrophotometer (Beckman Instruments Ltd., Glenrothes, Fife), so that the latter was used simply as a monochromator. The temperature was controlled by circulating ethylene glycol from a thermostatically controlled bath (±0·1°) through two plates on either side of the cell compartment. The temperature variation within any one cell was about ±0·3°, with a maximum temperature difference of 0·9° between cells. A period of 15–20min. was required for thermal equilibrium.

For some experiments, a Unicam SP.500 spectrophotometer (Unicam Instruments Ltd., Cambridge) was used in conjunction with a heating block (A. Adkins and Son Ltd., Leicester), which was electrically heated and thermostatically controlled and which fitted into the cell housing of the spectrophotometer. The temperature variation was ±0·1° at all temperatures.

The temperature in the spectrophotometer cells was recorded by measuring the resistance of a bead thermistor (VA 2106; Mullard Ltd., London, W.C. 1), which had been soldered to a hearing-aid lead and mounted inside a glass envelope. This fitted very tightly into a hole through a Teflon (du Pont Co. Ltd., London, W.C. 2) stopper for a cell and projected below the stopper and into the solution in the cell. The thermistor was in contact with the hemispherical end of the envelope, the top of which was filled as far as possible with moulding Araldite (Ciba Ltd., Duxford, Cambs.) to exclude any salt solution that might force itself up between the glass envelope and the Teflon stopper. The hearing-aid lead was connected to a Wheatstone-bridge apparatus, to measure the resistance of the thermistor. The thermistors were calibrated against mercury-in-glass thermometers (25° range in 0·05° graduations; Baird and Tatlock Ltd., Chadwell Heath, Essex) in a large water bath, and were checked periodically, when they were usually found to be within 0·05° of the original calibration. Temperatures in the cells could be measured to ±0·05°, which was less than the temperature variation within a cell.

Viscosities of solvents were determined with an Ubbelohde viscometer, and chloride concentrations with an EEL chloride meter (Evans Electroselenium Ltd.).

Kinetic studies. At the beginning of any renaturation experiment, three of the spectrophotometer cells in the cell compartment were filled with 2·5ml. of solvent (0·4M-, 0·6M- or 1·0M-NaCl), which had been filtered through a no. 4 sintered-glass filter, and allowed to attain thermal equilibrium (at a temperature between 60° and 90°). The fourth cell contained a solution of sodium salicylate, which was used as a standard to allow for any fluctuations in the base line of the instrument or in the brightness of the lamp. The extinction at 259mμ of this cell was stable to better than 1% for 24hr. at 80°, and in an experiment the extinction was adjusted to within 0·05 of that expected for the cells which would contain DNA solutions. The extinction of this cell, and those of the three cells containing solvent were measured relative to air at the beginning of the experiment.

In most experiments, about 0·5ml. of DNA solution (0·5–1mg. of DNA/ml. in 1mM-NaCl), which had been clarified by centrifugation at 90000*g*, was heated in a test tube in a boiling-water bath for 10min. About 0·1ml. of the hot denatured DNA was transferred, without cooling, into each of the three cells containing solvent by means of a hot tuberculin syringe with a no. 20 needle, and, after the solutions had been mixed thoroughly by a Perspex plunger, Teflon stoppers were inserted. The extinction at 259mμ of each of the four cells was measured in turn for 15–60sec., from within 1min. of the mixing of the solutions. This method of denaturation and renaturation is referred to below as the standard procedure. The time, t, from which the slow renaturation process actually observed was regarded as proceeding, was measured from the actual point of mixing; this is therefore referred to as 'zero time'.

In a few experiments, the procedure was varied slightly to examine the effect of different methods of denaturation: (A) the DNA was denatured as described above and then cooled rapidly in ice and kept at 2° until needed, when it was allowed to warm to room temperature and 0·1ml. was added to 2·5ml. of hot solvent (0·4M-, 0·6M- or 1·0M-NaCl at 65° or 80°) by a tuberculin syringe; (B) the DNA was denatured in 0·4M-NaCl, instead of 1mM-NaCl, and then renatured by the standard procedure at 65° in 0·4M-NaCl; (C) the DNA (0·33mg. of DNA/ml. in 1mM-NaCl) was denatured at 80° for 15min. in a spectrophotometer cell, and then 0·5ml. of 2M-NaCl at 80° was added and the renaturation continued at 80° (the chloride concentration of the resulting solution was measured with an EEL chloride meter at the finish of the experiment); (D) DNA (0·55mg. of DNA/ml. in 1mM-NaCl) was denatured by bringing the pH to 11·6 and then adding 0·1ml. of this solution to 2·5ml. of 0·4M-NaCl at 70°, which contained just enough acid to neutralize it (the pH of the final solution was checked at the end of the experiment).

Extinctions were recorded continuously for at least 5hr. and then intermittently for a further 20hr. The total concentration of DNA, [P], as g.atoms of P l.$^{-1}$, in each cell was measured at the end of the experiment by heating the cell to 100° and reading the extinction at 259mμ. The extinctions of the DNA solutions after various times of renaturation were calculated by measuring from the recorder chart the differences in extinction between the salicylate standard and the DNA solutions, adding to these differences the extinction of the standard solution and

subtracting the extinctions of the solvent blanks (measured at the beginning of the experiment).

In all experiments allowance was made for the thermal expansion of water by correcting readings to 25°, and all results, except those for rate as a function of concentration, were expressed as relative extinction, R_t at time t, which was defined as the extinction at 259 mμ of a particular DNA solution, corrected to 25°, divided by the extinction, at 25°, of a solution of native DNA of identical concentration. Extinctions could be measured with an accuracy of $\pm 0 \cdot 002$, so that R_t values were accurate to $\pm 0 \cdot 02$. Thus it was possible to plot renaturation curves (R_t versus t), which were analysed as described below.

Spectrophotometric studies. Spectra of DNA samples were read manually from 235 to 290 mμ, in 5 mμ increments, with the Beckman–Gilford spectrophotometer. For the initial studies on samples of native *E. coli* DNA the spectra at 25° were recorded, and the solutions were then heated to 100° and the spectra again recorded. Extinction values were corrected for the extinction of blanks and for thermal expansion.

In the experiments on the renaturation of *E. coli* DNA, the DNA was denatured and renatured by the standard procedure in 0·4 M-NaCl at 60°, 70° or 80°. The spectrum of each sample of DNA was recorded at various times during the renaturation and, 24 hr. after renaturation was begun, the sample was heated to 100° in the spectrophotometer and the spectrum of the denatured DNA recorded.

KINETIC STUDIES

Kinetic analysis. In a first-order reaction:

$$\ln(c_0/c) = k_1 t \quad (1)$$

and in a second-order reaction:

$$(c_0 - c)/c = k_2 c_0 t \quad (2a)$$

where c_0 and c are the concentrations of reactant in the solution at zero time and at time t respectively, and k_1 and k_2 are first-order and second-order rate constants. Eqn. (2a) may also be expressed as:

$$(c_0 - c)^{-1} = [1 + (k_2 c_0 t)^{-1}]/c_0 \quad (2b)$$

or as:

$$c_0/c = k_2 c_0 t + 1 \quad (2c)$$

In a third-order reaction:

$$1/c^2 - 1/c_0^2 = 2k_3 t \quad (3)$$

where k_3 is the third-order rate constant.

The relative extinction at the beginning of the experiment, R_0, was measured by extrapolating the curve to zero time. The relative extinction at infinite time, R_∞, was more difficult to measure accurately and two methods were used, which usually gave results to within 0·02 of each other: (i) R_t was plotted against t^{-1}; (ii) $(R_t)^{-1}$ was plotted against t^{-1}; and both plots were extrapolated to infinite time. These methods usually involved steep extrapolations, and the importance of errors in R_∞ is discussed below.

Some DNA (usually about 25%) had always renatured by the time measurements of extinction were started in any experiment. This initial renaturation occurred in less than a minute during the process of cooling to the renaturation temperature, and it was not possible to follow its kinetics. Some denatured DNA remained at the end of the experiment, and it was assumed that neither this DNA nor the rapidly renatured DNA interfered with the renaturation that was observed during the experiment. This assumption is justified by the work of Subirana & Doty (1966) and Walker & McCallum (1966), who showed that addition of native DNA to renaturing DNA did not affect the renaturation process. It was consequently incorrect to use the total concentration of DNA, [P], in the solution as the original concentration of 'reactant', c_0, for the 'reactant' was that DNA which actually renatured during the period of observation. Since the reactant concentration at infinite time must approach zero, assuming that there is no back reaction, the reactant was defined in these experiments as the DNA whose extinction actually decreased during the experiment. Though this DNA is only a part of the total that renatured (since some renatured very rapidly in being cooled from the denaturation to the renaturation temperature), a perfectly valid test of the order of the process actually observed can be obtained from this partial curve and a true value of the rate constant determined. The concentration, c_0, of this DNA was directly proportional to the decrease in extinction and was equal to $(R_0 - R_\infty)[P]/0 \cdot 40$, where the factor 0·40 represents the increase in relative extinction at 259 mμ on heating *E. coli* DNA (EC1) to 100°. Thus c was equal to $(R_t - R_\infty)[P]/0 \cdot 4$, and $(c_0 - c)$ to $(R_0 - R_t)[P]/0 \cdot 4$. The influence of single-strand stacking of the denatured DNA must be considered since, if this is at all significant, there will be a decrease in extinction on cooling the denatured DNA from 100° to the temperature of renaturation because of hypochromism associated with single-strand stacking. The factor 0·40 would then be in error and, though this would not affect the order or the activation energy, it would affect both k_2, which would have an error about the same as that in the factor 0·40, and to a much smaller extent the entropy of activation. However, it is known that there is only a small amount of stacking in polyadenylic acid at 80° (Holcomb & Tinoco, 1965), and some results of Cox & Kanagalingam (1968) indicate that when DNA is denatured, shock-cooled to 4° and then reheated in 0·1 M-Na^+ to 60° only about 10% of the hypochromism of native DNA remains. In the present experiments the DNA was cooled directly to temperatures between 60 and 80° and so the hypochromism must be less than this 10%,

making the error in the factor 0·40 no greater than 5%.

Eqn. (1) was used for the first-order analysis of the renaturation curves, and eqn. (2a) for second-order analysis. Some curves were also analysed for third-order behaviour by plotting $(R_t - R_\infty)^{-2}$ against t. A rate constant for each experiment was obtained and the experimental activation energy, E, was calculated by means of the usual Arrhenius plot and the entropy of activation, $\Delta S^{\ddagger}$, from the absolute theory of reaction rates, in which:

$$k_2 = (\boldsymbol{k}T\mathrm{e}/\boldsymbol{h})\mathrm{e}^{-E/\boldsymbol{R}T}\mathrm{e}^{\Delta S^{\ddagger}/\boldsymbol{R}} \quad (4)$$

where $\boldsymbol{k}$ is Boltzmann's constant, $\boldsymbol{h}$ is Planck's constant, T is the absolute temperature and $\boldsymbol{R}$ is the gas constant.

Renaturation curves (R_t versus t) were constructed from the theoretical kinetic equations for first-, second- and third-order reactions, with quantities of approximately the same magnitude as those obtained from actual experiments. The purpose of these calculations was to examine, with hypothetical results obeying a given rate law, the extent to which it was possible to discriminate between different orders by means of the various plots and to see how sensitive to the value of R_∞ any conclusions might be. The four essential parameters of these calculations were: total DNA concentration, $[\mathrm{P}] = 6{\cdot}46 \times 10^{-5}$g.atom of P l.$^{-1}$; relative extinction at zero time, $R_0 = 1{\cdot}30$; relative extinction at infinite time, $R_\infty = 1{\cdot}03$; time of half reaction = 7500sec. (= 125min.).

The rate constants consistent with these parameters were calculated to be: $k_1 = 7{\cdot}24 \times 10^{-5}$sec.$^{-1}$; $k_2 = 3{\cdot}06$ l.mole^{-1}sec.$^{-1}$; $k_3 = 1{\cdot}05 \times 10^{5}$ l.^{2}mole^{-2} sec.$^{-1}$; and, from these values, hypothetical renaturation curves were constructed. Each of these curves was then analysed for first-, second- (by eqn. 2a) and third-order behaviour, and it was noted that there was no chance of confusing the order provided that the plots were extended as far as $t = 200$min.

It was known that R_∞ was likely to be inaccurate by possibly $\pm 0{\cdot}02$, and so R_∞ was altered by this amount and the theoretical curves for each order were again analysed for all orders. The linearity of the curves from the kinetic analyses depended on the value assumed for R_∞, as expected, but this dependence was not so great that it would be possible to confuse the order of a reaction if the value of R_∞ was within 0·02 of its correct value and the analysis was continued for at least twice the time of half reaction (250min. in this case).

The value of $(R_0 - R_t)^{-1}$ was plotted against $1/t$ according to the second-order equation (2b) by using R_t and t values obtained from the first-, second- and third-order curves: all gave linear plots for the first 500min., and only deviated markedly at longer times ($t^{-1} < 1/500$min.$^{-1}$) when the process is more than 80–90% complete. Hence it is concluded that this plot could not have discriminated convincingly in practice between first- and second-order processes when the extent of reaction was less than 90%, as it appears to have been in many of the published results of Subirana & Doty (1966), who based all of their conclusions on this type of plot (eqn. 2b). Once the second-order character of the process had been established by other types of analysis, the values of k_2 calculated by this method were in good agreement with the k_2 values derived from these other analyses, but the initial problem was to decide what the order was. The hypothetical curves were also analysed by the dimensionless plot of eqn. (2c), which has been used by Ross & Sturtevant (1960) and Cavalieri *et al.* (1962). The reciprocal of the fraction remaining unchanged, c_0/c, is plotted against the product of the time and of the initial rate of increase of c_0/c with time, which is equal to k_2c_0. A second-order reaction gives a linear plot of unit slope and and intercept. When the value of R_∞ was incorrect by 0·02, the plots for the second-order theoretical curve were only slightly curved, whereas the plots for the first- and third-order curves deviated very markedly from linearity with all values of R_∞. However, this method is difficult to apply in practice because of the inaccuracy in measuring the initial rate of change of c_0/c.

The conclusions that have been drawn from these calculations are only valid for the particular chosen values of the constants, though these are, in fact, representative of the experimental constants. However, they illustrated the inherent uncertainties and difficulties in the interpretation of such kinetic experiments, and pointed to obedience to eqn. (2a) as the most suitable test for second-order behaviour.

Extent of renaturation of E. coli *DNA*. (*a*) Dependence on salt concentration and temperature. The total fraction of renaturable *E. coli* DNA, as measured by R_∞, was dependent on both salt concentration and temperature and had a broad maximum between 65° and 75° (Table 2; and Fig. 1 of Thrower & Peacocke, 1966). The value of R_0 varied little with temperature or salt concentration (Table 2) and was constant at about 1·3 (the value for totally denatured *E. coli* DNA being 1·40). Thus about 25% of the DNA renatured very quickly indeed during the cooling process.

Four samples of EC 1 DNA, denatured separately, were renatured in 0·4M-sodium chloride at 65° to test the reproducibility of the results. This was seen to be satisfactory (Table 2, * and first footnote) so that one run was considered adequate in subsequent experiments.

(*b*) Dependence on method of denaturation. There was no significant difference between the

Table 2. *Renaturation of* E. coli *DNA* (*EC* 1)

DNA stock solution (0·5–1·0mg. of DNA/ml. in 1mM-NaCl) was denatured at 100° for 10min. and then about 0·1ml. was added directly to 2·5ml. of solvent at the temperature indicated in the first column.

Temp.	Concn. of NaCl (M)	$10^5 \times$ Concn. of DNA (g.atoms of P l.$^{-1}$)	R_0	R_∞	$R_0 - R_\infty$	k_2 (l. mole^{-1} sec.$^{-1}$)
60°	0·4	6·8	1·31	1·06	0·25	1·2
	0·6	6·8	1·29	1·05	0·24	1·5
	1·0	7·0	1·28	1·05	0·23	1·7
65	0·4*	—	1·30	1·03	0·27	$1{\cdot}4_3 \pm 0{\cdot}0_2$
	0·6	7·2	1·29	1·03	0·26	1·8
	1·0	7·1	1·27	1·00	0·27	2·1
67·5	0·4	6·0	1·30	1·03	0·27	1·6
	0·6	7·0	1·29	1·03	0·27	2·0
	1·0	7·9	1·28	1·02	0·26	3·0
70	0·4	6·4	1·31	1·03	0·28	1·4
	0·6	6·5	1·26	0·99	0·27	1·9
	1·0	5·9	1·30	0·99	0·31	2·2
72·5	0·4	6·0	1·31	1·03	0·28	1·5
	0·6	6·7	1·32	1·01	0·31	1·7
	1·0	7·2	1·31	1·02	0·29	2·6
75	0·4	7·4	1·30	1·09	0·21	2·2
	0·6	7·2	1·30	1·04	0·26	1·9
	1·0	7·1	1·29	1·00	0·29	2·5
77·5	0·4	7·7	1·31	1·07	0·24	2·0
	0·6	8·0	1·30	1·03	0·27	2·1
	1·0	7·7	1·31	1·03	0·28	2·9
80	0·4	6·8	1·31	1·10	0·21	2·2
	0·6	7·7	1·30	1·05	0·25	2·7
	1·0	9·5	1·30	1·03	0·27	2·9
90	0·4	5·6	1·28	1·22	0·06	—†
	0·6	7·6	1·32	1·17	0·15	3·9
	1·0	7·7	1·32	1·08	0·24	4·1

* Mean results of four experiments at different DNA concentrations. The standard deviation of R_0, R_∞ and $(R_0 - R_\infty)$ was $\pm 0{\cdot}01$.

† Insufficient renaturation to determine k_2.

parameters of renaturation, R_∞ and $(R_0 - R_\infty)$, observed after denaturation by various methods. DNA denatured at 80° for 15min. in 1mM-sodium chloride had lower values of R_0 and R_∞ than in the standard procedure, although $(R_0 - R_\infty)$ was almost the same; perhaps the DNA was not completely denatured under these less vigorous conditions.

(*c*) Dependence on viscosity of the solvent. DNA was denatured and renatured at 70° in 0·4M-sodium chloride by the standard procedure, except that the viscosity of the solvent for renaturation was varied by the addition of sucrose (10–30g./100ml. of solution). These solvents were clarified by filtration and the viscosities relative to 0·4M-sodium chloride determined at 70°. The value of $(R_0 - R_\infty)$ appeared to decrease with increasing viscosity (Table 3), but this could be attributed to the lower rate so that after 24hr. the value of R_t was higher and there was a greater chance of error in extrapolation to R_∞.

(*d*) Dependence on DNA concentration. The effect of DNA concentration on the extent of renaturation in 0·4M-sodium chloride at 65° was studied. It was concluded that the extent was independent of the concentration of DNA over the range 1×10^{-5}–14×10^{-5}g.atoms of P l.$^{-1}$.

Order and rate of renaturation of E. coli *DNA*. (*a*) Dependence on salt concentration and temperature. Fig. 1 shows a typical set of renaturation curves in 1·0M-sodium chloride at 60°, 70° and 80°, and Fig. 2 the second-order analyses of these curves by eqn. (2a). A second-order kinetic law was obeyed for at least 5hr. in these runs and in almost all other runs, although, as previously reported (Thrower & Peacocke, 1966), a few did deviate after about 3hr. The second-order rate constant

Table 3. *Effect of solvent viscosity on the renaturation of* E. coli *DNA (EC 1) in 0·4M-sodium chloride at 70°*

DNA stock solution (0·55mg. of DNA/ml. in 1mM-NaCl) was denatured at 100° for 10min. and then about 0·1 ml. was added directly to 2·5ml. of 0·4M-NaCl in 0–30% (w/v) sucrose at 70°. Viscosities of the solvents at 70° were measured by an Ubbelohde capillary viscometer, and are given as relative to the viscosity of 0·4M-NaCl at 70°. Rate constants are relative to that of renaturation in 0·4M-NaCl. By the method of least squares a plot of the relative rate constant, $(k_2)_{rel.}$ versus $\eta_{rel.}$ has a slope of $-0·3 \pm 0·1$, and that of $(k_2)_{rel.}$ versus $(\eta_{rel.})^{-1}$ has a slope of $0·7 \pm 0·1$.

Concn. of sucrose (%, w/v)	$\eta_{rel.}$	$10^5 \times$ Concn. of DNA (g.atoms of P l.$^{-1}$)	R_0	R_∞	$R_0 - R_\infty$	k_2 (l. mole^{-1} sec.$^{-1}$)	$(k_2)_{rel.}$
0	1	5·9	1·32	1·05	0·27	1·6	1·0
10	1·21	6·7	1·32	1·08	0·24	1·3	0·8
15	1·35	8·8	1·32	1·08	0·24	1·3	0·8
20	1·59	8·0	1·30	1·08	0·22	1·0	0·6
30	2·27	9·4	1·30	1·08	0·22	1·0	0·6

calculated from these curves (Table 2) increased with both the salt concentration and the temperature of renaturation. Analyses of some of the curves at 60°, 70° and 80° by eqns. 2(b) and (2c) confirmed that the order was second and the rate constants so calculated were in good agreement with those given in Table 2.

Energies and entropies of activation have been calculated (Table 4). The value of the activation energy, E, did not change significantly within the limits of error, which were conditioned by the inevitably narrow temperature range over which the renaturation could be studied. There was a variation of only 0·2cal.deg.$^{-1}$mole^{-1} in the entropies of activation, $\Delta S^{\ddagger}$, calculated from E and the corresponding value of k_2 at temperatures from 60° to 90°.

(*b*) Dependence on method of denaturation. In all of the experiments, renaturation was second-order for at least 150min., and most showed second-order behaviour for at least 300min. Thus the method of denaturation had little effect on the order of the subsequent renaturation. The rate constants were mainly unaffected, although those for the renaturation of shock-cooled DNA at 80° ($k_2 = 3·8 \pm 0·6$ l. mole^{-1}sec.$^{-1}$), and also for renaturation after denaturation at 80° in 1mM-sodium chloride ($k_2 = 3·9 \pm 0·3$ l. mole^{-1}sec.$^{-1}$), were considerably higher than those obtained when the procedure was standard.

(*c*) Dependence on viscosity of the solvent. When the viscosity of the solvent was increased by addition of sucrose, the rate of renaturation decreased, although the order was still second for times up to 300min. (Table 3). The results have been analysed by the method of least squares for the relative rate constant as a function of $\eta_{rel.}$ and of $(\eta_{rel.})^{-1}$. The former analysis gave a line of slope $-0·3 \pm 0·1$, whereas the latter slope was $0·7 \pm 0·1$. If the reaction is diffusion-controlled it would be expected that the rate should be a linear function

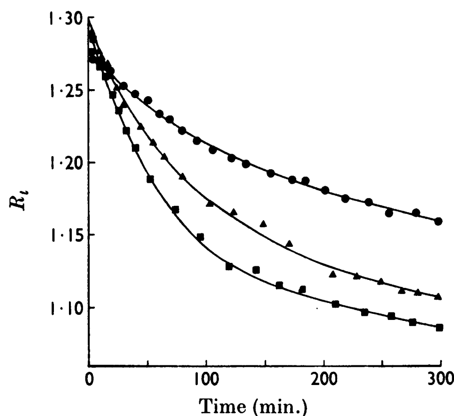


Fig. 1. Renaturation curves, plotted as R_t against t, of *E. coli* DNA (EC1) denatured in 1mM-NaCl at 100° for 10min. and renatured by the standard procedure in 1M-NaCl at 60° (●), 70° (▲) and 80° (■). For second-order kinetic analysis of these curves see Fig. 2.

of $(\eta_{rel.})^{-1}$, since diffusion coefficients are also a function of $(\eta_{rel.})^{-1}$.

Order of the renaturation of E. coli *DNA from initial rates.* Two series of experiments were performed to confirm the order of the renaturation by studying initial rates. In the first, *E. coli* DNA solution in 1mM-sodium chloride was heated at 100° for 10min. and then renatured by the standard procedure in 0·4M-sodium chloride at 65°. It has already been shown that the relative extent of renaturation is independent of DNA concentration, and the initial rate of reaction, i.e. the rate of decrease in the actual concentration (in g.atoms of P l.$^{-1}$) of renaturable DNA present, was measured as a function of DNA concentration over an eight-fold range. The slope of the logarithmic plot of these results (Fig. 3) was $1·9 \pm 0·1$ by the method of least

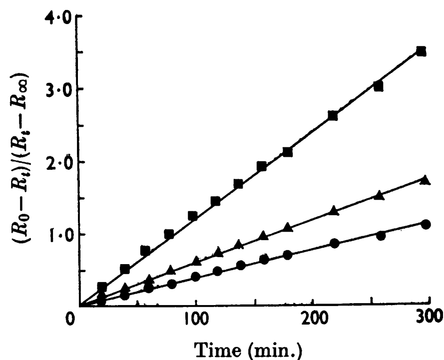


Fig. 2. Second-order analysis of the curves shown in Fig. 1 for renaturation in 1 M-NaCl at 60° (●), 70° (▲) and 80° (■).

Table 4. *Energy and entropy of activation of the renaturation of* E. coli *DNA (EC 1)*

The energy of activation, E, was calculated by the method of least squares from the Arrhenius equation, by using the values of k_2 given in Table 2, and the entropy of activation ($\Delta S^\ddagger$) was calculated by eqn. (4) from the values of E and k_2 at 70°.

Concn. of NaCl (M)	E (kcal. mole^{-1})	$\Delta S^\ddagger$ (cal. deg.$^{-1}$ mole^{-1})
0·4	6·8 ± 1·2	−40 ± 4
0·6	6·8 ± 1·2	−40 ± 3
1·0	6·1 ± 1·1	−41 ± 3

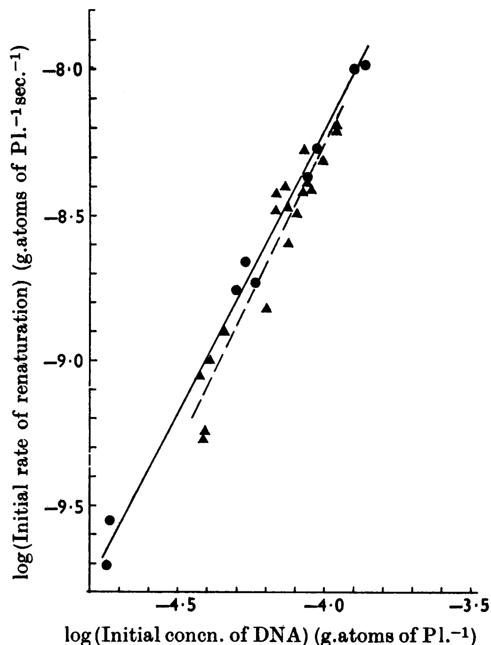


Fig. 3. Effect of initial concentration of DNA on initial rate of renaturation. *E. coli* DNA (EC1) was denatured in 1 mM-NaCl at 100° for 10 min. (●) or 80° for 15 min. (▲), and then renatured by the standard procedure in 0·4 M-NaCl at 65°. The lines were calculated by the method of least squares and have slopes of 1·9 ± 0·1 (●——●), and 2·1 ± 0·1 (▲— —▲).

squares and the value of k_2 was 0·7 ± 0·1 (S.D.) l. mole^{-1}sec.$^{-1}$. In a second series of experiments, in which the DNA was denatured in 1 mM-sodium chloride at 80° for 15 min. and renatured in 0·4 M-sodium chloride at 65°, the slope (Fig. 3) was 2·1 ± 0·1 and k_2 was 0·6 ± 0·1 (S.D.) l. mole^{-1}sec.$^{-1}$.

The renaturation of DNA is confirmed as a second-order process, although the actual values of the second-order rate constants do not agree very well with those obtained by other methods.

Renaturation of DNA from other sources. (*a*) *B. pertussis* DNA. DNA was denatured and renatured in 0·4 M-sodium chloride at 65° by the standard procedure in two of the preliminary experiments in this investigation. Readings were taken continuously for only 3 hr. and a further reading after 24 hr. was taken as the extinction at infinite time. The extent of renaturation was less than with *E. coli* DNA ($R_0 - R_\infty$ = 0·14 and 0·16) although the order was still second, with k_2 about tenfold higher (13 and 22 l. mole^{-1}sec.$^{-1}$).

(*b*) T7-phage DNA. DNA was denatured and renatured in 0·4 M-sodium chloride at 65° by the standard procedure in six experiments. The order was again second for up to 45 min., after which the process was almost complete. The rate constants were all very much higher than with *E. coli* DNA (240 ± 40 l. mole^{-1}sec.$^{-1}$). The value of R_∞ (1·02 ± 0·02) was not very constant, but the average value was similar to that for *E. coli* DNA. It was difficult to measure R_0 (1·22 ± 0·01) very accurately, as the renaturation was so rapid that there was a very steep extrapolation to zero time, but it seems that there was more renaturation initially than with *E. coli* DNA.

SPECTROPHOTOMETRIC STUDIES

Spectral analysis. The spectra of native and denatured *E. coli* DNA samples (EC2) and the hyperchromic spectra were analysed by the method of Felsenfeld & Hirschman (1965) and Hirschman & Felsenfeld (1966). Three-term analyses were used to calculate the DNA concentration, C (in g.atoms of Pl.$^{-1}$), base composition, ϕ (as mole fraction of A–T*), and deviations from randomness δ.

* Abbreviations: A–T, adenine–thymine base pair; G–C, guanine–cytosine base pair.

The spectra of native and denatured DNA were analysed by means of the parameters given in Tables 1 and 3 respectively of Hirschman & Felsenfeld (1966). However, since measurements at wavelengths below 235 mμ were not very reproducible, in the analysis of the hyperchromic spectra (Hirschman & Felsenfeld, 1966; Table 5) extinctions at wavelengths from 220 to 230 mμ were omitted from the calculations. Therefore the parameters $S_1 \cdots\cdots S_6$ were recalculated from:

$$\begin{pmatrix} S_6 & S_1 & S_4 \\ S_1 & S_2 & S_3 \\ S_4 & S_3 & S_5 \end{pmatrix} = \begin{pmatrix} \sum_i \alpha_i^2 & \sum_i \alpha_i\beta_i & \sum_i \alpha_i\gamma_i \\ \sum_i \alpha_i\beta_i & \sum_i \beta_i^2 & \sum_i \beta_i\gamma_i \\ \sum_i \alpha_i\gamma_i & \sum_i \beta_i\gamma_i & \sum_i \gamma_i^2 \end{pmatrix}^{-1}$$

by using the values of α_i, β_i and γ_i given by Hirschman & Felsenfeld (1966; Table 5) for wavelengths from 235 to 290 mμ. The values were: $S_1 = 0{\cdot}8306 \times 10^{-7}$; $S_2 = 0{\cdot}6805 \times 10^{-7}$; $S_3 = 0{\cdot}2421 \times 10^{-7}$; $S_4 = 0{\cdot}5583 \times 10^{-7}$; $S_5 = 0{\cdot}2743 \times 10^{-7}$; $S_6 = 3{\cdot}1424 \times 10^{-7}$. The inversion of the right-hand matrix was performed on an Elliott 803 computer with a programme written by Mr J. F. Bithell.

Native E. coli *DNA*. The spectra of solutions of native DNA (EC2) were recorded as described in the Experimental section. The values of C, ϕ and δ for each solution of DNA were calculated separately, from the spectra of native and denatured DNA and the hyperchromic spectra (Table 5). The molar extinction coefficient at 260 mμ was calculated for each spectrum from the extinction, or difference in extinction, at 260 mμ and the calculated value of C. The values of the molar extinction coefficients (Table 5) agree well with some quoted previously (Felsenfeld & Hirschman, 1965), and the values of C derived from the three spectra of any one solution were very similar. The mean value of ϕ was close to that obtained by chemical analysis (0·50), and values of δ were, within the limits of error, close to zero.

Some similar experiments on T7-phage DNA and calf thymus DNA gave values of C and ϕ similar to those obtained by other methods. It was concluded from these initial experiments that both the method of spectral analysis and the particular sample of *E. coli* DNA were suitable for further studies on the formation and structure of renatured DNA.

Renaturation of E. coli *DNA*. Samples of DNA (EC2) were renatured and the spectra recorded as described in the Experimental section. From the spectrum of the fully denatured DNA, the values of C, ϕ and δ for that particular sample of DNA were calculated. In order to calculate the values of C, ϕ and δ of the DNA that had renatured after any particular time, t, the spectrum at t was subtracted from the spectrum of the fully denatured DNA and the resulting hyperchromic spectrum analysed. The following assumptions, which are essential in attempting to apply spectral analysis to the renaturation process, were made: (i) there were only two species of DNA in the solution that contributed to the spectrum, i.e. fully renatured (which, as will appear, was equivalent to native) and fully denatured DNA; (ii) DNA which was still denatured at time t made the same contribution to the spectrum then as to the spectrum at 100° of the fully denatured DNA, i.e. the only change on heating the solution to 100° was caused by the denaturation of renatured DNA; (iii) although DNA was renaturing, and hence altering the spectrum while it was being recorded, this change was not so great as to confuse the results. The only factor that might undermine (i) and (ii) is the possibility that there may be present in the DNA at the temperature of renaturation a small amount of single-strand stacking, which has a hypochromic effect. There is no accurate method of allowing for this over the spectral range used in the present experiments. Reasons have already been given in the section on Kinetic analysis that errors in the extinction due to stacking are probably no greater than 5%. In order to investigate the third

Table 5. *Values of the base composition, ϕ, the molar extinction coefficient, $\epsilon_{(P),260}$, and the deviation from randomness, δ, of native* E. coli *DNA (EC2), calculated from the spectra*

The spectra of samples of native DNA at 25° were recorded from 235 to 290 mμ, in 5 mμ increments. The solutions were heated to 100° and the spectra again recorded, allowance being made for thermal expansion and for solvent blanks. Values of ϕ (mole fraction of A–T), δ (deviation from randomness) and C (g.atoms of P l.$^{-1}$) were calculated from each spectrum, as described in the text. Errors are given as standard deviations.

	Spectrum of native DNA	Spectrum of denatured DNA	Hyperchromic spectrum
Total no. of experiments	23	34	21
ϕ	0·48 ± 0·05	0·49 ± 0·03	0·48 ± 0·04
δ	0·03 ± 0·05	0·06 ± 0·05	0·06 ± 0·09
$\epsilon_{(P),260}$	6610 ± 90	9280 ± 40	2620 ± 50

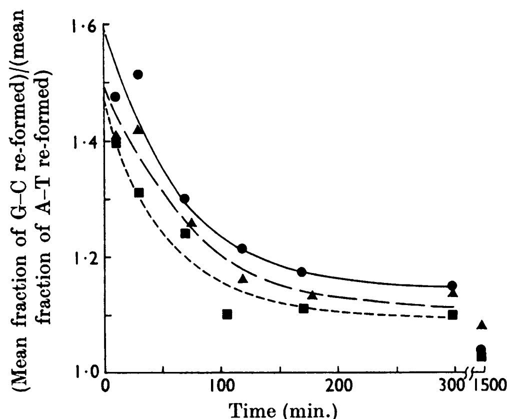


Fig. 4. Change of (mean fraction of G–C re-formed)/(mean fraction of A–T re-formed) with time as *E. coli* DNA (EC2), denatured in 1mM-NaCl at 100° for 10min., renatured by the standard procedure in 0·4M-NaCl at 60° (●——●), 70° (▲— —▲) and 80° (■----■). Spectra from 235 to 290 mμ, in 5 mμ increments, were recorded at intervals and the concentration, C, base composition, ϕ, and deviation from randomness, δ (see Fig. 5) of the renatured DNA calculated by the method described in the text.

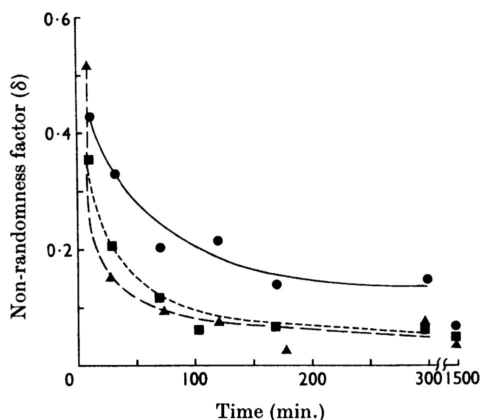


Fig. 5. Change of deviation from randomness, δ, with time as *E. coli* DNA (EC2), denatured in 1mM-NaCl at 100° for 10min., renatured by the standard procedure in 0·4M-NaCl at 60° (●——●), 70° (▲— —▲) and 80° (■----■).

assumption, some calculations were made on a hyperchromic spectrum. It was assumed that the spectrum was both changing and being scanned at constant rates, and so the extent of the change at any wavelength was proportional both to extinction at that wavelength and the time taken to reach that wavelength from the start of the scan. It was shown that C, ϕ and δ changed negligibly with the

Table 6. *Second-order rate constants for the re-formation of G–C, A–T and G–C+A–T during the renaturation of* E. coli *DNA (EC2) in 0·4M-sodium chloride at 60°, 70° and 80°*

DNA stock solution (0·6mg. of DNA/ml. in 1mM-NaCl) was denatured at 100° for 10min. and then about 0·1ml. was added directly to 2·5ml. of 0·4M-NaCl at 60°, 70° or 80°. The spectrum from 235 to 290 mμ, in 5 mμ increments, of each of the three cells was recorded soon after the DNA had been added and thereafter at intervals for up to 24hr., when the cells were heated to 100° and the spectrum was again recorded. The extents of re-formation of A–T and G–C and also of G–C+A–T were calculated as described in the text, and these results were analysed for second-order kinetic behaviour. Rate constants were also calculated from the change of R_t (at 260 mμ) with time. Each result is the average of three experiments, the spread of results about the mean being about ± 1 l. mole^{-1} sec.$^{-1}$ in all cases.

Temp.	k_2 (l. mole^{-1} sec.$^{-1}$) for re-formation of G–C	A–T	G–C + A–T	Change of R_t (260 mμ)
60°	6·8	4·5	2·7	2·3
70	8·8	7·4	4·0	3·8
80	7·0	5·4	3·1	3·0

rate of change of spectrum and rate of scanning actually used in the present experiments.

The values of C, ϕ and δ of the renatured DNA in solution at various times were therefore calculated. From these, the fractions of A–T and G–C re-formed (as fractions of total A–T and G–C in the solution) were obtained and the averages taken of three experiments at each temperature. Thus the value of (mean fraction of G–C re-formed)/(mean fraction of A–T re-formed) was calculated as a function of the time of renaturation, and the result is shown in Fig. 4. The values of δ for the three experiments were also averaged and these are given in Fig. 5.

It was also possible to examine the kinetics of re-formation of G–C, A–T and total (G–C+A–T) base pairs by assuming that the values of C and ϕ after 24hr. were the same as those for infinite time, and by using the same concentration terms etc. as defined previously (see the Kinetic Studies section). The kinetics of re-formation of G–C, A–T and total (G–C+A–T) base pairs were second-order with the rate constants given in Table 6. Rate constants were also calculated from the change of relative extinction at 260 mμ with time (Table 6), and these agree with those calculated for the re-formation of total base pairs.

The significant features in these observations are the initially very high values of both δ and the ratio of the mean fraction of G–C re-formed to

mean fraction of A–T re-formed. The values of δ and ϕ were reasonably reproducible in three distinct runs and the changes of these quantities with time were significant, though the very high values of δ may have affected the calculation of ϕ slightly. It is clear that the initially renatured DNA was different from native DNA in both its degree of randomness and in its base composition, and, with increasing times of renaturation, renatured DNA gradually approached native DNA in these two respects.

There is no reason to suppose that the values of k_2 for the re-formation of G–C and A–T are grossly inaccurate, and so it may be noted that G–C re-formed faster than A–T at all temperatures, although the rate was relatively slower at 70°, and 80°, than at 60°. There were insufficient results to calculate the values of the activation energy for the re-formation of G–C and A–T.

DISCUSSION

It is concluded from the spectrophotometric studies that the renatured DNA at equilibrium after 24hr. is very similar to native DNA, with respect to both its base composition (Fig. 4) and its degree of randomness (Fig. 5). This agrees with other studies, which have shown that renatured and native DNA have almost the same biological activity, 'melting' temperature, ultraviolet extinction (Doty *et al.* 1960; Marmur & Lane, 1960; Marmur & Doty, 1961) and buoyant density (Doty *et al.* 1960; Ageno, Dore & Frontali, 1966; Subirana, 1966) and appear similar in electron micrographs (Thomas & MacHattie, 1964). So the process with which we are here concerned can properly be regarded as renaturation.

Kinetics of renaturation. It was demonstrated that the process of renaturation was second-order for at least 5hr., though occasionally deviations occurred after about 3hr. All methods of analysis of the time-course of the process supported this conclusion, which was confirmed by the dependence of the initial rate of renaturation on the second power of the DNA concentration. Moreover, from the spectrophotometric studies it was concluded that G–C and A–T also re-formed individually by a second-order process, and that G–C re-formed more rapidly than A–T (Table 6). These results support the conclusions of Subirana & Doty (1966), Bolton *et al.* (1966) and Ageno *et al.* (1966) that the process is second-order throughout, rather than those of Cavalieri *et al.* (1962), and are more cogent than these earlier results for methodological reasons. Analysis of hypothetical reaction curves showed that the method used by Subirana & Doty (1966) could not have discriminated sufficiently between first-order and second-order to settle the matter. The studies of Bolton *et al.* (1966) are difficult to compare with others because they always used shear-degraded DNA and their graphical presentation of results does not allow a ready comparison with first-order kinetics. Ageno *et al.* (1966) used a different method of following renaturation, of alkali-denatured DNA, which was based on density-gradient analysis of the extent of renaturation, and their results provide valuable corroboration of our own, on a different basis.

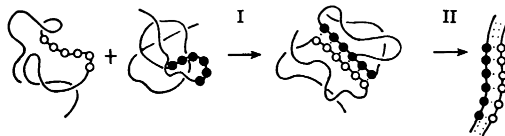


Fig. 6. Possible mechanism for the renaturation of DNA. Process I is a diffusion-controlled nucleation step to form a nucleus of a few base pairs (○•●) between complementary chains, and process II is a 'zipper-like' step to form a complete double helix from the nucleus.

Since the process is second-order it is therefore presumably bimolecular, through the coming together of two separated complementary strands and the formation of a hydrogen-bonded nucleus of a few base pairs between them (I, Fig. 6). Subsequently, 'intramolecular crystallization' would then spread from this nucleus with the formation of a complete double helix (II, Fig. 6). It is unlikely that this process II is rate-limiting for several other reasons. First, it is known that the rate constant for helix formation between complementary polynucleotide chains in high ionic strength solvents is very high indeed, of the order of 10^5l.mole^{-1}sec.$^{-1}$ (Ross & Sturtevant, 1960; Blake & Fresco, 1966), and that it decreases as the temperature approaches the 'melting' temperature (Ross & Sturtevant, 1960; Inman & Baldwin, 1964; Blake & Fresco, 1966). The rate constant of the renaturation of *E. coli* DNA is very much lower than this and it increases with temperature up to 5° below the 'melting' temperature. Secondly, the rate of renaturation falls as the viscosity of the solvent is increased (Table 3), indicating that the process is controlled by diffusion, which can only mean that process I, and not process II, is rate-determining.

There is also further evidence which positively supports the hypothesis that process I is rate-limiting. The observed rate constant increases only slowly with temperature, and consequently the activation energy is small (Table 4) and of the order of magnitude that is characteristic of diffusion-controlled reactions, namely 2–5kcal.mole^{-1} (Laidler, 1965). Any decrease of the rate of process II with temperature must be masked by the increase of the rate of process I, since process II is so much

faster than I under all conditions. Moreover, the decrease of the observed rate with increase in the viscosity of the solvent also indicates that the overall renaturation process is diffusion-controlled, and again therefore that I is rate-determining.

Usually the entropy of activation is very small for diffusion-controlled processes (Glasstone, Laidler & Eyring, 1941), but it is very negative in the present instance for reasons peculiar to the DNA system with its need to match complementary regions. Various factors combine to decrease the non-exponential term in the rate equation [see eqn. (5) below], which is equivalent to making $\Delta S^{\ddagger}$ large and negative. Thus the chance of complementary chains meeting, as required in process I, may be quite small and even when they do collide there is only a small probability that complementary sequences will come into register. There is also some loss of configurational entropy in process I, which would therefore be expected to occur with a negative value of ΔS [and low value of PZ, eqn. (5) below]. Hence it seems clear that process I, rather than II, is rate-determining.

Some observations of previous workers (Marmur & Doty, 1961; Wada & Yamagami, 1964; Bolton *et al.* 1966), which show that the rate of renaturation goes through a maximum with increase of renaturation temperature, are apparently a combination of the extent of renaturation, which in our view has such a maximum, and the rate of renaturation, which rises with temperature. The two do not seem to have been clearly distinguished since, in the kinetic plots, the DNA actually renaturing appears to have been taken as the total DNA present, i.e. the total [P] instead of our $(R_0 - R_\infty)[P]/0{\cdot}4$, whereas about 25%, in our runs, of the total DNA actually renatured during the rapid cooling to the temperature of the experiments and some of the DNA never renatured at all.

Relationship between the rate of renaturation and the source of the DNA. The energy of activation would be expected to be similar for all DNA samples, and the only factor that might alter the entropy of activation is the chance of complementary chains meeting. Taking the energy of activation to be the same (6·8 kcal.mole^{-1}) as for *E. coli* DNA it is possible to calculate $\Delta S^{\ddagger}$ values for *B. pertussis* DNA and T7-phage DNA (Table 7). Alternatively, one can use the equation:

$$k_2 = PZe^{-E/RT} \qquad (5)$$

where P is a steric, or probability, factor and Z is a collision number. Taking E as 6·8 kcal.mole^{-1} and the values of k_2 for renaturation in 0·4 M-sodium chloride at 65° given in Table 7, the values of PZ for the renaturation of *E. coli* DNA, *B. pertussis* DNA and T7-phage DNA can be estimated (Table 7). It is possible to calculate the chance of a strand colliding with its complement if the weight of all the DNA (assumed to be in one piece) per cell and the molecular weight of the extracted DNA is known, and if it is assumed that all the DNA strands have been degraded identically.

It is known that most of the strands of T7-phage DNA may be recovered as one linear helix (Davison & Freifelder, 1962), so that in a solution of the denatured DNA there are only two types of strands. It is relevant that the sedimentation coefficient for the present sample of T7-phage DNA (Table 1) agrees well with that obtained by other workers (Davison & Freifelder, 1962).

The sample of *B. pertussis* DNA had a molecular weight of $17{\cdot}4 \times 10^6$ (Table 1), and each cell contains between 6×10^8 and 3×10^9 daltons of DNA (Bacon *et al.* 1967), so that a solution of denatured DNA contains between 70 and 350 different types of strands. Similarly, *E. coli* DNA has a molecular weight *in vivo* of about 4×10^9 (Cairns, 1962) whereas that of the DNA extracted was $19{\cdot}1 \times 10^6$ (Table 1), so that there were about 420 different types of single-stranded DNA.

Thus in a solution of denatured T7-phage DNA the chance of two complementary strands meeting is 1 in 2, for *B. pertussis* DNA it is 1 in 70–350 and for *E. coli* DNA it is 1 in 420. These chances,

Table 7. *Relationship between complexity of DNA and rate constant of renaturation*

Values of $\Delta S^{\ddagger}$ and PZ were calculated from the values of k_2 by using eqns. (4) and (5) and assuming a value for E of 6·8 kcal.mole^{-1}. The relative chance of complementary strands meeting, $(P)_{rel.}$, was calculated as described in the text.

Source of DNA	k_2 (l. mole^{-1} sec.$^{-1}$)*	$\Delta S^{\ddagger}$ (cal. deg.$^{-1}$ mole^{-1})	PZ (l. mole^{-1} sec.$^{-1}$)	$(PZ)_{rel.}$†	$(P)_{rel.}$†
T7 phage	240 ± 40	$-30{\cdot}4 \pm 0{\cdot}2$	$(4{\cdot}4 \pm 0{\cdot}7) \times 10^6$	1	1
B. pertussis	18 ± 5	$-(35{\cdot}6 \pm 0{\cdot}6)$	$(3{\cdot}2 \pm 0{\cdot}8) \times 10^5$	$(7{\cdot}3 \pm 1{\cdot}8) \times 10^{-2}$	$(1{\cdot}8 \pm 1{\cdot}2) \times 10^{-2}$
E. coli	$1{\cdot}43 \pm 0{\cdot}02$	$-40{\cdot}5 \pm 0{\cdot}1$	$(2{\cdot}6 \pm 0{\cdot}1) \times 10^4$	$5{\cdot}9 \times 10^{-3}$	5×10^{-3}

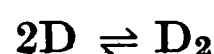
* k_2 for renaturation in 0·4 M-NaCl at 65°.
† Calculated relative to the value for T7-phage DNA.

expressed relative to T7-phage DNA are given in Table 7, together with the values of PZ, also expressed relative to T7-phage DNA. There is a surprisingly close correspondence between these last two columns of Table 7.

Extent and character of renaturation. The relative extent of renaturation was dependent on both temperature and on salt concentration, but was independent of DNA concentration (over a 14-fold range) and of the method of denaturation. The extent was maximal at 65–75° (Table 2; and Fig. 1 of Thrower & Peacocke, 1966), which accords with earlier observations (Marmur & Doty, 1961) that the optimum temperature of renaturation is about 25° below the 'melting' temperature. The extent of renaturation increased with salt concentration, as was to be expected, since renaturation depends on the close approach of very negatively charged polynucleotide chains.

A fairly consistent proportion of the DNA (about 25%) renatured quickly during the rapid cooling from the temperature of denaturation to that of renaturation. The decrease in extinction could only have been followed by rapid-rate methods. It is possible that some strands are held together by interchain bonds, which may well be the covalent bonds of the type detected in *Bacillus subtilis* DNA (Alberts, 1968; Alberts & Doty, 1968) and *H. influenzae* DNA (Chevallier & Bernadi, 1968; Mulder & Doty, 1968). They might also be residual interchain hydrogen bonds. In any case the spectrophotometric results clearly indicated that the G–C/A–T molar ratio (Fig. 4) in renatured DNA, soon after the beginning of renaturation, was about 1·5 (i.e. a molar base composition of 60% G–C) and also that the non-randomness factor, δ, of this DNA was unusually high (Fig. 5). This suggests that certain regions of the original DNA have a higher-than-average G–C content, and that these regions have longer runs of G–C than would be expected from a random distribution. Thus any interchain linkages, whether covalent or hydrogen bonds, must join regions of high G–C content so that, on cooling, the strands so joined re-form very quickly by a 'zipper-like' mechanism in a manner similar to that observed with 'reversible' DNA (Geiduschek, 1961).

The state of the DNA solution after 24hr. at the renaturation temperature is very stable and must represent a position of equilibrium characteristic of that solvent and temperature, even though it has been attained in two stages by an initial rapid cooling from the denaturation to the renaturation temperature, followed by a slow second-order process of renaturation. The equilibrium can be represented as:



$$2D \rightleftharpoons D_2$$

where D represents separated strands of denatured DNA and D_2 represents double-helical DNA, and the corresponding equilibrium constant is:

$$K = [D_2]/[D]^2$$

Since $[D] = (R_\infty - 1)[P]/0{\cdot}4$ and $[D_2] = (1{\cdot}4 - R_\infty)[P]/0{\cdot}4$, K can be written as:

$$K = \frac{0{\cdot}4(1{\cdot}4 - R_\infty)}{[P](R_\infty - 1)^2} \quad (6)$$

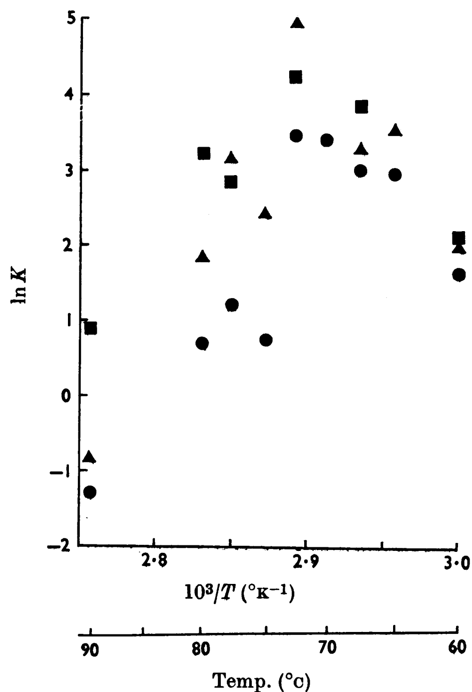


Fig. 7. Plot of ln (equilibrium constant, K) against reciprocal of the absolute temperature for renaturation in 0·4M-NaCl (●), 0·6M-NaCl (▲) and 1·0M-NaCl (■). Values of K were calculated from eqn. (6), by using the values of R_∞ given in Table 2. When $R_\infty \leqslant 1{\cdot}00$ (because of experimental error) values of K are meaningless and these points have been omitted from the Figure.

Hence K can be calculated from the experimental results covering the temperature range 60–90° with 0·4M-, 0·6M- and 1·0M-sodium chloride as solvent, and in Fig. 7 $\ln K$ is plotted against the reciprocal of the absolute temperature. The points display a maximum at about 70°. Since the slope of this plot is equal to $-\Delta H/\boldsymbol{R}$, where ΔH is the enthalpy of double-helical DNA minus that of denatured DNA, it must be concluded that ΔH is positive up to about 70°, zero near to this temperature and negative at higher temperatures. Hence, $\partial(\Delta H)/\partial T = \Delta C_p$ is negative, where ΔC_p is the corresponding difference

in heat capacities. The heat capacity of denatured, single-stranded, DNA is therefore always greater than that of double-helical DNA at 60–90° in these salt solutions. This is to be expected, since the motion of the separated polynucleotide chains is more rotational and translational, with a correspondingly greater C_p, than that of the double-helical structure, in which rotational and translational modes must be replaced by vibrational, since the two strands are then cross-linked by hydrogen bonds. This is in itself an adequate explanation of the sign of ΔC_p, of the change of ΔH with temperature and also therefore of the existence of a maximum in the variation of K with temperature. An analogous thermodynamic situation arises with the carboxylic acids, whose dissociation constants also have a maximum at a particular temperature (Hambly, 1965).

Note added after submission. Since the preparation of this paper was completed another investigation on the renaturation of DNA has been published (Wetmur & Davidson, 1968). The authors agree that the process is second-order and further state that the rate constant reaches a maximum at 15–30° below the 'melting' temperature, T_m. We have already made clear why we think it necessary to use as 'c_0' in the kinetic analysis the concentration of the DNA that is actually renaturing in the observed process, since there is undoubtedly a rapid (first-order?) renaturation during the shock-cooling that inevitably precedes renaturation. We observed that the rate of renaturation increases with temperature, but that the extent, which represents a kind of equilibrium position, passes through a maximum. Nevertheless, we have applied the method of calculation of Wetmur & Davidson (1968) to our results, and thus have taken R_0 to be 1·4 and R_∞ as 1·0. The value of k_2 calculated on this basis then has a maximum at about 70°. To this extent our observations agree with theirs, but our interpretations differ since we regard this maximum as the effect of combining an increasing rate and an extent with a temperature maximum, factors that are not distinguished by their method of calculation. They also observed that the rate constant increased slightly with increasing G–C content of the DNA sample. In this connexion, we note that G–C were observed to renature faster than A–T (Table 6). The results in Table 7 also agree with theirs that the rate of renaturation is inversely related to the complexity of the DNA sample.

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