The Theory of the Deoxyribonucleic Acid–Ribonucleic Acid Hybridization Reaction

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A general equation is derived describing data of DNA–RNA hybridization in the presence of a competing self-annealing reaction of RNA. The well known double-reciprocal relation and the Scatchard equation are shown to be limiting cases of this general equation. Some new hybridization data at various temperatures are presented and analysed by using the new equation. The results can only be explained if we assume that the behaviour of DNA towards single RNA molecules is the same as that towards the annealed form, (RNA)₂. The variation of the equilibrium constant of the hybridization reaction with temperature is small, indicating a small heat of reaction. The maximum amount of hybridized RNA at equilibrium appears to be independent of temperature.

DNA–RNA hybridization data are usually obtained by annealing for a constant time a constant amount of DNA, immobilized on membrane filters, with various amounts of RNA in solution. It was empirically found that a double-reciprocal plot, i.e. a plot of $1/[\text{RNA hybridized}]$ against $1/[\text{RNA added}]$, is a useful way of organizing and presenting these data, since it usually generates a straight line. Lavallé & de Hauwer (1968) gave a sound explanation of this by assuming that, after a certain time, a chemical equilibrium is established between the DNA–RNA hybrid on the one hand, and free DNA and RNA on the other. By equating the rates of the forward and reverse reactions at equilibrium and making use of the approximation $[\text{RNA added}] - [\text{RNA hybridized}] \simeq [\text{RNA added}]$ they derived the linear double-reciprocal relationship theoretically.

The above equilibrium interpretation, however, was criticized by Bishop (1970), who argued mainly on the basis of the discrepancies between the value of the equilibrium constant, K , calculated as the ratio of rate constants k_{-1}/k_{+1} of the reverse and forward reactions, and the value of K found by direct estimation from the equilibrium concentration of the hybrid. The latter was determined either after 24 h of annealing or by extrapolation to infinite time. The discrepancy between k_{-1}/k_{+1} and the direct estimate of K after 24 h of annealing was attributed to non-establishment of equilibrium after 24 h, whereas three alternative explanations were offered by Bishop (1970) for the discrepancy between k_{-1}/k_{+1} and the value of K found from the extrapolated concentration of the hybrid. It was concluded that the equilibrium interpretation of the linear reciprocal relationship is unsatisfactory.

We, however, suggest a fourth explanation for the

discrepancy described above, without discarding or even weakening the equilibrium interpretation. This explanation is based on the principle of microscopic reversibility (Laidler, 1965). It is a conclusion of this principle that the ratio of rate constants in forward and reverse directions is equal to the equilibrium constant, provided that we are dealing with an elementary reaction. For a complex reaction this is true only if the measured rate constants are those that apply when the system is at equilibrium. The ratio of initial rate constants for the forward and reverse reactions is not in general equal to the equilibrium constant when the mechanism of the reaction is complex. Initial rate constants were used by Bishop (1970) in his ratio k_{-1}/k_{+1} , and there is evidence (Spiegelman *et al.*, 1973) that the mechanism of the DNA–RNA hybridization reaction is complex. Thus the discrepancy between k_{-1}/k_{+1} and K is easily explained without abandoning the idea of an equilibrium condition, established after certain time.

Marsh & McCarthy (1973), adopting the equilibrium condition, analysed hybridization data by using the method of Scatchard (1949) for the binding of small ions by proteins. This leads to a linear plot of $[\text{RNA hybridized}]/[\text{RNA free}]$ against $[\text{RNA hybridized}]$ and has certain advantages over the double-reciprocal plot, especially when RNA samples are heterogeneous or where non-specific reactions are present.

In the present paper we develop a more general equation describing hybridization data in the presence of a competing self-annealing reaction of RNA. The double-reciprocal relation and the Scatchard equation are shown to be limiting cases of this general equation. Some new hybridization data at various temperatures are presented and analysed by using the

new equation derived. Thus the variation with temperature of the equilibrium constant of DNA-RNA hybridization is studied for the first time.

Theory

Consider an RNA solution in contact with denatured strands of complementary DNA adsorbed on filter paper. We assume that after a certain time two simultaneous equilibria are established:

The first of these represents the hybridization reaction, and the second is the self-annealing reaction of RNA.

Equilibrium constants K and K' for the two reactions (1) and (2), respectively, can be expressed in terms of the following: c_1 = equilibrium concentration of RNA in solution ($\mu\text{g/ml}$); c_2 = equilibrium concentration of $(\text{RNA})_2$ in solution ($\mu\text{g/ml}$); θ = fraction of reactive sites in DNA hybridized at equilibrium; V = total volume of solution (ml); α = total initial amount of RNA in solution (μg); x = amount of RNA hybridized at equilibrium (μg); x_m = amount of RNA required to block all reactive sites in $5\mu\text{g}$ of DNA, i.e. maximum amount of hybridized RNA (μg). Then we can write for the two simultaneous equilibria:

$$K = \frac{\theta}{(1-\theta)c_1} \quad (3)$$

$$K' = \frac{c_2}{c_1} \quad (4)$$

The fact that concentrations are expressed in $\mu\text{g/ml}$, rather than mol/litre, has the effect of multiplying K and K' by a constant which depends on the molecular weight, M_1 , of RNA; e.g. K becomes $K(10^{-3} \times M_1)$ if c_1 and c_2 are expressed in mol/l.

Conservation of mass requires that,

$$c_1 V + c_2 V + x = \alpha \quad (5)$$

Eliminating c_1 and c_2 between eqns. (3), (4) and (5), we obtain:

$$(\alpha - x) \left(\frac{1-\theta}{\theta} \right) = \frac{V}{K} + V \left(\frac{K'}{K} \right)^2 \left(\frac{\theta}{1-\theta} \right) \quad (6)$$

Finally, by setting $\theta = x/x_m$, eqn. (6) becomes:

$$(\alpha - x) \left(\frac{x_m}{x} - 1 \right) = \frac{V}{K} + V \left(\frac{K'}{K} \right)^2 \left(\frac{x_m}{x} - 1 \right)^{-1} \quad (7)$$

Now, α and x are easily determined experimentally, and if x_m can be estimated, a plot of the left-hand side of eqn. (7) versus $[(x_m/x) - 1]^{-1}$ should be linear,

giving the value of V/K from the intercept and that of $V(K'/K)^2$ from the slope. The total volume of the solution, V , being known, K and K' can be calculated. Conceivably the maximum amount of RNA that can be hybridized, x_m , corresponds to an infinite RNA concentration or, V being held constant, to an infinite value of α . Thus x_m can be found from a plot of $1/x$ against $1/\alpha$ by extrapolating back to zero value of $1/\alpha$. As mentioned in the introduction, this plot is approximately linear and from its intercept $1/x_m$ is obtained. The value of x_m so determined is then used to plot eqn. (7) as described above.

It can be now shown that the double-reciprocal relationship (Lavallé & de Hauwer, 1968) and the Scatchard equation (Marsh & McCarthy, 1973) are limiting cases of eqn. (7). The first approximation needed here is that $K' \ll K$, i.e. at equilibrium the amount of self-annealed RNA is negligible compared with that which is hybridized. Then the last term of eqn. (7) can be ignored, leaving

$$(\alpha - x) \left(\frac{x_m}{x} - 1 \right) = \frac{V}{K} \quad (8)$$

which, after division by $(x_m - x)$ and inversion, gives

$$\frac{x}{\alpha - x} = \frac{K}{V} (x_m - x) \quad (9)$$

This is a Scatchard equation, equivalent to that derived by Marsh & McCarthy (1973). Clearly a plot of $x/(\alpha - x)$ against x should give a straight line, which will permit the calculation of K from the slope and of x_m from the intercept on either axis.

A further approximation is now adopted, namely that the fraction of RNA hybridized is small, i.e. $x \ll \alpha$, from which it follows that $\alpha - x \simeq \alpha$. Then, substituting α for $\alpha - x$ in eqn. (8), dividing by αx_m and rearranging, we obtain the double-reciprocal relationship

$$\frac{1}{x} = \frac{1}{x_m} + \frac{1}{K x_m} \cdot \frac{V}{\alpha} \quad (10)$$

showing that, at equilibrium, $1/(\text{amount of RNA hybridized})$ is a linear function of V/α , which is equal to $1/(\text{initial concentration of RNA})$.

It is worth summarizing the conditions under which eqn. (10) is valid. These are: (a) the extent of the self-annealing reaction should be negligible, and (b) the fraction of RNA hybridized should be small. Only condition (a) is required for eqn. (9).

A new equation, subject only to condition (a), can be derived from eqn. (8) after division by x_m and rearrangement:

$$\frac{\alpha - x}{x} = \frac{V}{K x_m} + \frac{1}{x_m} (\alpha - x) \quad (11)$$

This provides another method of plotting hybridization data, namely $(\alpha - x)/x$ against $\alpha - x$, which will

permit the calculation of x_m from the slope and K from the ratio slope/intercept = K/V . A comparison between eqns. (9) and (11) is made in the Discussion section.

Materials and Methods

Materials

Unlabelled ribonucleoside triphosphates were obtained from Koch-Light Ltd., Colnbrook, Bucks., U.K. [$5\text{-}^3\text{H}$]UTP was supplied by The Radiochemical Centre, Amersham, Bucks., U.K. Spray-dried cells of *Micrococcus lysodeikticus* came from Miles Seravac (Pty) Ltd., Maidenhead, Berks., U.K. RNA polymerase (step V) was prepared from *M. lysodeikticus* by the method of Nakamoto *et al.* (1964). Nitrocellulose membranes (type MF 50) were purchased from Membranfilter-Gesellschaft, Göttingen, Germany. Sephadex G-50 (medium) was from Pharmacia, Uppsala, Sweden. Crystalline pancreatic ribonuclease was obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Electrophoretically pure deoxyribonuclease (ribonuclease-free) came from Worthington Biochemical Corp., Freehold, N.J., U.S.A., and Pronase from Calbiochem A.G., Lucerne, Switzerland. Swiss Albino mice were used. HeLa cells were purchased from Flow Laboratories, Irvine, Ayrshire, U.K.

Preparation of DNA

This was prepared from a whole mouse embryo by a modified Marmur (1961) procedure as described by Paul & Gilmour (1968).

Synthesis of labelled RNA *in vitro*

Incubation mixtures were set up to contain the following components in a final volume of 3 ml: 150 μmol of Tris/HCl, pH 7.5; 7.5 μmol of MnCl_2 ; 2.4 μmol of ATP; 2.4 μmol of CTP; 2.4 μmol of GTP; 2.4 μmol of [$5\text{-}^3\text{H}$]UTP (1 Ci/mmol); 400–500 μg of DNA; 100–150 units of RNA polymerase. A unit of enzyme activity is defined as the amount catalysing the incorporation of 1 nmol of labelled UTP into the acid-insoluble fraction during 10 min of incubation at 37°C with whole mouse embryo DNA.

The reaction mixtures were incubated at 37°C for 90 min and the reaction was terminated by placing the tubes in ice. The mixtures were treated with 20 μg of deoxyribonuclease/ml at 37°C for 20 min. The solutions were shaken with water-saturated phenol containing 0.1% 8-hydroxyquinoline and centrifuged at 2000g for 10 min. This step was repeated until no interface remained. The extract was then passed through a column (1.2 cm $\times$ 35 cm) of Sephadex G-50, equilibrated with water, and the fractions which

contain the RNA synthesized *in vitro* were freeze-dried. The resulting powder was dissolved in 0.01 M-Tris/HCl, pH 7.5. The size of RNA produced was greater than 150 nucleotides, as estimated by the pattern obtained on polyacrylamide-gel electrophoresis (2.4%, w/v, acrylamide and 0.12% bisacrylamide; Loening, 1967).

Hybridization procedure

The technique used was essentially that described by Gillespie & Spiegelman (1965). Purified DNA was denatured by heating and 5 μg portions of this were immobilized on nitrocellulose filters (13 mm diameter) which then dried overnight at room temperature (24°C), followed by baking at 80°C for 4 h. The filters were then incubated with various amounts of ^3H -labelled RNA in 4 $\times$ SSC* contained in small tightly stoppered vials thermostatically maintained at various temperatures ($\pm 0.05^\circ\text{C}$) for 17 h. The final volume of each hybridization mixture was 250 μl .

After incubation the vials were cooled in ice; the filters were then washed on both sides and treated with pancreatic ribonuclease as specified by Gillespie & Spiegelman (1965). The filters were air dried and incubated with 1 ml of 1 M-HClO₄ for 1 h at 80°C. Finally, they were dissolved in a 10 ml mixture containing 1 part of Triton X-100 and 2 parts of toluene-based scintillator [0.42% 2,5-diphenyloxazole, 0.021% 1,4-bis-(5-phenyloxazol-2-yl)benzene]. The resulting samples were counted in a Beckman liquid-scintillation counter, corrections for background radiation etc. being made.

Self-annealing of RNA

The RNA was incubated in 4 $\times$ SSC, pH 7.0 at 67.0°C for 20 h at a concentration of 80 $\mu\text{g}/\text{ml}$. This was measured spectrophotometrically at 260 nm by using a value of $E_{1\%}^{1\text{cm}} = 285.7$, which means that 1 unit of E_{260} corresponds to 35 $\mu\text{g}/\text{ml}$. At various time-intervals, four 50 μl samples were removed and diluted to give a final concentration of 2 $\times$ SSC, pH 7.0. Two of them were digested with heat-treated pancreatic ribonuclease (20 $\mu\text{g}/\text{ml}$) for 20 min at 37°C and the other two served as controls of RNA before enzymic digestion. All samples were then precipitated by addition of 0.1 ml of bovine serum albumin (4 mg/ml) and 0.1 ml of 50% (w/v) trichloroacetic acid. The samples were mixed and 3 ml of 5% trichloroacetic acid was added. After standing for 10 min on ice, the samples were centrifuged at 2000g for 10 min at 4°C. The pellets were drained, dissolved in 0.3 ml of 1 M-NaOH and transferred to vials containing 0.5 ml of 1 M-HCl. The tubes were rinsed with 0.2 ml of water, which was also

* Abbreviation: SSC, saline/sodium citrate, i.e. 0.15 M-NaCl/0.015 M-sodium citrate. '2 $\times$ SSC' and '4 $\times$ SSC' mean respectively two or four times these concentrations.

transferred to the vials. Finally, 10ml of the scintillation mixture described under 'Hybridization procedure' was added and the samples were counted for radioactivity.

Retention of DNA on the filters

Labelled DNA was prepared from HeLa cells by using [^3H]thymidine ($5\text{--}10\mu\text{Ci/ml}$). Samples ($5\mu\text{g}$) were denatured and immobilized on filters, which were then subjected to a complete hybridization and washing procedure and counted for radioactivity. In one set, radioactive RNA was incubated in the hybridization mixture. It was found that 85–90% of the immobilized DNA is retained on the filters throughout the whole procedure.

Results

Hybridization reaction

The amount of RNA hybridized (μg) was calculated by simply dividing the corrected counting rate of each sample (c.p.m.) by the specific radioactivity of the RNA solution used (c.p.m./ μg of RNA) determined under identical counting conditions.

After a 17h incubation period the amount of hybridized RNA remained practically constant at all temperatures used. These 17h values can then be considered as representing the equilibrium amounts, x . Table 1 gives equilibrium data of hybridization at various temperatures.

For the evaluation of the above data, it should be always taken into account that, as found by Britten & Kohne (1968) in higher organisms and under the conditions used for DNA–RNA hybridization experiments, the method is not sensitive enough to detect that part of the genome that occurs in only a few

copies but only the so-called 'intermediate genomic portions'.

Self-annealing reaction of RNA

This was studied at one temperature only (67°C). Fig. 1 depicts the resistance of RNA to ribonuclease as a function of time, and shows that after 6h about 50% of RNA became ribonuclease resistant and this proportion remained practically constant after that, up to about 20h. From these data strong evidence is provided for the existence of complementary strands of the RNA synthesized by *Micrococcus* RNA polymerase and a DNA primer. This is in agreement with previous reports (Geiduschek *et al.*, 1962; Bishop & Robertson, 1969). More support comes from the thermal-denaturation analysis of self-annealed RNA (Fig. 2). The un-incubated sample shows a broad range of thermal-denaturation temperatures (T_m), with a mean of 56°C . The incubated sample, however, exhibits an altered 'melting' profile, and a sharp transition in the thermal denaturation curve is obtained with $T_m = 73^\circ\text{C}$. These results provide evidence for the formation of RNA–RNA double strands during incubation.

Fig. 1. Time-course of conversion of [^3H] RNA into acid-insoluble form

The RNA was incubated in $4\times\text{SSC}$, pH 7.0, at 67°C . At various time-intervals $50\mu\text{l}$ samples were removed and digested with pancreatic ribonuclease (final concn. $20\mu\text{g/ml}$) at 37°C for 20 min. The samples were then precipitated by addition of 0.1 ml of bovine serum albumin (4mg/ml in water) and 0.1 ml of 50% trichloroacetic acid. See the Materials and Methods section (under 'Self-annealing of RNA') for further details. □, Acid-precipitable RNA (% of total) (left ordinate); ●, radioactivity (c.p.m.) of acid-precipitable RNA (right ordinate); ○, radioactivity (c.p.m.) of control RNA, i.e. without ribonuclease (right ordinate).

Table 1. Equilibrium amount of RNA hybridized with $5\mu\text{g}$ of DNA as a function of the total initial amount of RNA per $250\mu\text{l}$ of solution, at various temperatures

See under 'Hybridization procedure' for details.

RNA added (α) (μg)	RNA hybridized (x) (μg)				
	60°C	67°C	70°C	75°C	80°C
8	0.425	—	0.228	—	—
10	—	0.525	—	0.350	—
15	—	—	—	0.420	—
16	0.548	—	0.340	—	0.240
20	0.574	0.640	0.410	0.475	0.280
24	0.644	—	0.460	—	0.324
25	—	0.687	—	0.540	—
30	—	0.726	—	0.570	—
32	0.686	—	0.516	—	0.380
35	—	—	—	0.580	—
40	—	0.747	—	—	—

Fig. 2. Absorbance/temperature profiles of RNA in $0.015 \times \text{SSC}$

○, RNA incubated at 67.0°C for 6h and slowly cooled to room temperature; ●, un-incubated RNA. The absorbance changes with temperature of the two preparations were recorded in parallel on a Gilford 2400-S automatic recording spectrophotometer.

Discussion

Having demonstrated experimentally that both hybridization of RNA with DNA and self-annealing of RNA take place simultaneously, we can apply eqn. (7), derived in the Theory section, to determine K and K' . For this purpose only the hybridization data of Table 1, but not self-annealing data, are required. As suggested in the Theory section, the x_m values can first be found from plots of $1/x$ against $1/\alpha$ by extrapolating to zero value of $1/\alpha$, i.e. to infinite value of α . Since this is equivalent to plotting the approximate eqn. (10), it is preferable to use the more accurate eqn. (11) to find x_m from the slopes of the plots of $(\alpha - x)/x$ against $\alpha - x$. Such plots are shown in Fig. 3, and the values of x_m , calculated by standard least-squares procedures, are given in Table 2. In any case the error in using eqn. (10) should be small, since it is found experimentally (see Table 1) that x never exceeds the amount corresponding to 5.31% of RNA input.

The biggest difference between the x_m values found at the various temperatures is $0.92 - 0.81 = 0.11$, and this is only about 1.5 times the error of the bigger value (0.07). Thus, without even going into proper statistical tests, it can be concluded that the differences in the x_m values given in Table 2 are not at all statistically significant, showing that the number of sites in DNA reactive for hybridization is independent of temperature, at least within the range of temperatures used in the present work. Moreover, this number is

Fig. 3. Plots of experimental data of Table 1 according to eqn. (11), for the hybridization reaction of RNA with DNA

For details, see under 'Hybridization procedure' in the Materials and Methods section. α is the total initial amount of RNA in solution (μg) and x the amount of RNA hybridized at equilibrium (μg). Arrows indicate the ordinate (left or right) to which each plot corresponds.

Table 2. Maximum amount of RNA hybridized with 5 μg of DNA (x_m) and equilibrium constants (K) for the hybridization reaction at various temperatures

Errors given in the last two columns are standard errors calculated from regression analysis.

Temperature (°C)	x_m (μg of RNA)	$10^2 \times K$ (ml/ μg)
60	0.86 ± 0.05	2.9 ± 0.4
67	0.87 ± 0.02	3.8 ± 0.4
70	0.92 ± 0.08	1.0 ± 0.1
75	0.81 ± 0.03	1.9 ± 0.1
80	0.92 ± 0.07	0.6 ± 0.2

rather small, since the maximum amount of hybridized RNA is less than 20% of the amount of DNA, in spite of the fact that input amounts of RNA as high as eight times the amount of DNA were used.

The value of x_m having been determined, the more accurate and general eqn. (7) can now be used to determine K and K' as described in the Theory section. However, when the left-hand side of eqn. (7) was calculated from the experimental data, values were obtained which differed little from each other at the same temperature, their difference being within

Fig. 4. Data from Table 1 at 75°C plotted according to the Scatchard method (eqn. 9)

α is the total initial amount of RNA in solution (μg) and x the amount of RNA hybridized at equilibrium (μg).

the limits of experimental error. Therefore the left-hand side of eqn. (7) can be considered as a constant, the contribution to it of the variable last term of the right-hand side, $V(K'/K)^2 [(x_m/x) - 1]^{-1}$, being negligible. Since $[(x_m/x) - 1]^{-1}$ varies considerably with α at the same temperature, the independence of eqn. (7) from the last term means that K'/K has a negligibly small value or is even zero. This could happen if $K' \ll K$, i.e. if self-annealing of RNA (eqn. 2) took place to a negligible extent compared with the extent of its hybridization with DNA (eqn. 1). This, however, is not supported by experimental evidence, since the results on DNA-RNA hybridization and on self-annealing of RNA, as summarized in Table 1 and Fig. 1 respectively, show that a much higher proportion of RNA is self-annealed than hybridized with DNA.

Facing the above conflict, we are tempted to conclude that the two equilibria (1) and (2) are independent of each other. This seems to be the only explanation for the invariance of the left-hand side of eqn. (7) in spite of the fact that both reactions (1) and (2) are taking place experimentally to a considerable extent. It is probable that the independence of the two equilibria means that for the behaviour of DNA it is irrelevant whether RNA is found as single molecules or in the annealed form, $(\text{RNA})_2$. In other words, reaction (1) seems to 'ignore' completely the existence of reaction (2), so that the demands for RNA during the hybridization reaction can be satisfied by either kind of RNA, provided, of course, that the equilibrium constants of the hybridization with

single RNA and with $(\text{RNA})_2$ are nearly the same. If this tentative conclusion is correct, the first condition for the derivation of eqns. (9), (10) and (11) from eqn. (7), i.e. that the extent of the self-annealing reaction is negligible, is no longer necessary. There may be cases, however, where this is not true, e.g. when the left-hand side of eqn. (7) is not invariable.

The discussion so far indicates that, under the conditions used in our experiments, only K , but not K' , can be determined at various temperatures. This can be done either from the mean values of the left-hand side of eqn. (7) without taking into account the last term on the right-hand side, i.e. eqn. (8), or from the intercepts and the slopes of the plots of eqn. (11), which has been derived directly from eqn. (8). Such plots have been given in Fig. 3. The values of K at various temperatures, calculated from these plots, are given in Table 2.

Table 2 shows that the variation of K with temperature is small and irregular, indicating that the heat of the hybridization reaction is small. An explanation of this is offered by the possibility that a great proportion of the hybrid is formed by the reaction

Such an 'exchange' reaction is expected to have a small ΔH value. Moreover, it is consistent both with the high proportion of self-annealed RNA found and with the independence of the hybridization reaction from the self-annealing equilibrium, mentioned earlier.

Finally, it would be interesting to compare our calculated values of K and x_m with those obtained by fitting experimental results of Table 1 to the Scatchard eqn. (9), since it is claimed (Marsh & McCarthy, 1973) that this is the best available method of plotting DNA-RNA hybridization data. As an example, we have plotted our data at 75.0°C (Fig. 4). The slope of the line is $-(7.58 \pm 0.68) \times 10^{-2}$, and is equal to $-K/V$, according to eqn. (9). Multiplication by the volume of the solution (250 μl) and changing sign gives the value of K as $(1.9 \pm 0.2) \times 10^{-2} \text{ ml}/\mu\text{g}$. From the y intercept we find that $(K/V) \cdot x_m = (6.17 \pm 0.33) \times 10^{-2}$. Division by '-slope' gives $x_m = 0.81 \pm 0.08 \mu\text{g}$ of RNA.

Comparing these values with those given in Table 2 for 75°C, we can conclude that our eqn. (11) gives results identical with those from the Scatchard plot. However, the errors in the K and x_m values calculated by the latter plot are considerably bigger than those given in Table 2, and this seems to suggest that eqn. (11) provides a superior method of plotting DNA-RNA hybridization data than eqn. (9). This is also apparent on comparing the plot of Fig. 4 with the plot at 75°C of Fig. 3.

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