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Molecular Hybridization of Ribonucleic Acid with a Large Excess of Deoxyribonucleic Acid

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When RNA is annealed in solution with a sufficiently large excess of DNA, the kinetics of DNA–RNA hybridization are relatively simple. Methods are described for following the course of both DNA renaturation and DNA–RNA hybridization in this system. To explore the characteristics of the reaction a series of model systems was used. Each one utilized DNA (sheared to constant size) from a bacterium or bacteriophage and homologous cRNA, i.e. RNA synthesized *in vitro* on a template of the same DNA. Temperature optima were determined for the hybridization of *Escherichia coli* nucleic acids in 2×SSC and 3×SSC–50% formamide buffers, and of *Proteus mirabilis* nucleic acids in 2×SSC buffer. Rate-constants for DNA–RNA hybridization were measured by two methods. These gave somewhat different results, but in all cases the rate-constant of DNA–RNA hybridization was clearly less than that of DNA renaturation. Thus hybridization is a slower reaction than DNA renaturation. Nevertheless, in some cases, with a high concentration of DNA and a long annealing time, 90–95% of the added RNA became resistant to ribonuclease. Experiments are described which show that it is possible to deduce the analytical complexity of DNA with reasonable accuracy from its hybridization with complementary RNA. Similarly, it is possible to estimate the reiteration frequency of multiple DNA sequences (such as ribosomal DNA) from the hybridization of the total DNA with RNA complementary to the multiple sequences. The effect on the system of various DNA/RNA ratios from 100 to 1 is described.

In most cases it is not possible to isolate the complementary strands of DNA, and molecular hybridization experiments have to be done when both DNA strands are present. When the DNA is annealed with a similar quantity of RNA, the ensuing reaction is very complex, with RNA and DNA sequences competing for the same complementary DNA sites. For this reason several methods have been developed to prevent DNA renaturation. Even the most successful of these imposes a severe limit on the concentration of DNA that can be used. This presents no problems for work with viral DNA, but for work with higher organisms, which contain a very large number of different DNA sequences, the limitation on DNA concentration is an important restriction.

In looking for a solution to this problem it was discovered that if DNA and RNA are annealed together in solution, provided the concentration ratio of DNA sequences to complementary RNA sequences is very high, the kinetic treatment of the DNA–RNA hybridization reaction becomes more straightforward, and the time and concentration dependence of hybridization is related in a fairly simple way to that of DNA renaturation. In principle, it should be possible to estimate the rate constant of DNA renaturation from the hybridization of complementary RNA sequences provided that a sufficient excess of the DNA is added. This result could be of great value for the study of DNA organization in higher organisms. For example, by hybridizing a specific species of RNA to the total DNA it should be possible to deduce the reiteration frequency of the DNA sequences to which the RNA is complementary.

The results of a series of experiments that were done to investigate the properties of the system are given in the present paper. For these experiments the relatively simple DNA complements of bacteria and bacterial viruses were used. In most cases, the DNA was annealed with RNA synthesized *in vitro* on a template of native complementary DNA. For convenience the method is termed 'hybridization in vast DNA excess'. A preliminary account of the method has already been published (Melli *et al.*, 1971). Experiments with excess of DNA have also been described by Gelderman *et al.* (1971).

Methods

Preparation of nucleic acids

DNA was prepared from the bacteria *Escherichia coli* M.R.E. 600 and *Proteus mirabilis* PM-1, from bacteriophage T4 and from bacteriophage λ. Bacterial DNA was purified as follows. First, 100g of packed cells, harvested during exponential growth, was resuspended at 0°C in 1 litre of 0.1M-NaCl–2mM-EDTA–10mM-tris–HCl buffer, pH7.5 at 0°C, and

50ml of lysozyme solution (10mg/ml in the same buffer) was added. After 5min at 0°C the suspension was transferred to a water bath at 37°C. When the temperature of the suspension reached 10°C, 100ml of 10% (w/v) sodium dodecyl sulphate in water was added, followed by 50ml of Pronase solution (8mg/ml in water). Complete lysis occurred within 30min, then 250ml of 5M-$NaClO_4$, 100ml of 3M-tris–HCl buffer, pH8.5 at 20°C, and 300ml of water-saturated redistilled phenol were added, and the mixture was shaken on a rotary shaker at 37°C for 20min. Chloroform (300ml) was added and the mixture shaken by hand for 10min. The aqueous and phenol phases were separated by centrifugation at 8000*g* for 30min and the aqueous phase was removed and kept. The phenol phase was discarded, and the interphase was resuspended in 400ml of 1% (w/v) sodium lauryl sulphate in 0.3M-tris–HCl buffer, pH8.5 at 20°C, by blending for 2–3s in the MSE Ato-Mix. Then 100ml of 5M-$NaClO_4$ and 200ml of water-saturated phenol were added and the mixture shaken as before. Then 200ml of chloroform was added and after shaking for 10min the aqueous phase was removed as before. The aqueous phases were pooled and the DNA was spooled out after adding an equal volume of ethanol, and dissolved in 400ml of DSC buffer (DSC buffer is a 1-in-10 dilution of SSC buffer; SSC buffer is 0.15M-NaCl–15mM-trisodium citrate, pH7.5). The solution was dialysed against 2×SSC (twofold-concentrated SSC buffer) with $ZnCl_2$-treated dialysis tubing (see Frame & Bishop, 1971) until the phenol concentration was negligible. Heat-treated ribonuclease (10mg/ml, boiled for 7min) was added to a final concentration of 100μg/ml, and the solution was dialysed against 20vol. of 2×SSC buffer for 16h at 37°C. Pronase solution (8mg/ml) was added to a final concentration of 0.4mg/ml, and the solution was incubated for 1h at 37°C. To the digest 0.25vol. of 5M-$NaClO_4$ and 0.1vol. of 3M-tris–HCl buffer, pH8.5 at 20°C, were added and extraction with phenol and chloroform was done as before. After centrifugation the aqueous phase was removed and the remainder discarded. The aqueous phase was re-extracted twice more with phenol and chloroform and then dialysed exhaustively first against 2×SSC buffer, then against DSC buffer. The solution was passed through a French Pressure Cell (Ivan Sorvall and Co., Norwalk, Conn., U.S.A.) at 345$MN \cdot m^{-2}$ (50000lb/in^2) to shear the DNA, the temperature at the needle valve being kept between 5 and 15°C. The sheared DNA was precipitated by adding 0.1vol. of 2M-sodium acetate, pH5, and 2vol. of ethanol. After being left overnight at −20°C the precipitate was collected by centrifugation and dissolved in 20ml of water. The solution was adjusted to a vol. of 40ml in 0.3M-NaCl – 0.01M-sodium acetate buffer, pH5.5, and passed, in 10ml lots, through a column (2.5cm× 35cm) of Sephadex SE-50 or SP-50 [Pharmacia (G.B.) Ltd., London W.5, U.K.], that was washed and developed with the same buffer. The peak eluted at the void volume was collected and precipitated by adding 2vol. of ethanol and then leaving overnight at −20°C. The precipitate was collected by centrifugation, drained for 30min to remove ethanol, and dissolved in DSC buffer to a final concentration greater than 20mg/ml. The final preparation was completely free from alkali-labile and cold-acid-soluble material. The single-strand molecular weight of several preparations was measured by the method of Studier (1965). Molecular weights from 160000 to 180000 were observed.

To prepare bacteriophage DNA a suspension of purified bacteriophage in 0.1M-NaCl–2mM-EDTA–10mM-tris–HCl buffer, pH7.5, was adjusted to 1M-$NaClO_4$ and 0.1vol. of 3M-tris–HCl buffer, pH8.5, was added. A portion (10ml) of this mixture was placed in a 250ml conical flask in an ice bath and 5ml of water-saturated redistilled phenol was added. The flask was swirled gently by hand intermittently for 10min, then 5ml of chloroform was added and the contents of the flask were mixed, then immediately centrifuged for 10min at 10000*g*. The phenol–chloroform layer was removed and the aqueous layer and interphase were returned to the conical flask. Extraction with phenol and chloroform and centrifugation were repeated twice. After the final centrifugation the aqueous phase was gently removed and dialysed exhaustively first against 2×SSC buffer then against DSC buffer. The DNA was then passed through the French Pressure Cell and through Sephadex as described for bacterial DNA.

cRNA (RNA synthesized *in vitro*) was synthesized on a template of unsheared DNA and purified as described by Melli & Bishop (1969) with the following modifications: RNA polymerase was purified from *E. coli* by the procedure of Chamberlin & Berg (1962), then subjected to glycerol-gradient centrifugation at a low salt concentration as described by Burgess (1969); the labelled substrate was [α-^{32}P]ATP from The Radiochemical Centre, Amersham, Bucks., U.K., specific radioactivity 100–1000Ci/mol; 1.6mM-K_2HPO_4 was added to the reaction mixture. ^{32}P-labelled ribosomal RNA from *E. coli* was prepared from cells labelled with [^{32}P]P_i and 'chased' with unlabelled P_i for two generations. The cells were collected by centrifugation and 1–2g was resuspended in 100ml of ice-cold 0.45M-sucrose–1mM-EDTA–15mM-tris–HCl buffer, pH8 at 0°C, containing 0.5mg of egg-white lysozyme/ml. After 10min at 0°C, the spheroplasts were collected by centrifugation at 10000*g* for 10min. The spheroplasts were resuspended in 10ml of ice-cold 0.1M-KCl–10mM-$MgCl_2$–5mM-tris–HCl buffer, pH7.5 at 0°C, containing 5μg of polyvinyl sulphate/ml and homo-

genized briefly in a power-driven Teflon-in-glass homogenizer. Dextran sulphate (0.1ml of a 5mg/ml solution) was added and cell debris was removed by centrifugation for 10min at 15000*g*. Ribosomes were collected from the supernatant by centrifugation for 2h at 60000rev./min (260000$g_{av.}$) and 0°C in the M.S.E. 10×10 titanium rotor, and resuspended in 1ml of 30mM-KCl–2mM-$MgCl_2$–10mM-tris–HCl buffer, pH7.5 at 0°C. To this was added 4ml of a solution containing 6% sodium 4-aminosalicylate, 1% NaCl, 1% sodium tri-isopropyl naphthalene sulphonate and 6% butan-2-ol (Parish & Kirby, 1966) and 5ml of a mixture of water-saturated redistilled phenol (100ml), *m*-cresol (10ml) and 8-hydroxyquinoline (0.1g). The mixture was shaken for 5min at room temperature and then centrifuged for 10min at 10000*g* and 0°C. The aqueous layer was removed and re-extracted as before with the phenol–cresol mixture. After centrifugation 2vol. of ethanol was added and after 1h at 0°C the RNA precipitate was collected by centrifugation. The pellet was dissolved in 4ml of 0.2M-sodium acetate, pH5, and immediately 2vol. of ethanol was added. After 1h at 0°C the RNA was again collected by centrifugation and again dissolved and reprecipitated as before. Finally the pellet was washed twice with 10ml of ethanol–ether (1:2, v/v), drained and dissolved in 1ml of ice-cold 0.1M-NaCl–2mM-EDTA–10mM-tris–HCl buffer, pH7.5 at 0°C, containing 0.5% sodium dodecyl sarcosinate (Geigy Industrial Chemicals Corp., Ardsley, N.Y., U.S.A.). Then 0.5ml of this was layered on a 16ml 5–20% (w/v) sucrose density gradient based on the same buffer, and centrifuged for 24h at 24000rev./min (69000$g_{av.}$) and 0°C in the M.S.E. 6×15ml swing-out rotor. The 16S and 23S RNA peaks were located, pooled and precipitated with 2vol. of ethanol.

DNA renaturation and DNA–RNA hybridization

Either DNA alone or DNA and RNA together, dissolved in DSC buffer, were heated for 5min in a boiling-water bath to effect complete denaturation, and a control sample was immediately withdrawn and diluted greatly with ice-cold DSC buffer. The remainder was placed in an oil bath at the temperature chosen for annealing, and after 30s the salt concentration was adjusted. This moment was taken as zero time. At subsequent times samples were diluted into ice-cold water or dilute SSC buffer to give a final salt concentration equal to that of DSC buffer in each case. In most cases two annealing mixtures were used, with DNA concentrations of 0.5 and 5mg/ml. For experiments without formamide, sodium dodecyl sulphate was added in the first instance in an amount such that at the time of ribonuclease treatment (see below) the concentration would not exceed 0.01%. In experiments with formamide, all of the formamide needed was added before denaturation. Salt concentrations were adjusted by using a convenient portion of a suitable dilution of 20×SSC buffer that had been adjusted to pH5.5 (measured with a pH meter) with HCl. When diluted tenfold the pH of the solution (2×SSC buffer) was 6.3–6.4. In special cases a stock solution of 5M-$NaClO_4$–0.075M-trisodium citrate was used. When diluted fivefold the pH of the solution (1M-$NaClO_4$) was 6.8. Oil-bath temperatures were precisely adjusted and monitored by means of thermistor probes (G. H. Zeal and Co., London S.W.19, U.K.) that were compared routinely with thermometers calibrated by the National Physical Laboratories, Teddington Middx., U.K.

Thermal denaturation

DNA and RNA were mixed, denatured by heat (see above) and annealed to $C_0t = 1500$–3000 in 2×SSC buffer at 70°C. The mixture was chilled and a sample was suitably diluted and adjusted to the required solvent concentrations. Portions of this sample were dispensed and controls were kept cold or put in a boiling-water bath for 7min. Other portions were kept at different temperatures for 7min. All heated samples were immediately chilled and diluted with ice-cold water or water and DSC buffer to a final salt concentration equal to that of DSC buffer.

Estimation of the proportion of DNA in the duplex form

The procedure was the same for renaturation and denaturation experiments. The samples, in DSC buffer, were adjusted with DSC buffer to a concn. of 25–50μg of DNA/ml. The spectrum of each sample was recorded from 240 to 320nm, first at 50°C, then at 90°C in a Unicam SP.800 spectrophotometer equipped with a constant-temperature cell-housing. The extinction of the sample at 260nm was taken from the two recordings, giving $E_{260(50)}$ and $E_{260(90)}$. When formamide was present $E_{270(50)}$ and $E_{270(90)}$ were used. Between 50°C and 90°C in DSC buffer the increase in extinction at 260 or 270nm is mainly due to denaturation of DNA duplexes (but see below). As a first measure, the proportion of the total DNA in the form of duplex d/C_0 was calculated from:

$$d/C_0 = [E_{(90)} - E_{(50)}]/[H/(1+H)]E_{(90)} \qquad (1)$$

where H is the proportional increase in extinction of native DNA from the same source treated in the same way [i.e. $(1+H) = E_{(90)}/E_{(50)}$ for native DNA]. H was found to equal 0.39, 0.38 and 0.42 respectively for *P. mirabilis* DNA at 260nm, *E. coli* DNA at 260nm, and *E. coli* DNA at 270nm.

Estimation of the proportion of RNA hybridized

Again, the same procedure was used for denaturation and renaturation experiments. The sample was adjusted to 2×SSC and four equal portions were removed. To two of these, 20μg of pancreatic ribonuclease was added/ml. All four portions were incubated for 20min at 37°C and then chilled in ice. Bovine serum albumin solution (5mg/ml) was added to give a final concentration of 100 or 200μg/ml, and cold 50% trichloroacetic acid was added to a final concentration of 7%. After 20min in ice the precipitates were collected on 3cm diameter glass-fibre filters (Whatman GF/C) and washed with 10ml of cold 5% trichloroacetic acid. The filters were transferred to 20ml vials, and these were placed in a vacuum oven under reduced pressure at 80°C for 20min. Toluene-based scintillation-counting fluid (15ml) was added and the radioactivity of the samples was determined by using a Packard liquid-scintillation counter. As a first measure, the proportional hybridization was taken to be the ratio of the sum of the ribonuclease-treated samples to the sum of the untreated samples.

Rate constant of DNA–RNA hybridization from initial-rate measurements

The annealing mixtures were prepared and sampled and the amount of hybrid formed was estimated as described above, except that final DNA concentrations of 25 and 50μg/ml were used and annealing times were chosen so that less than 5% of the DNA would renature during the experiments. Rate constants were calculated from the relationship (Nygaard & Hall, 1964):

$$k^h = (dH/dt)/R_0 \cdot C_0 \quad (2)$$

where dH/dt is the initial rate of incorporation of RNA into ribonuclease-resistant hybrid, k^h is the rate constant of DNA–RNA hybrid formation and R_0 and C_0 are the initial concentrations (mol of nucleotide/l) of the RNA and DNA respectively. As suggested by Nygaard & Hall (1964) it is convenient to express dH/dt and R_0 in arbitrary units of RNA quantity. The experiments with *E. coli* nucleic acids in 2×SSC buffer and in 3×SSC–50% formamide buffer were performed by taking a series of samples at different times for each temperature. In the experiment with *P. mirabilis* nucleic acids two samples were taken at zero time and two after 14min of annealing.

Kinetics of DNA–RNA hybridization in vast DNA excess (Melli et al., 1971)

The course of the DNA renaturation reaction may be described by the equation:

$$d/C_0 = 1 - [1/(k^d C_0 t + 1)] \quad (3)$$

where d/C_0 is the proportion of the DNA incorporated into duplex at time t (d is the concentration of DNA duplex, C_0 the initial DNA concentration, both expressed as mol of nucleotide/l). k^d is the rate-constant of DNA-renaturation. The course of DNA–RNA hybridization is described by the analogous equation:

$$r/R_0 = 1 - [1/(k^d C_0 t + 1)^{k^h/k^d}] \quad (4)$$

where r/R_0 is the proportion of RNA incorporated into hybrid at time t, and k^h is the rate-constant of DNA–RNA hybridization. For convenience r/R_0 is sometimes called the 'hybridization level'.

As shown by Britten & Kohne (1966, 1968) when d/C_0 is plotted against $\log(C_0 t)$, an ideal second-order reaction generates a sigmoid curve. All ideal second-order reactions generate a curve of the same shape, and the position of each relative to the abscissa [$\log(C_0 t)$] is determined mainly by the analytical complexity of the DNA under study. Each curve can be conveniently characterized by the value of $C_0 t$ at which d/C_0 equals one half (called $C_0 t_{\frac{1}{2}}^d$).

When r/R_0 is plotted against $\log(C_0 t)$ a sigmoid curve is again obtained. In this case the shape of the curve is determined by the ratio (k^h/k^d), called the 'k ratio' (Melli *et al.*, 1971). Each curve can be partly characterized by the value of $C_0 t$ at which r/R_0 equals one half (called $C_0 t_{\frac{1}{2}}^h$).

k^d and $C_0 t_{\frac{1}{2}}^d$ are related by the equation (Britten & Kohne, 1966, 1968):

$$k^d = 1/C_0 t_{\frac{1}{2}}^d \quad (5)$$

and k^h and $C_0 t_{\frac{1}{2}}^h$ by the equation:

$$k^h = (k^d \log 2)/\log(k^d C_0 t_{\frac{1}{2}}^h + 1) \quad (6)$$

When compared with homologous renaturation curves, hybridization curves are generally displaced to higher values of $C_0 t$. The displacement may be described by the '$C_0 t$ ratio', which is the ratio $C_0 t_{\frac{1}{2}}^h / C_0 t_{\frac{1}{2}}^d$.

Results

Normalization

Heated and quenched samples never give a completely zero value of either hybridization or renaturation. Also, neither reaction is ever complete at the highest $C_0 t$ values. An example is shown in Fig. 1(*a*). DNA from *P. mirabilis* was annealed with cRNA synthesized *in vitro* on a template of *P. mirabilis* DNA. Such RNA contains sequences complementary to all or nearly all of the *P. mirabilis* DNA sequences. Most important for the present purpose, it does not contain a large proportion of RNA (Robinson *et al.*, 1964).

The heated and quenched samples give an apparent renaturation of about 0.13 and an apparent hybrid-

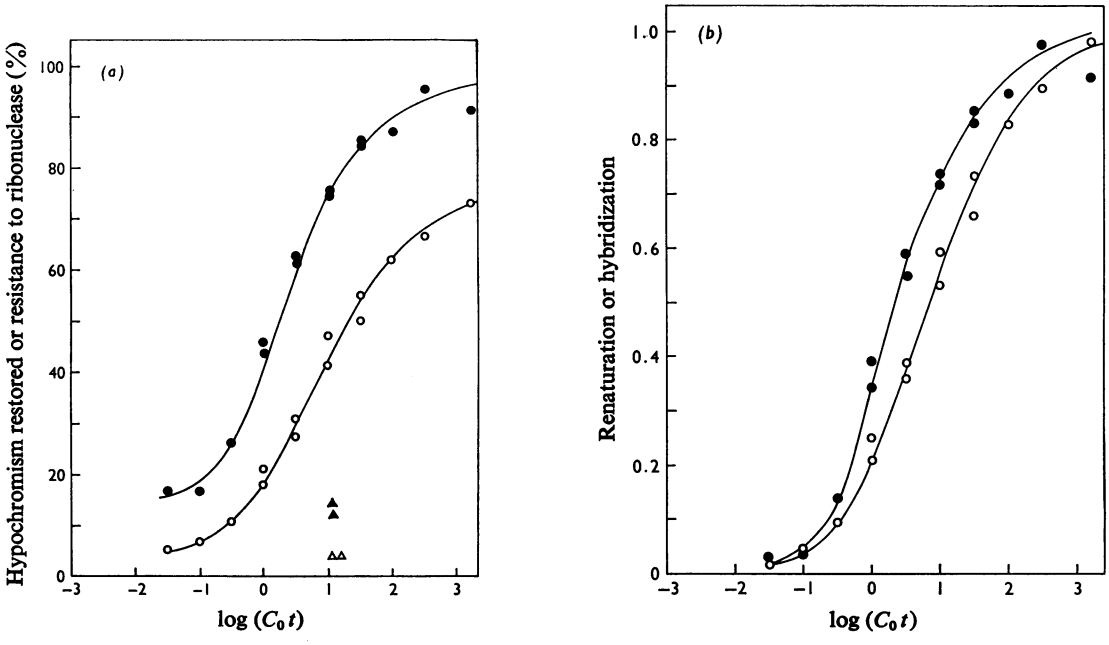

Fig. 1. *Renaturation of P. mirabilis DNA, and hybridization with homologous cRNA in vast DNA excess*

(*a*) shows the results, (*b*) the same results after normalization as described in the text. ●, Renaturation; ○, hybridization; ▲ and △, corresponding controls that were heated and quenched. DNA/RNA ratio 10000; 2×SSC buffer at 65°C.

ization of about 0.04. The apparent renaturation may be partly due to the rapid renaturation of reiterated RNA sequences (Chiscou & Kohne, 1970), to renaturation during handling and to the renaturation of sequences held in register by cross-linkage (Alberts & Doty, 1968). The main effect here, however, is the hypochromism of single-stranded DNA at 50°C at a Na^+ concentration of 0.02 g-equiv./l (Studier, 1969). This is demonstrated by the experiment shown in Fig. 2. DNA from *E. coli* at a concentration of 50 μg/ml or 1 mg/ml was heated and quenched in DSC buffer and then transferred to a spectrophotometer at 50°C. After temperature equilibration the temperature was steadily raised to 92°C at a rate of 2°C/min and readings were made. There was a steady rise in the E_{260}, levelling off at about 90°C. There is no indication in Fig. 2 of the sharp transition at 72–73°C shown by native and renatured *E. coli* DNA in this solvent (Doty *et al.*, 1960; Marmur & Doty, 1962).

A hybridization level of 0.03 in the quenched sample is typical of experiments with cRNA and with cellular mRNA. Values were between 0.02 and 0.05. rRNA gave a value of approx. 0.25. Samples heated and quenched in the absence of DNA showed a similar degree of ribonuclease resistance in every case. The effect is evidently caused by ribonuclease-resistant radioactive material in these RNA preparations.

Fig. 1(*a*) also shows that neither reaction goes to completion. This is a well-known feature of DNA renaturation, and is probably due to some sequences becoming inaccessible to further renaturation during the reassociation of randomly sheared molecules.

In this case about 75% of the RNA becomes ribonuclease-resistant at the highest C_0t values. This was not significantly changed by increasing the salt concentration during enzyme digestion from 2×SSC to 5×SSC, by decreasing the ribonuclease concentration from 20 to 1 μg/ml, or by lowering the digestion temperature from 37° to 20°C. There are several possible reasons for this effect. (1) The formation of RNA 'tails', for one reason or another, at the ends of paired sequences. (2) Partial ribonuclease sensitivity of hybridized RNA. (3) The presence of polyribonucleotide sequences that are not complementary to sequences present in the DNA. (4) The failure of some RNA sequences to hybridize because thermal scission makes them too small. In the experiments to be described here, *E. coli* cRNA (about 50% G+C) and ribosomal RNA (about 53% G + C) showed about 95% resistance to ribonuclease after annealing to high C_0t values, *P.*

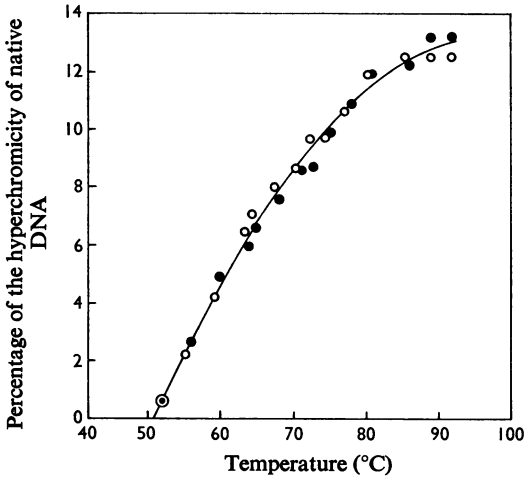

Fig. 2. *Dependency of the extinction (E_{260}) of denatured E. coli DNA on temperature*

E. coli DNA in DSC buffer was heated in a boiling-water bath for 7min at a concentration of 50μg/ml (●) or 1mg/ml (○). The 50μg/ml sample was chilled in an ice bath, and the 1mg/ml sample was diluted with 19vol. of ice-cold DSC buffer. Both samples were brought quickly to 50°C and placed in the spectrophotometer. The temperature of the solutions was raised at the rate of 2°C/min and the E_{260} was recorded intermittently.

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mirabilis (38% G+C) and bacteriophage λ (50% G+C) cRNA showed about 75% resistance, and bacteriophage T4 cRNA (34% G+C) about 60% resistance. There is evidently no correlation between ribonuclease resistance and base composition.

In all cases we have made the assumption that the DNA or RNA observed to be incorporated into the duplex form accurately reflects the course of the reaction. Thus we take the $C_0t_{\frac{1}{2}}$ of the reaction to be the C_0t value at which renaturation or hybridization is half-way between the quenched control and the value reached at a high C_0t. It is important that the determination of the latter value is subjective (Fig. 1*a*) and this will necessarily introduce some error into the estimation of $C_0t_{\frac{1}{2}}^{d}$ and particularly $C_0t_{\frac{1}{2}}^{h}$.

To make clearer the relationships between DNA renaturation and DNA–RNA hybridization under different conditions, the results have in many cases been normalized by setting the difference between the quenched control value and the estimated final value to equal 1. An example is shown in Fig. 1(*b*) This figure demonstrates the general characteristics of hybridization in vast DNA excess. The hybridization reaction is slower than the renaturation reaction. In the experiments reported here $C_0t_{\frac{1}{2}}^{h}$ is always greater than $C_0t_{\frac{1}{2}}^{d}$ and k^h is always less than k^d.

Estimation of the rate constants from renaturation and hybridization results

Curves drawn like those shown in Fig. 1 are a very useful visual representation of the results. However, their evaluation is often difficult because of the subjective element involved in positioning the curve. It is well-known that eqn. (3) may be rearranged in several ways to give a linear relationship between D (the concentration of single-stranded DNA at time t) and C_0t. These have the disadvantage that at low values of D large errors in estimation are encountered. Unfortunately, the same points contribute disproportionately to the slope of the line because D bears a reciprocal relationship to C_0 and t. A compromise that greatly decreases this effect is given by the relationship:

$$\log(d/D) = \log C_0t + \log k^d \qquad (7)$$

Here, k^d may be obtained from the intercept on the $\log C_0t$ axis of the plot of $\log(d/D)$ against $\log C_0t$. Also, $C_0t_{\frac{1}{2}}^{d} = 1/k^d$ may be obtained as the value of C_0t at which $d/D = 1$ and $\log(d/D) = 0$. All ideal second-order renaturation reactions have the same slope, the location of the line relative to the abscissa being determined by the value of k^d. (In the same way all ideal second-order reactions have the same shape when d is plotted against $\log C_0t$, and the position of the curve on the $\log C_0t$ scale is determined by k^d (Britten & Kohne, 1966, 1968).

If $\log(d/D)$ is plotted against $\log C_0t$, deviations from ideal second-order kinetics immediately become obvious. An example is shown in Fig. 3. Deviations are most marked in the early and late parts of the reaction. The logarithmic ordinate scale compresses renaturation values from 0.09 to 0.91 between ordinate values of -1 and $+1$. If these values only are considered, the estimate will reflect the progress of the major part of the reaction. Very similar estimates of $C_0t_{\frac{1}{2}}^{d}$ are obtained by fitting the best line to the values between $\log(d/D) = -1$ and $\log(d/D) = 1$ and by fitting the ideal second-order reaction line to the same values. A least-squares estimate is not required here, since:

$$\log k^d = (1/N)[\sum \log(d/D) - \sum \log(C_0t)] \qquad (8)$$

where N is the number of determinations for which $-1 < \log(d/D) < 1$.

There is no rearrangement of eqn. (4) analogous to eqn. (8). The following linear equation, however:

$$k^d \cdot \log(R_0/R) = k^h \cdot \log(k^d \cdot C_0t + 1) \qquad (9)$$

allows the determination of k^h from the plot of $k^d \cdot \log(R_0/R)$ against $\log(k^d \cdot C_0t + 1)$. R represents

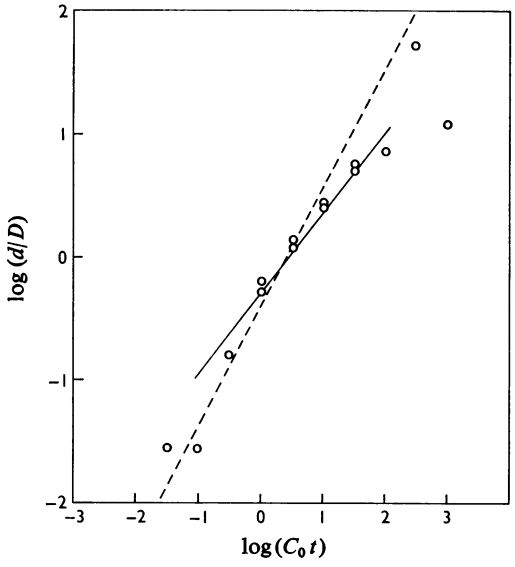

Fig. 3. *Double-logarithmic plot of DNA renaturation*

The values, for *P. mirabilis* DNA, are taken from Fig. 1. The broken line has the slope that would be expected if the reaction were ideally second-order, and is fitted by eye. The solid line is fitted by least-squares to the values between $\log(d/D) = -1$ and $\log(d/D) = +1$.

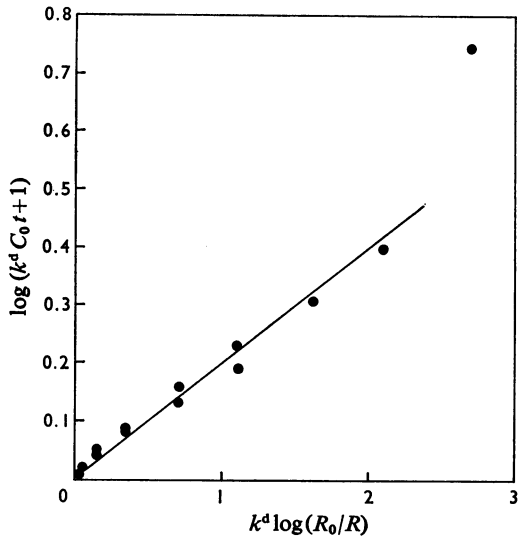

Fig. 4. *Double-logarithmic plot of DNA–RNA hybridization*

The values, for *P. mirabilis* DNA and cRNA, are taken from Fig. 1.

the concentration of non-hybridized RNA at time t. In this case low values of R tend to produce the greatest errors. The best statistical estimate of k^b is given by:

$$k^h = k^d \sum \log(R_0/R) / \sum \log(k^d \cdot C_0 t + 1) \quad (10)$$

An example plotted according to eqn. (9) is shown in Fig. 4. Usually, beyond a value of $R = 0.1$ the points become considerably more scattered. For this reason k^h was estimated from eqn. (10) by using only values of $\log(R_0/R)$ less than 1.

Temperature-dependence of hybridization in 2×SSC buffer: E. coli DNA and cRNA

The temperature optima for DNA renaturation and DNA–RNA hybridization are closely related to the mean melting temperature T_m of the DNA (Marmur & Doty, 1961; Nygaard & Hall, 1964). The thermal stability of DNA–RNA hybrid in 2×SSC buffer was measured by using a quenched assay. That is, after a time at elevated temperature, each sample was chilled in ice and diluted to a final salt concentration equal to that of DSC buffer. Quenched and direct assays of DNA denaturation give different results (Geiduschek, 1962), although the difference in T_m is rather small (1–2°C) when the DNA, like that used in these experiments, is small in size. As a control, renatured DNA duplex was melted in a parallel quenched assay. The results are shown in Fig. 5. The renatured DNA duplex 'melted' with a T_m of 95°C, which is 1–2°C lower than the T_m expected for native DNA of the same size in a quenched assay. The first part of the curve shows a more gradual decrease in hyperchromicity, indicating imperfect base-pairing. About 60% of the total duplex seems to be perfectly base-paired. The DNA–RNA hybrid 'melted' slightly less sharply, with a T_m of 91°C, 4°C below the DNA T_m. This is in agreement with measurements made on DNA–RNA hybrid synthesized enzymically on a template of single-stranded bacteriophage φX174 DNA (Chamberlin & Berg, 1964), and suggests that the hybrid duplexes are as well, or nearly as well, matched as the DNA duplexes under these annealing conditions. The hybrid begins to 'melt' at about 70°C in 2×SSC buffer and about 10% is melted at 80°C.

Rate constants of DNA renaturation are also shown in Fig. 5. The 60° and 70°C points were determined directly, and the others estimated relative to these from the values of Wetmur & Davidson (1968), which show the optimum temperature for DNA renaturation to be about 25°C below the T_m of the DNA.

Rate constants of DNA–RNA hybridization were

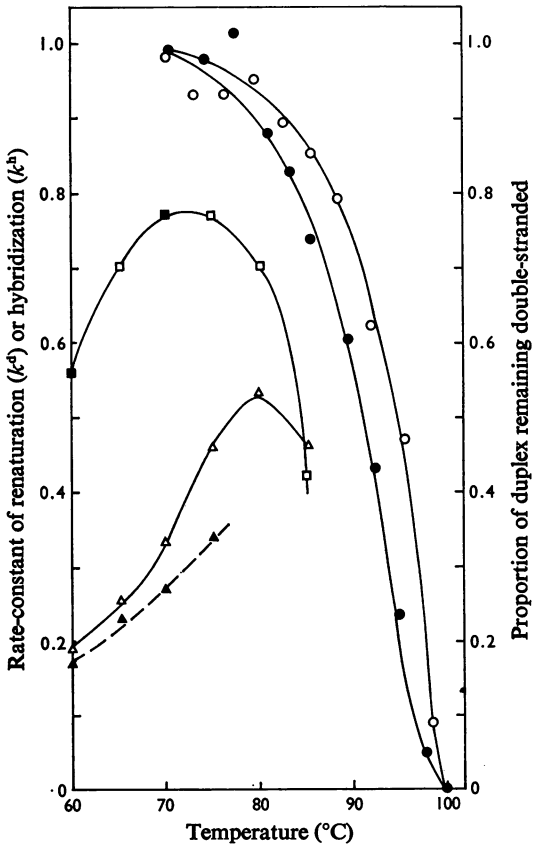

Fig. 5. *Temperature-dependence curves for E. coli nucleic acid in 2×SSC buffer*

Melting profiles of DNA duplex (○) and DNA–RNA hybrid (●) were determined on duplexes formed by annealing. The rate constant of DNA renaturation was determined at 60° and 70°C (■) and the remaining points were interpolated from the results of Wetmur & Davidson (1968). Rate constants of DNA–RNA hybridization were measured by initial-rate kinetics (△) and from hybridization results with vast DNA excess (▲).

determined both from initial reaction rates and from hybridization experiments with vast DNA excess. From the initial reaction rates the temperature optimum is at about 80°C, 15°C below the DNA T_m, and 8–10°C above the optimum temperature for DNA renaturation. This is in agreement with the findings of Nygaard & Hall (1964). The optimum temperature is thus only about 10°C below the T_m of the DNA–RNA hybrid, and, as shown above, 10% of the hybrid formed at 70°C is melted at 80°C.

Rate constants of DNA–RNA hybridization determined from experiments with vast DNA excess are lower than those determined from initial-rate experiments. The optimum temperature cannot be determined because the reaction is incomplete at 80°C (75%) and 85°C (55%). The most rapid complete reaction was observed at 75°C. Evidence presented below shows that the optimum temperature for the hybridization of (G+C)-rich sequences is higher than for (A+T)-rich sequences. This provides a possible explanation for the difference between the rate constants determined by the two methods. At each temperature some sequences will hybridize more rapidly than others. The rapidly hybridizing sequences will affect most the value of the rate-constant obtained from the initial reaction rate, but as the reaction proceeds they will be more rapidly depleted and the reaction rate will fall.

For interpretation of hybridization experiments with vast DNA excess the most useful measurements are $C_0t_{\frac{1}{2}}^h$ and the C_0t ratio (the ratio $C_0t_{\frac{1}{2}}^h/C_0t_{\frac{1}{2}}^d$). The values found for *E. coli* DNA and cRNA are listed in Table 1. At a given DNA concentration the midpoint of the hybridization reaction is reached at 75°C in about one-third of the time required at 60°C.

P. mirabilis DNA and cRNA

The method of hybridization with vast DNA excess was developed with a view to studying DNA–RNA homologies in higher organisms. The DNA of higher organisms has a rather high A+T content (approx. 60%). The melting temperature and optimal renaturation temperature of DNA is markedly dependent on its A+T content, suggesting that the same might be true of DNA–RNA hybridization. *P. mirabilis* DNA was chosen as a model because its A+T content is 62% (Marmur & Doty, 1962) and the renaturation rate of DNA from *Proteus* species is similar to that of *E. coli* (Kohne, 1968; Frame & Bishop, 1971; Bak *et al.*, 1970). The results of experiments done in 2×SSC buffer with *P. mirabilis* DNA and homologous cRNA are shown in Fig. 6. The mean melting temperature of the renaturated DNA duplex, 90°C, bears the expected relationship to the T_m of the *E. coli* DNA duplex (Marmur & Doty, 1962). The T_m of the DNA–RNA hybrid, 84°C, is 5°C lower than that of the DNA duplex. The optimum hybridization temperature, from the initial reaction rate, is about 70°C. This is about 14°C below the T_m of the DNA–RNA hybrid, a difference rather greater than that found for *E. coli*. Rate constants from experiments with vast DNA excess were determined at 65° and 70°C. Again they were well below the initial-rate determinations. The $C_0t_{\frac{1}{2}}$ measurements are listed in Table 2.

Table 1. *Characteristics of DNA renaturation and DNA–RNA hybridization: E. coli DNA and cRNA in 2×SSC buffer*

The values given in parentheses were calculated relative to the other values for the same parameters from the results of Wetmur & Davidson (1968).

Temperature (°C)	$C_0t_{\frac{1}{2}}^{d}$ (mol·l^{-1}·s)	$C_0t_{\frac{1}{2}}^{h}$ (mol·$_{DNA}$l^{-1}·s)	C_0t ratio	k^d (l·mol^{-1}·s^{-1})	k^d (l·mol^{-1}·s^{-1})	k ratio
60	1.8	16.1	9	0.56	0.17	0.30
65	(1.4)	11.5	8.2	(0.70)	0.23	0.33
70	1.3	8.1	6.3	0.77	0.27	0.35
75	(1.3)	5.1	3.9	(0.77)	0.34	0.44

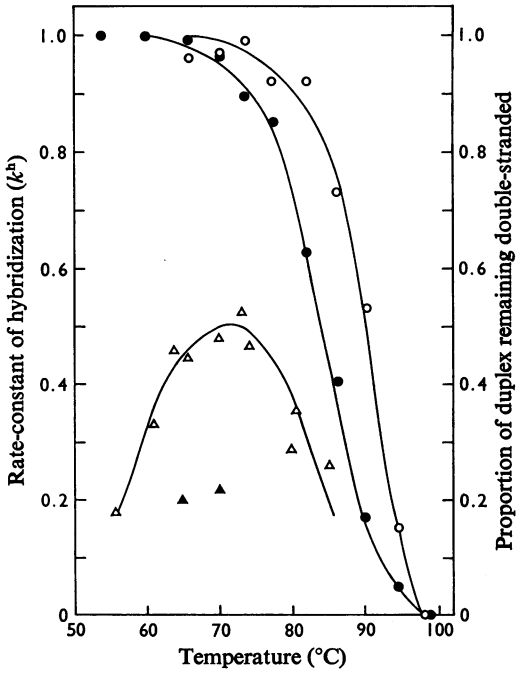

Fig. 6. *Temperature-dependence curves for P. mirabilis nucleic acids in 2×SSC buffer*

'Melting' profiles of DNA duplex (○) and DNA–RNA hybrid (●) were determined on duplexes formed by annealing. Rate constants of DNA–RNA hybridization were measured by initial rate kinetics (△) and from hybridization results with vast DNA excess (▲).

Effect of formamide

The denaturing effect of formamide lowers the temperature optimum of DNA denaturation and DNA–RNA hybridization (Helmkamp & T'so, 1961; Marmur & T'so, 1961; Bonner *et al.*, 1967; McConaughy *et al.*, 1969). If the rate of the reaction is not too greatly decreased, the use of formamide might be advantageous in hybridization experiments because RNA degradation will be less at lower temperatures. The effect of using 50% formamide in 3×SSC buffer was studied, with the results shown in Fig. 7.

The T_m of renatured *E. coli* DNA in the quenched assay was found to be 61°C. Compared with the T_m in 2×SSC buffer, this agrees well with the relationship established by McConaughy *et al.* (1969): a decrease in T_m of 0.72°C for each 1% of formamide present. Renatured *P. mirabilis* DNA has a T_m of 57°C, in reasonable agreement with the relationship. Surprisingly, in both cases the T_m of the DNA–RNA hybrid was higher than the T_m of the DNA duplex, 4°C higher in the case of *E. coli*, 2°C higher in that of *P. mirabilis*. This corresponds to a decrease of roughly 0.6°C for each 1% of formamide.

The renaturation rate-constant of *E. coli* DNA between 40° and 50°C was found to be 0.33–0.35, just under two times less than at 70°C in 2×SSC buffer.

From the initial reaction rates, the temperature optimum of DNA–RNA hybridization is close to 50°C, about 15°C below the T_m of the DNA–RNA hybrid and 11°C below the T_m of the DNA duplex.

In this case the temperature optimum is sufficiently far below the T_m of the hybrid that it allows complete reaction of the *E. coli* cRNA in conditions of vast DNA excess (Fig. 8). The rate constant calculated from this experiment is about half that calculated from the initial reaction rate.

The $C_0t_{\frac{1}{2}}$ values are listed in Table 3. At the temperature optimum $C_0t_{\frac{1}{2}}^{h}$ is 20, or about 4 times greater than the $C_0t_{\frac{1}{2}}^{h}$ in 2×SSC buffer at 75°C. Thus to reach any given stage in the reaction, with the same DNA concentration, requires a 4 times greater duration in 3×SSC–50% formamide buffer at 50°C.

Effects of analytical complexity

Doty *et al.* (1960) and Marmur & Doty (1961) showed the effect of analytical complexity on DNA renaturation and detailed studies were done by

Table 2. *Characterization of renaturation and hybridization: P. mirabilis DNA and cRNA in 2×SSC buffer*

Temperature (°C)	$C_0t_{\frac{1}{2}}^d$ (mol·l^{-1}·s)	$C_0t_{\frac{1}{2}}^h$ (mol·$_{DNA}$l^{-1}·s)	C_0t ratio	k^d (l·mol^{-1}·s^{-1})	k^h (l·mol^{-1}·s^{-1})	k ratio
65	2.5	6.5	2.6	0.4	0.22	0.55
70	2	8.5	4.2	0.5	0.21	0.42

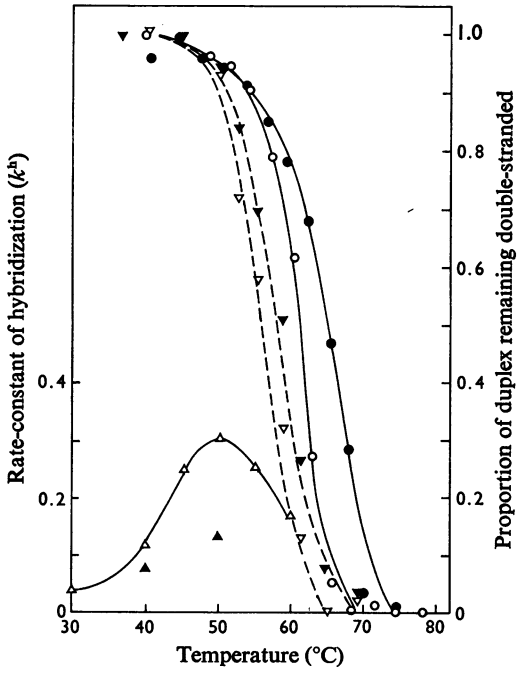

Fig. 7. *Temperature-dependence curves for P. mirabilis and E. coli nucleic acids in 3×SSC–50% formamide buffer*

'Melting' profiles of DNA duplex (○, *E. coli*; ▽, *P. mirabilis*) and DNA–RNA hybrid (●, *E. coli*; ▼, *P. mirabilis*) were determined on duplexes formed by annealing. Rate constants of DNA–RNA hybridization for the *E. coli* nucleic acids were measured by initial rate kinetics (△) and from hybridization results with vast DNA excess (▲).

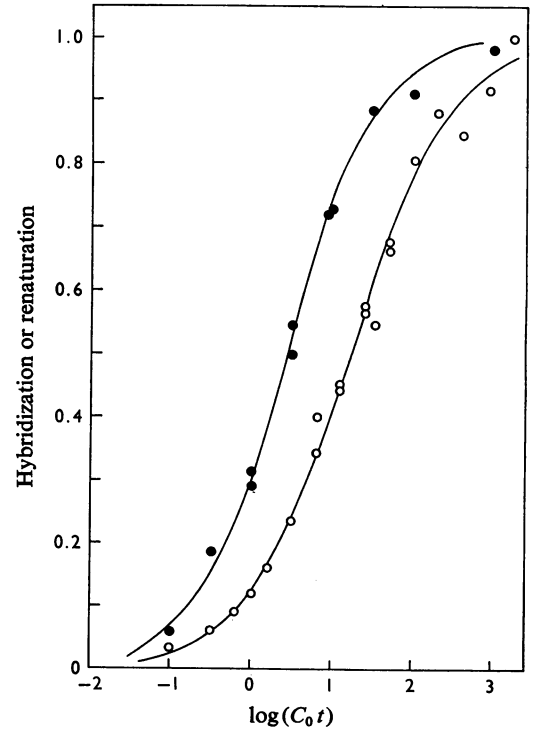

Fig. 8. *Renaturation and hybridization of E. coli DNA and cRNA in 3×SSC–50% formamide buffer at 50°C*

●, Renaturation; ○, DNA–RNA hybridization at a DNA/RNA ratio of 4500. The results have been normalized.

Wetmur & Davidson (1968) and Britten & Kohne (1966, 1968). Similarly Laird & McCarthy (1968) and Bishop (1969*a*) showed the effect of analytical complexity on DNA–RNA hybridization. The effect ought to be apparent in the system with vast DNA excess, and is, as shown by Fig. 9. In this experiment, bacteriophage λ DNA, bacteriophage T4 DNA and *E. coli* DNA were hybridized with homologous cRNA in vast DNA excess, and the results were normalized in the usual way. The general rule that a lower analytical complexity gives a faster reaction is clearly obeyed. A more detailed analysis is shown in Table 4. Interestingly, as observed by Bishop (1969*a*), the hybridization relationship between bacteriophage λ and *E. coli* reflects almost perfectly their analytical complexities, while the rate of hybridization found with bacteriophage T4 is unexpectedly high.

To examine this observation further, the renaturation of bacteriophage T4 DNA was studied. As shown in Table 4 the renaturation of this DNA was slower than expected from its analytical complexity (in agreement with Wetmur & Davidson, 1968;

Table 3. *Characteristics of renaturation and hybridization: E. coli DNA and cRNA in 3×SSC–50% formamide buffer*

Temperature (°C)	$C_0 t_{\frac{1}{2}}^{d}$ (mol·l^{-1}·s)	$C_0 t_{\frac{1}{2}}^{h}$ (mol·$_{DNA}$l^{-1}·s)	$C_0 t$ ratio	k^d (l·mol^{-1}·s^{-1})	k^h (l·mol^{-1}·s^{-1})	k ratio
30	6.0	160	27	0.17	0.035	0.21
40	3.0	64	21	0.33	0.075	0.23
50	2.8	20	7.1	0.35	0.13	0.37

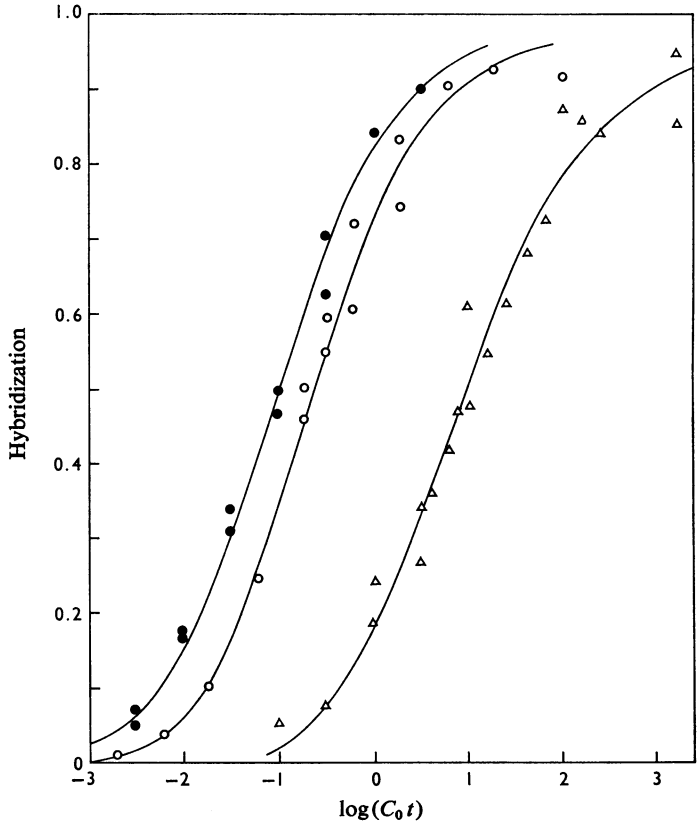

Fig. 9. *Effect of analytical complexity on DNA–RNA hybridization in vast DNA excess*

Bacteriophage λ (●), bacteriophage T4 (○) and *E. coli* (△) DNA were separately annealed with homologous cRNA in 2×SSC buffer at 70°, 65° and 70°C respectively. The results have been normalized.

Flavell & Jones, 1970). This is understandable in terms of the dependence of DNA renaturation on (G+C)-content (Wetmur & Davidson, 1968; but see Gillis *et al.*, 1970).

The low renaturation rate and high hybridization rate of bacteriophage T4 DNA and cRNA reflect the fact that k^d and k^h are very similar in this case ($k^d = 10$; $k^h = 6.3$). It has been assumed here that this reaction is anomalous, due perhaps to the unusual base-composition and glucosylation of bacteriophage T4 DNA.

Effect of DNA sequence reiteration

It follows that, provided that a vast excess of complementary DNA sequences is present, RNA sequences complementary to reiterated DNA sequences should hybridize more rapidly than

Table 4. *Characteristics of renaturation and hybridization: bacteriophage T4 and bacteriophage λ DNA and homologous cRNA in 2×SSC buffer*

	Temperature (°C)	$C_0t_{\frac{1}{2}}^d$ (mol·l^{-1}·s)	$C_0t_{\frac{1}{2}}^h$ (mol·$_{DNA}$l^{-1}·s)	C_0t ratio	k^d (l·mol^{-1}·s^{-1})	k^h (l·mol^{-1}·s^{-1})	k ratio
Bacteriophage T4	65	0.10	0.20	2	10	6.3	0.63
Bacteriophage λ	70		0.10				

Values relative to *E. coli* system (set as 1)

	Analytical complexity	$C_0t_{\frac{1}{2}}^d$	$C_0t_{\frac{1}{2}}^h$	k^d	k^h
Bacteriophage T4	0.044	0.077	0.025	13	23
Bacteriophage λ	0.011		0.012		

non-reiterated RNA and DNA sequences. The *E. coli* DNA sequences complementary to ribosomal RNA are reiterated between 5 and 10 times (Gillespie & Spiegelman, 1965; Attardi *et al.*, 1965; Kennell, 1968). The hybridization of 16S and 23S ribosomal RNA is shown in Fig. 10. The two species of RNA have the same hybridization curve. From the $C_0t_{\frac{1}{2}}^h$, relative to that of cRNA, the reiteration frequency of the ribosomal DNA sequences is calculated to be 6–7. This compares with a value of 7–8 found in experiments done in 1×SSC buffer at 65°C (Melli *et al.*, 1971).

Effect of the DNA/RNA ratio

Experiments with *E. coli* DNA and 16S rRNA were done to obtain an estimate of what 'vast DNA excess' actually means in practice. With the assumption that there are seven 16S rRNA cistrons/genome equivalent of *E. coli* DNA, annealing mixtures were prepared with DNA/RNA complementary sequence ratios of 70, 7 and 1.4 (Fig. 11). Two effects are seen. First, as the sequence ratio falls, the percentage of the RNA that becomes hybridized at the highest C_0t values also falls. This effect is quite clear at a sequence ratio of 7, and is very marked at a ratio of 1.4. Secondly, the apparent $C_0t_{\frac{1}{2}}$ of the observed reaction becomes smaller as the sequence ratio is decreased. This means that failure to reach an effective vast excess of DNA will result in an overestimation of k^h. The results of these experiments are summarized in Table 5.

Pulse-labelled RNA from E. coli

In a growing culture of *E. coli* 97–98% of the total RNA is of stable types (rRNA and tRNA) and 2–3% is mRNA (Lieve, 1965; Mangiarotti & Schlessinger, 1967). During a short pulse of radioactive uracil, however, only two-thirds of the newly synthesized RNA is rRNA and tRNA, and one-third is mRNA (Mangiarotti & Schlessinger, 1967).

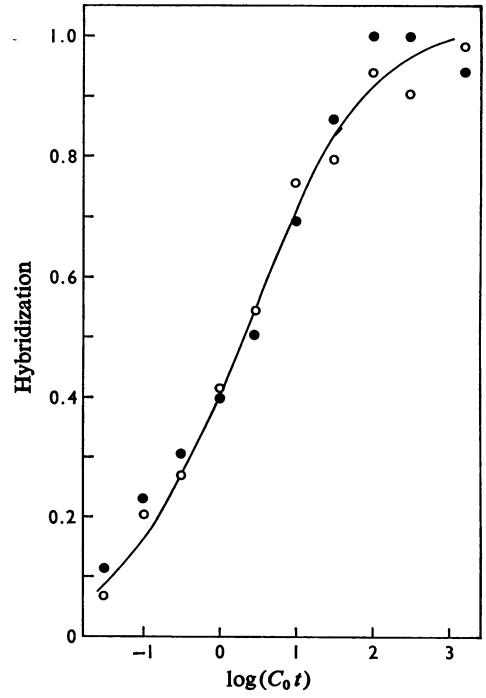

Fig. 10. *Hybridization of E. coli DNA with E. coli rRNA*

○, 16S rRNA; ●, 23S rRNA. In each case the ratio of complementary DNA to RNA sequences was approximately 140:1. The results have been normalized.

Total RNA extracted from such cells thus contains 97–98% of stable RNA with a low specific radioactivity and 2–3% of mRNA with a high specific radioactivity.

The rRNA and tRNA are complementary to a very small part of the total DNA (approx. 0.5%),

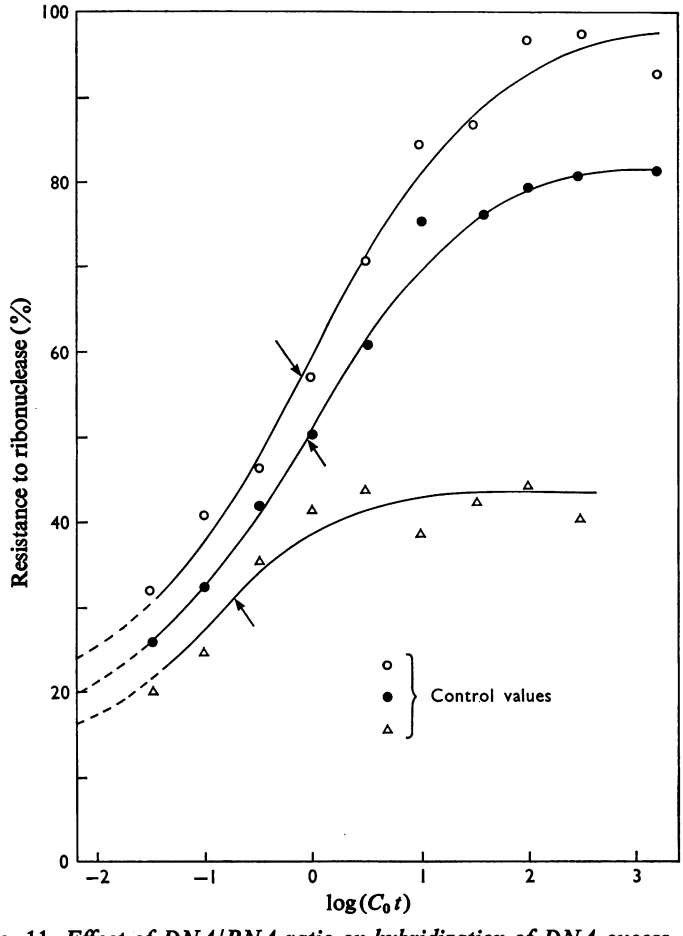

Fig. 11. *Effect of DNA/RNA ratio on hybridization of DNA excess*

E. coli DNA was annealed with *E. coli* 18S rRNA (2×SSC buffer at 70°C) at complementary DNA/RNA sequence ratios of 70:1 (○), 7:1 (●) and 1.4:1 (△), calculated on the assumption that there are 7 rDNA cistrons per 2.7×10^9 daltons of DNA. The mid-point of each transition is indicated by an arrow.

whereas the mRNA is complementary to a large part (14–28%; Bishop, 1969*b*). This makes it possible to mix the DNA and RNA in such proportions that the stable RNA is in excess of the complementary DNA sequences while at the same time the DNA sequences complementary to the mRNA are in excess. Under these circumstances only a negligible amount of stable RNA will become hybridized, and the mRNA will closely follow the kinetics of vast DNA excess. Assuming, for example, that mRNA is complementary to 14% of the DNA (a minimum assumption) and stable RNA to 0.5%, that 3% of the cellular RNA is mRNA and 97% is stable RNA; then, with a gross DNA/RNA ratio of 10:1, the overall DNA/RNA sequence ratios is 0.02 for the stable RNA and 50 for the mRNA.

An experiment with total cellular RNA from *E. coli* pulse-labelled with radioactive uracil is shown in Fig. 12. About 40% of the RNA became hybridized at a DNA/RNA ratio of 10:1. The $C_0t_{\frac{1}{2}}^h$ value of the reaction, 6, was slightly less than found with cRNA (8.1).

Discussion

The purpose of the work presented in this paper is to define suitable conditions for DNA/RNA hybridization experiments in vast DNA excess, and to provide a basis for the interpretation of results obtained with complex systems.

The work is mainly concerned with temperature

Table 5. *Effect of varying the DNA/RNA complementary sequence ratio*

The experimental hybridization curves are shown in Figs. 11 and 12.

Nominal complementary sequence ratio DNA/RNA	Percentage of the RNA hybridized at high C_0t (approximate)	$C_0t_{\frac{1}{2}}^h$ of the observed reaction
140	95	1.2
70	95	0.8
7	82	0.8
1.4	44	0.2

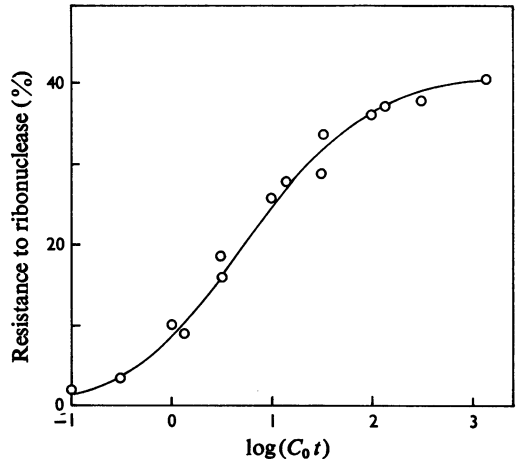

Fig. 12. *Hybridization of pulse-labelled E. coli RNA with the rRNA in excess of the complementary rDNA*

Only the mRNA hybridizes to a significant extent. The annealing conditions were 2×SSC buffer at 70°C. The overall DNA/RNA ratio = 10.

optima. The 2×SSC buffer was chosen as a compromise between lower and higher salt concentrations. At lower salt concentrations the reaction is slower, although the precision of base-pair formation is probably greater. At higher salt concentrations the reaction is more rapid, but the precision of base-pairing is probably less. In the case of higher organisms, base-pairing precision is an important consideration.

The most relevant measurements are summarized in Table 6. The hybridization of *E. coli* cRNA with *E. coli* DNA has twice as great a $C_0t_{\frac{1}{2}}^h$ in 1×SSC buffer at 65°C as in 2×SSC buffer at 70°C. The $C_0t_{\frac{1}{2}}^h$ is nearly the same in 2×SSC buffer at 75°C as in 1 M-$NaClO_4$ at 70°C. These temperatures are 20° and 22°C respectively below the DNA T_m (Wetmur & Davidson, 1968). At the temperature optimum (50°C) in 3×SSC–50% formamide buffer, the $C_0t_{\frac{1}{2}}^h$ is greater than in 1×SSC buffer at 65°C, and about 4 times greater than in 2×SSC buffer at 75°C. Unless the rate of thermal breakage of RNA is more than 4 times less in 3×SSC–50% formamide buffer at 50°C than in 2×SSC buffer at 75°C, no advantage will be gained by using formamide.

The mean (G+C) content of the DNA of vertebrates is about 42%, but individual messenger RNA species may diverge quite widely from this value. The (G+C)-content of duck haemoglobin mRNA, for example, is reported to be 50–58% (Scherrer & Marcaud, 1968; Attardi *et al.*, 1970). There is a special problem in interpreting results where the (G+C) content of the reactive species is not known. The results shown in Table 6 offer a compromise in cases where the (G+C) content is between 38 and 50% (assuming that the analytical complexity of *E. coli* and *P. mirabilis* DNA is similar). Although 70°C is not optimum in either case, the $C_0t_{\frac{1}{2}}^h$ values for the two species are close (8.1 and 8.5). Sequences within this range of G+C content will therefore follow a very similar hybridization pattern. The C_0t ratios are also similar (6.3 and 4.2). If a C_0t ratio of 5 is used to calculate $C_0t_{\frac{1}{2}}^d$ from the $C_0t_{\frac{1}{2}}^h$ of an RNA sample within this range of G+C content, an accuracy of ±20% may be expected.

Further experiments are needed to define conditions for RNA sequences outside this range. However, the information obtained with *E. coli* may be used as an approximate guide. A 10% increase in G+C content gives an increase in T_m of 4.1°C (Marmur & Doty, 1962). Annealing sequences with 60% G+C at 70°C is therefore roughly equivalent to annealing the *E. coli* system at 65°C. Accordingly, the observed $C_0t_{\frac{1}{2}}^h$ will be about 1.5 times too great and $C_0t_{\frac{1}{2}}^d$ will be incorrectly estimated by about the same factor. Sequences with a G+C content below 38% will also hybridize more slowly. Furthermore, the reaction will be incomplete to an extent that depends on the precise G+C content of individual sequences.

One factor of potential importance that has not yet been investigated is the effect of the molecular size of the RNA molecules on the reaction rate. Because DNA can always be sheared to the same size, its molecular size is not a practical problem in these

Table 6. *Summary of $C_0t_{\frac{1}{2}}$ values*

Organism	Annealing conditions: Buffer	Temperature (°C)	$C_0t_{\frac{1}{2}}^{d}$ (mol·l^{-1}·s)	$C_0t_{\frac{1}{2}}^{h}$ (mol·$_{DNA}$l^{-1}·s)	C_0t ratio
E. coli	1×SSC	65	4	15.9	4.0
	2×SSC	70	1.3	8.1	6.3
	2×SSC	75	1.3	5.1	3.9
	1 M-$NaClO_4$	70	0.74	5.2	7.0
	3×SSC–50% formamide	50	2.8	20	7.1
P. mirabilis	2×SSC	65	2.5	6.5	2.6
	2×SSC	70	2.0	8.5	4.2

experiments. In the calibration experiments described here, in which cRNA was used, the RNA is relatively constant in size, with a mean sedimentation coefficient of 5–6S (mol.wt. approx. 50000). RNA isolated from cells and tissues, on the other hand, varies very widely in size.

The susceptibility of RNA to breakage presents problems to an analysis of the effect of its size on the rate of hybridization, especially because initial-rate measurements do not agree well with measurements made at later stages of the reaction. At the same time, provided the rate of breakage per nucleotide is constant, a relatively high rate will tend to bring different sizes of RNA molecules quite quickly to a similar size. For example, if the rate is 1 break/h per 1500 nucleotides, after 10h RNA molecules with an initial molecular weight of 2×10^6 will have an average molecular weight of about 5×10^4. In the same time molecules with an initial molecular weight of 5×10^4 will decrease in size to approx. 2.5×10^4. The ratios at the outset and after 10h are 40 and 2 respectively.

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