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Ribonucleic Acid–Deoxyribonucleic Acid Hybridization as a Second-Order Reaction

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Certain features of RNA–DNA hybridization can be accounted for in terms of second-order-reaction theory. These include the use of annealing kinetics to estimate RNA complexity and the occurrence of approximately linear double-reciprocal plots.

The formation of specific RNA–DNA hybrid molecules has been widely used to study relationships between RNA and DNA sequences. Although this reaction probably involves mechanisms similar to those of DNA renaturation, the analysis of its kinetics is more complicated because the relative concentrations of complementary RNA and DNA sequences are often unknown. Moreover during hybridization the DNA is often immobilized to prevent renaturation.

Nygaard & Hall (1964) studied the formation of ribonuclease-resistant RNA–DNA duplexes in solution and concluded that the initial reaction rate was proportional to the product of the initial RNA and DNA concentrations over a 10000-fold range. It has also been shown that the rate of hybridization is approximately proportional to the complexity of the complementary sequences that take part in the reaction (Laird & McCarthy, 1968; Bishop, 1969; Birnstiel *et al.*, 1972). An identical relationship governing DNA renaturation was observed by Doty *et al.* (1960) and Marmur & Doty (1961), and was more formally stated by Wetmur & Davidson (1968) and Britten & Kohne (1968), who both demonstrated that DNA renaturation is a second-order reaction. The purpose of our paper is to investigate some consequences of the application of second-order-reaction theory to RNA–DNA hybridization.

Second-order-reaction theory

To calculate a theoretical time-course for hybridization we assume that the rate of hybridization, dH/dt, is proportional to the product of total DNA concentration, D, and total RNA concentration, R. This assumption is applicable only if DNA renaturation is prevented, and hence the following analysis can only be applied to experiments in which the DNA has been denatured and immobilized.

The second-order rate equation is given by:

$$\frac{dH}{dt} = kRD \qquad (1)$$

where k is the rate constant. If it is assumed arbitrarily that the initial RNA concentration, R_0, is greater than the initial DNA concentration, D_0, the hybridization time-course may be found by integration of eqn. (1) to give:

$$H = \frac{R_0 D_0 (1 - e^{(R_0 - D_0)kt})}{D_0 - R_0 e^{(R_0 - D_0)kt}} \qquad (2)$$

If however, the initial concentrations are such that $R_0 = D_0$, it can be shown similarly by integration that the time-course is given by:

$$H = \frac{R_0 D_0 kt}{1 + D_0 kt} \qquad (3)$$

This equation may also be used to describe DNA renaturation, for which the initial concentrations of the strand and anti-strand DNA must always be equal (Britten & Kohne, 1968).

Application of theory

Complexity of RNA. Britten & Kohne (1968) have shown that for DNA renaturation the parameter $C_0 t_{\frac{1}{2}}$ (C_0 = DNA concentration in mol of nucleotides/litre, $t_{\frac{1}{2}}$ = time for half-reaction in s) is proportional to DNA complexity. Hence with the appropriate control experiments measurement of $C_0 t_{\frac{1}{2}}$ may be used to estimate the analytical complexity of a particular DNA species. The proportionality between $C_0 t_{\frac{1}{2}}$ and DNA complexity may be deduced from two facts. First, the concentration of a particular DNA sequence in a fixed amount of unique DNA is inversely proportional to genome size and therefore to complexity. Secondly, for a second-order reaction with equal initial concentrations (as described by eqn. 3) the product of initial concentration and $t_{\frac{1}{2}}$ is constant (equal to the reciprocal of the rate constant).

The use of a similar parameter, $R_0 t_{\frac{1}{2}}$, to measure the complexity of an RNA has been examined in detail by Birnstiel *et al.* (1972), using filter hybridization techniques. It was observed empirically that, for hybridization of excess of RNA to DNA, $R_0 t_{\frac{1}{2}}$ was substantially independent of the quantity of DNA on the filter and was approximately proportional to the analytical complexity of the RNA. These results

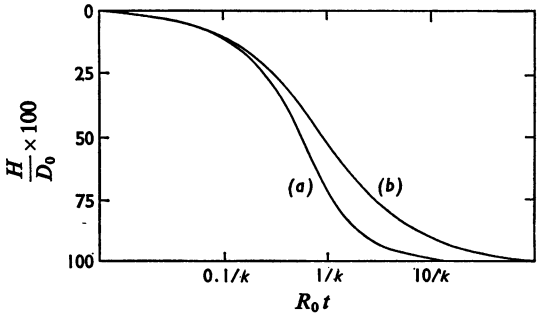

Fig. 1. *Ideal second-order reaction time-courses*

Curve (*a*) is from eqn. (2) for large RNA excess ($R_0 \gg D_0$) and curve (*b*) is from eqn. (3) for equal amounts of RNA and DNA ($R_0 = D_0$).

may be accounted for if, under these conditions, RNA–DNA hybridization behaves generally as a second-order reaction. If equal amounts of RNA and DNA are present, the proportionality between $R_0 t_{\frac{1}{2}}$ and RNA complexity may be deduced in the same way as the relationship between $C_0 t_{\frac{1}{2}}$ and DNA complexity. From eqn. (3) for $R_0 = D_0$ it can be shown that:

$$R_0 t_{\frac{1}{2}} = \frac{1}{k} \tag{4}$$

Under conditions of RNA excess, however, we have from eqn. (2):

$$R_0 t_{\frac{1}{2}} = \frac{1}{k} \cdot \frac{\log_e\left(2 - \frac{D_0}{R_0}\right)}{\left(1 - \frac{D_0}{R_0}\right)} \tag{5}$$

Hence, for large RNA excess $R_0 t_{\frac{1}{2}}$ has a limiting value of $0.69/k$. Decreasing the DNA concentration from $D_0 = R_0$ to $D_0 \ll R_0$ will result in a 31% decrease in $R_0 t_{\frac{1}{2}}$, most of this change occurring at RNA/DNA ratios less than 10:1. Thus the second-order interpretation predicts that hybridization of RNA to DNA at ratios greater than 10:1 will result in values of $R_0 t_{\frac{1}{2}}$ which are substantially independent of the RNA/DNA ratio and which are proportional to the complexity of the hybridizing system.

Hybridization time-course. DNA renaturation can be conveniently plotted as a function of $\log(C_0 t)$ (Britten & Kohne, 1968). In a similar manner two second-order reaction time-courses are plotted in Fig. 1 as functions of $\log(R_0 t)$. Curve (*a*) was calculated from eqn. (2) for a large RNA excess and curve (*b*) was calculated from eqn. (3) for equal concentrations of RNA and DNA. Curve (*b*) has the symmetrical sigmoid shape typical of DNA-renaturation curves, whereas curve (*a*) has an asymmetrical sigmoid shape. This asymmetry is due to the fact that, for large RNA excess, the theoretical reaction is pseudo-first-order because the RNA concentration is not significantly decreased by hybridization. These curves also illustrate the 31% decrease in $R_0 t_{\frac{1}{2}}$ produced by increasing the RNA/DNA ratio from 1 to ∞.

Double-reciprocal plots. It was shown by Paul & Gilmour (1966, 1968) and Bishop (1969) that, if the reciprocal of hybrid concentration is plotted as a function of the reciprocal of either time or RNA concentration, an approximately linear relationship is obtained. By extrapolating a straight line to the ordinate it was assumed that the amount of RNA–DNA hybrid that would be formed at infinite time or infinite RNA concentration could be estimated. It can be shown from eqn. (2) that a hybridizing system which behaves as a second-order reaction would produce curved double-reciprocal plots lying asymptotically between two intersecting straight lines.

The plot of $1/H$ versus $1/t$ has two asymptotes, which are given by:

$$\frac{1}{H} = \frac{1}{D_0} \tag{6}$$

$$\frac{1}{H} = \frac{1}{t}\left(\frac{1}{kR_0D_0}\right) + \frac{1}{2R_0} + \frac{1}{2D_0} \tag{7}$$

A second-order reaction time-course was calculated from eqn. (2) with arbitrary values of $R_0 = 2$, $D_0 = 1$ and $k = 1$, and is plotted in Fig. 2(*a*) as $1/H$ versus $1/t$. From eqns. (6) and (7) the two asymptotes for this curve are given by $1/H = 1$ and $1/H = (0.5/t) + 0.75$.

The second form of double-reciprocal plot, i.e. D_0/H versus D_0/R_0, also has two asymptotes, one of which is given by:

$$\frac{D_0}{H} = 1 \tag{8}$$

The other asymptote is given by:

$$\frac{D_0}{H} = \frac{D_0}{R_0}\left(\frac{e^z}{e^z - 1}\right) + \frac{(z-1)e^z + 1}{(e^z - 1)^2} \tag{9}$$

where $z = kD_0 t$. An example of this type of plot is illustrated in Fig. 2(*b*). D_0, k and t each have the value 1 and the amount of hybrid that would be formed for a particular RNA concentration was calculated by using eqn. (2). The term $kD_0 t$ equals 1, and hence from eqns. (8) and (9) the two asymptotes are given by $D_0/H = 1$ and $D_0/H = (1.58\, D_0/R_0) + 0.34$.

Discussion

The hybridization of RNA to DNA involves the association of complementary sequences of nucleotides. Molecules of more than one sequence are usually present in both RNA and DNA preparations, and the proportions of the RNA and DNA represented by a particular sequence are often unknown. Although the reaction between a single

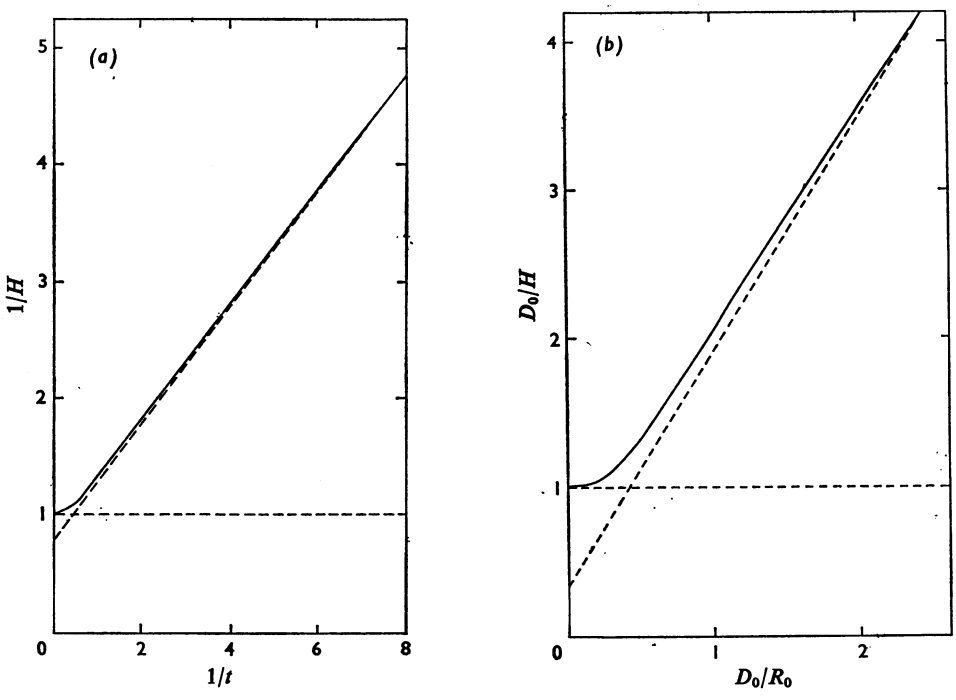

Fig. 2. *Double-reciprocal plots of second-order reaction*

(*a*) This plot was obtained from eqn. (2) with $R_0 = 2$, $D_0 = 1$ and $k = 1$. The asymptotes, indicated by broken lines, were derived from eqns. (6) and (7) (see the text). (*b*) This plot was obtained from eqn. (2) with $D_0 = 1$, $k = 1$ and $t = 1$. The asymptotes were derived from eqns. (8) and (9) (see the text).

RNA sequence and its DNA complement may be expected to be second-order, it does not follow that the sum of a number of such reactions, i.e. the hybridization time-course, is second-order. There are, however, several situations that would be expected to produce a second-order hybridization time-course. If non-repetitive DNA were transcribed into RNA in such a way that no part of the DNA was preferentially transcribed then hybridization of the RNA to its template DNA would be second-order. If transcription were non-uniform or the DNA contained repeated sequences, hybridization would deviate from true second-order kinetics. The hybridization of a homogeneous RNA species would also be expected to be a second-order reaction. The observation by Birnstiel *et al.* (1972) that for homogeneous RNA species the parameter $R_0 t_{\frac{1}{2}}$ is approximately proportional to the complexity of the hybridizing system supports the view that second-order theory is applicable to such situations.

Although double-reciprocal plots of second-order reactions have been shown to have slight curvature, this feature is not evident from experimental results. This is probably because the curvature is within the experimental error of double-reciprocal plots of hybridization experiments, and hence a straight line is a reasonable approximation to the experimental points. However, the general form of experimental plots agrees with second-order theory. For example, the plots of $1/H$ versus $1/t$ used by Birnstiel *et al.* (1972) clearly showed that the gradient was inversely proportional to RNA concentration, a feature that is also exhibited by the asymptote of the reciprocal plot of second-order reactions (see eqn. 7).

The theoretical dependence of D_0/H on D_0/R_0 for a particular incubation time is illustrated in Fig. 2(*b*) over a wide range of RNA/DNA ratios. Increasing the incubation time has no effect on the horizontal asymptote but causes the other asymptote to approximate to a line with a gradient of 1 through the origin. If the time chosen is sufficiently large the plot becomes indistinguishable from these two lines. In practice, such experiments are carried out with excess of RNA present and hence should produce points within a limited region near the ordinate. The theory described above, if applicable to these experiments, implies

that fitting a straight line to experimental points will overestimate the saturation value obtained for either infinite RNA concentration or infinite incubation time. This error, however, may be decreased to negligible proportions by obtaining values as close as possible to the ordinate.

Under normal incubation conditions hybridization may be assumed to be an irreversible process (Bishop, 1970). It can be shown that the double-reciprocal plot of a reversible second-order reaction also consists of a curve lying between two intersecting asymptotes. The horizontal asymptote in the plot of D_0/H versus D_0/R_0 in Fig. 2(*b*) becomes the line known as the Langmuir Adsorption Isotherm (Lavallé & De Hauwer, 1968) and the other asymptote is also modified by the occurrence of an equilibrium between the hybrid molecules and the RNA and DNA molecules. This type of reaction would only apply where the incubation temperature is near the 'melting' temperature of the hybrid.

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