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THE PROBLEM OF NUCLEASE ACTIVITY IN NUCLEIC ACID HYBRIDIZATION REACTIONS. THEORETICAL CONSIDERATIONS

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The presence of nucleases during DNA–DNA or DNA–RNA hybridization reactions alters the kinetics of hybrid formation. Unfortunately, while the effect (even of small amounts of nuclease) on the C_0t curve may be large, it may not be readily detectable. The effect of various types of nuclease is shown. As many nucleases cause a shift in the position rather than a change in shape of the curve, all studies involving nucleic acid hybridization should assay for the presence of nucleases and care must be taken to avoid their presence as contaminants.

1. Introduction

Since the initial work by Britten and Kohne in 1968 [1], the use of DNA–DNA hybridizations and DNA–RNA hybridizations has become common in molecular biology. Nucleic acid hybridization has been used to measure amounts of mRNA, to determine the presence of sequences in DNA, to determine sequence relationships between nucleic acids, to sequence RNA and to study transcription [1–10]. However, as in all studies involving nucleic acids, care must be taken to avoid nucleases. If a nuclease is present, the data may be uninterpretable. The appearance of a "normal" C_0t curve (the extent of hybrid formation plotted against the logarithm of the product of the initial nucleic acid concentration and time) cannot be used as an indicator that the system is free of problems. The major effect of many nucleases is to change the position of the curve rather than its shape so that its presence may not be readily apparent.

2. Experimental

2.1. Methods

The system of differential equations representing the competition of nucleic acid digestion and hybridization was solved explicitly or by numerical approximation using a subroutine of MLAB [11,12] on a DEC PDP/10 time sharing system. The curves were drawn on an on-line CRT display and hard copy obtained with a Calcomp plotter.

In the discussions that follow, C represents unhybridized DNA concentration, C_r, unhybridized RNA concentration and H represents DNA–DNA hybrid or DNA–RNA hybrid concentration as appropriate. The subscript, zero, represents initial concentrations. K_1 represents the rate constant of nucleic acid hybridization; K_2 represents the rate constant for nucleic acid degradation (which may be secondary to nuclease activity or to a physical loss of nucleic acids). In the examples provided, K_1 has been chosen as one for DNA–DNA hybridization and as 0.69315 for DNA–RNA hybridization so that the $C_0t_{1/2}$ (or $C_{r,o}t_{1/2}$) will be one. Plots of these reactions involve H/C_0 as the experimental parameter. This involves measuring C_0 but is frequently obtained experimentally by measuring the total DNA after the hybridization reaction has been stopped. In this case, if a DNase had been present, the measured total DNA concentration, C_T, will be less than C_0. Hopefully, gross differences between C_0 and C_T would be noticed by the experimenter.

The equations used to represent the degradation reaction and the hybridization reaction are both simplifications. It is assumed that the nuclease degrades entire molecules rather than causing one

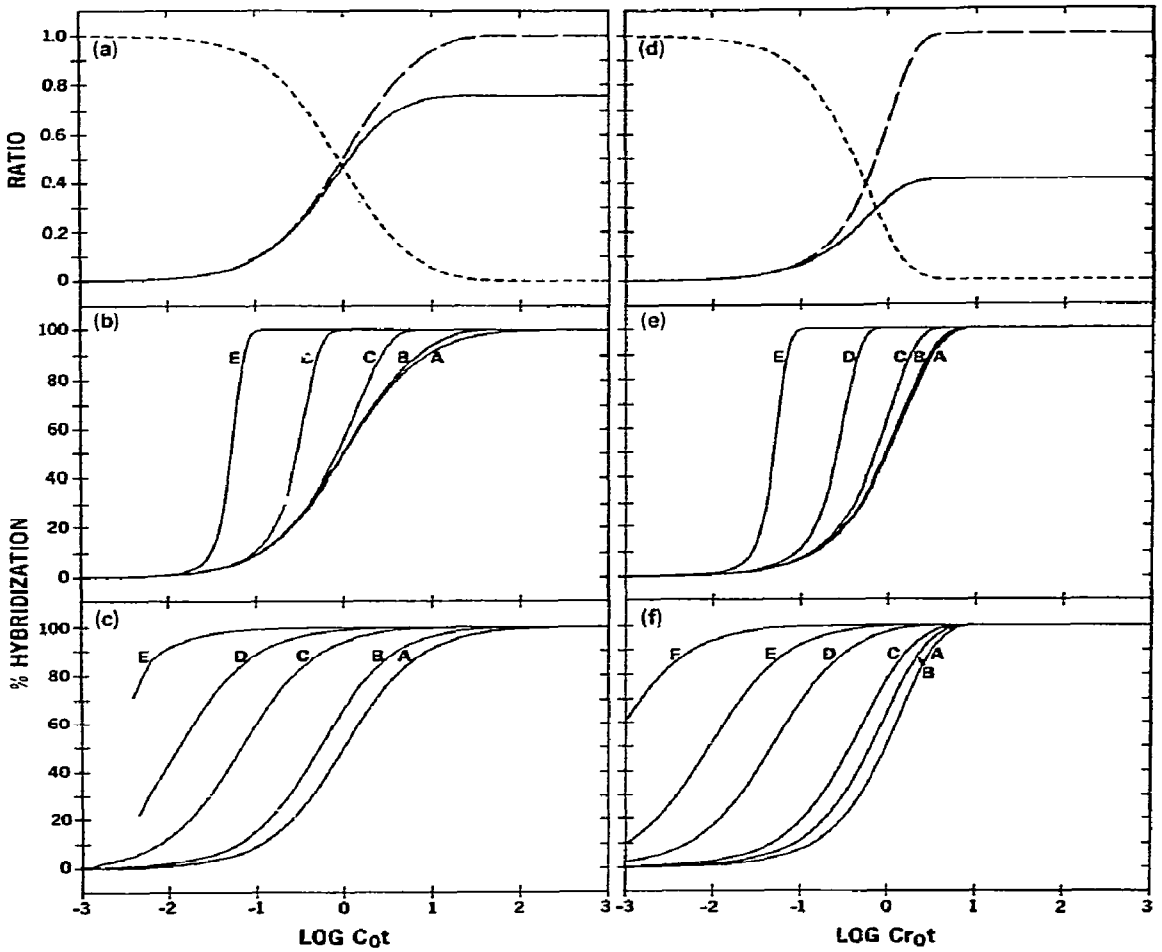

Fig. 1. The effect of a single strand specific DNase on hybridization. (a) The effect of DNase on DNA–DNA hybridization. In the example chosen here, $C_0 = 10^{-5}$ M and $K_2 = 10^{-6}$ s^{-1}. - - - - represents C/C_0, —— represents H/C_T and ——— represents H/C_0. (b) The effect of varying the initial DNA concentration on DNA–DNA hybridization. K_2 has been chosen as 10^{-5} s^{-1} and the initial DNA concentrations are 10^{-3} M for curve A, 10^{-5} for curve B, 10^{-6} for curve C, 10^{-7} for curve D, and 10^{-8} for curve E. The curves represent H/C_T. (c) The effect of varying the length of incubation on DNA–DNA hybridization. K_2 has been chosen as 10^{-5} s^{-1} and the length of incubation is 10 000 s or less for curve A, 200 000 for curve B, 500 000 for curve C, 700 000 for curve D and 10^6 s for curve E. Each curve is of the form H/C_T. (d) The effect of DNase on DNA–RNA hybridization. In this example, $C_{T,0} = 10^{-5}$ M and $K_2 = 10^{-5}$ s^{-1}. - - - - represents C/C_0, —— represents H/C_T and ——— represents H/C_0. (e) The effect of varying the initial RNA concentration on DNA–RNA hybridization. K_2 has been chosen as 10^{-5} s^{-1} and the initial RNA concentrations are 10^{-3} M for curve A, 10^{-4} for curve B, 10^{-5} for curve C, 10^{-6} for curve D and 10^{-7} for curve E. The curves represent H/C_T. (f) The effect of varying the length of incubation on DNA–RNA hybridization. K_2 has been chosen as 10^{-5} s^{-1} and the length of incubation is 10 000 s or less for curve A, 100 000 for curve B, 200 000 for curve C, 500 000 for curve D, 700 000 for curve E and 10^6 s for curve F. Each curve represents H/C_T.

chain cleavage at a time which would result in a change in the size distribution of the nucleic acid molecules. As K_1 is inversely proportional to the square-root of the size of the DNA molecule [13], K_1 is not constant. The hybridization reaction has been assumed to be a second order reaction. However, it has been shown that the observed kinetics differ significantly and empiric equations have been derived [14]. Also, the back reaction (separation of the hybrid into two strands) has been considered negligible in the discus-

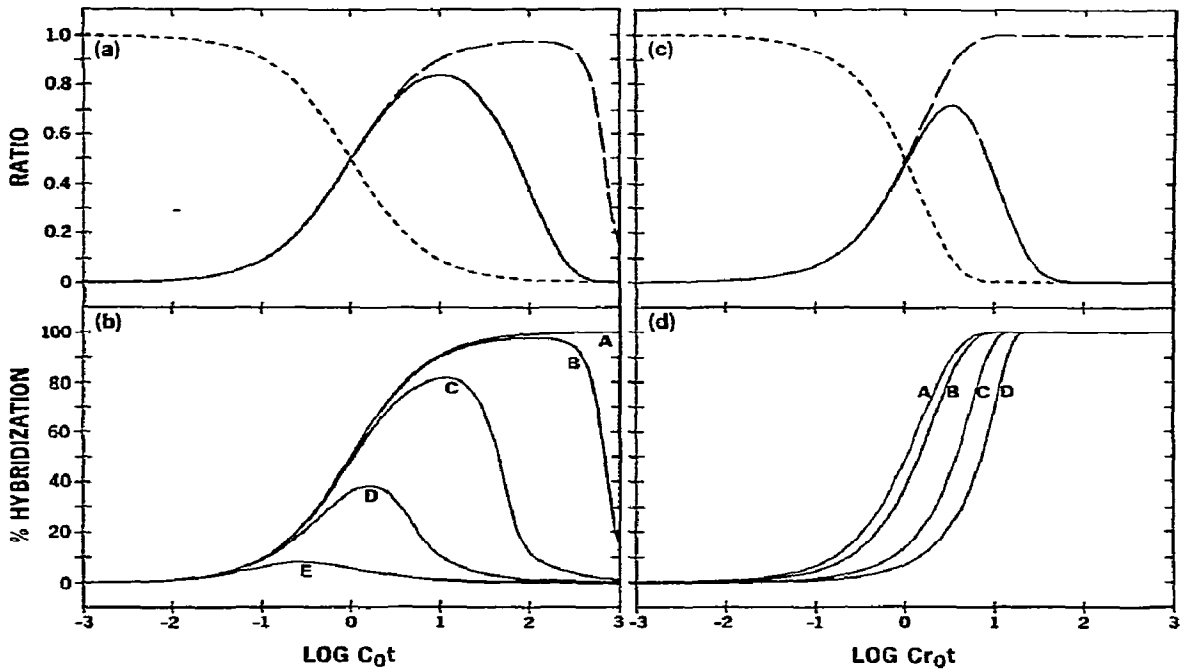

Fig. 2. The effect of a hybrid specific DNase on hybridization. (a) The effect on DNA–DNA hybridization. In the example chosen, $C_0 = 10^{-5}$ M and $K_2 = 10^{-7}$ s^{-1}. - - - represents C/C_0, ——— represents H/C_T and —— represent H/C_0. (b) The effect of varying the concentration of hybrid nuclease on DNA–DNA hybridization. C_0 has been chosen as 10^{-5} M and $K_2 = 10^{-8}$ s^{-1} for curve A, 10^{-7} for curve B, 10^{-6} for curve C, 10^{-5} for curve D and 10^{-4} for curve E. The curves represent H/C_T. (c) The effect on DNA–RNA hybridization. In the example here, $K_2 = 10^{-5}$ s^{-1} and $C_{r,0} = 10^{-5}$ M. - - - represents C/C_0, ——— represents H/C_T, and —— represents H/C_0. (d) The effect of varying the length of incubation on DNA–RNA hybridization. K_2 has been chosen as 10^{-5} s^{-1} and the length of incubation is 10 000 or less for curve A, 200 000 for curve B, 700 000 for curve C and 10^6 s for curve D. The curves in (b) and (d) represent H/C_T.

sions that follow although it is treated briefly in the section on hybrid specific RNase. The curves that follow all represent an "ideal" case and therefore demonstrate clearly the problems of nucleases. Obtaining more exact solutions by not making the above approximations represents an academic exercise as nucleases must be avoided and data from experiments containing a nuclease should not be corrected.

3. DNA–DNA hybridization

3.1. Single strand specific DNase

When a single strand specific DNase is present during hybridization the non-hybridized DNA is degraded. This loss of DNA has two effects. First, any degraded DNA (which would have hybridized) can no longer form a hybrid and the total amount of DNA hybridized will be reduced. At the same time, because of the removal of the nonhybridized DNA, the fraction of the DNA remaining that is hybridized will be increased. An example of this is shown in fig. 1a. The short-dashed line represents C/C_0; the concentration of DNA falls because of degradation of DNA by nuclease and because of formation of hybrid. The solid line represents H/C_0. In this example, only 86% of the initial DNA hybridized; the remaining 14% of the DNA had been degraded. The long-dashed line represents H/C_T (which is usually determined experimentally); hybridization appears to be complete as there is no single-stranded DNA remaining. However, presence of a single strand specific DNase shifts the C_0t curve to the left. The size of the shift increases with increasing nuclease concentration, time of incubation or decreasing initial DNA concentration (figs. 1b and 1c). The equation relating these variables is:

$$H = \frac{K_2}{K_1}\left[\ln \frac{K_2}{K_1C_0 + K_2 - K_1C_0e^{-K_2t}} + \frac{K_1C_0 + K_2}{K_2} - \frac{K_1C_0 + K_2}{K_1C_0 + K_2 - K_1C_0e^{-K_2t}}\right].$$

In the non-ideal case where hybridization is less than 100%, nuclease activity might be discovered because of an impossibly high fraction of DNA hybridizing.

3.2. Double strand specific DNase

When a double strand specific DNase is present during hybridization the hybrid is degraded. As the hybrid must form before it can be degraded, hybrid concentration first increases and then decreases with increasing time of incubation. This is shown in fig. 2a. The solid line represents H/C_0 and demonstrates the changes in hybrid concentration. The shape of the H/C_T curve may be abnormal. With increasing nuclease concentration (fig. 2b), less hybrid is formed and the $C_0t_{1/2}$ is decreased (shifted to the left). At higher nuclease concentrations than shown in fig. 2b ($K_2 > 10^{-4}\ s^{-1}$), no hybrid would be detected experimentally. If, instead of varying the time of incubation, one uses a fixed incubation time, the C_0t curve is shifted to the right; the shift increases with increasing time of incubation. The family of curves obtained resembles those shown in fig. 2d. Differences in C_0t curves obtained at constant DNA concentrations as compared to constant time of incubation are a hallmark of nuclease contamination. With constant DNA concentration, high C_0t values are obtained by long incubations so nuclease effects will be maximal at the high values. With constant time of incubation, high C_0t values are obtained by high DNA concentrations so nuclease effects will be minimal at the high values.

4. DNA–RNA hybridization with RNA excess

4.1. Single strand specific DNase

The effect of a single strand specific DNase in DNA–RNA hybridization is similar to its effect in DNA–DNA hybridizations as is shown in fig. 1d. The $C_{r,0}t$ curve is shifted more to the left by increasing the time of incubation (fig. 1e), or decreasing the initial RNA concentration (fig. 1f) but is independent of the initial DNA concentration. The differential equations are readily solved to give

$$C/C_0 = e^{-(K_1C_{r,0} + K_2)t}$$

and

$$H/C_0 = \frac{K_1C_{r,0}}{K_1C_{r,0} + K_2}\left[1 - \exp(-e^{-(K_1C_{r,0} + K_2)t})\right].$$

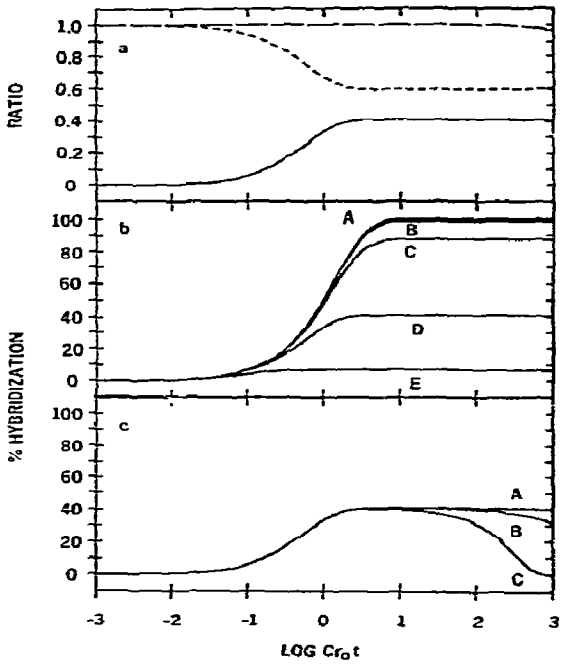

Fig. 3. The effect of RNase H on DNA–RNA hybridization. (a) In this example, $K_2 = 10^{-5}\ s^{-1}$, $R_0 = 10^{-5}$ M and $C_0 = 10^{-9}$ M. - - - represents C/C_0, —·— represents $C_r/C_{r,0}$ and —— represents H/C_0. (b) The effect of varying the initial RNA concentration on DNA–RNA hybridization. K_3 has been chosen as $10^{-5}\ s^{-1}$ and C_0 as 10^{-9} M. The initial RNA concentration is 10^{-2} M for curve A, 10^{-3} for curve B, 10^{-4} for curve C, 10^{-5} for curve D, and 10^{-6} for curve E. (c) The effect of varying the initial DNA concentration on DNA–RNA hybridization. K_2 has been chosen as $10^{-5}\ s^{-1}$ and $C_{r,0}$ as 10^{-5} M. The initial concentration of DNA is 10^{-10} M for curve A, 10^{-8} for curve B and 10^{-7} for curve C. Higher DNA concentrations are not shown since under those circumstances RNA is not in ten-fold excess. The curves in (b) and (c) represent H/C_T.

4.2. Hybrid specific DNase

The effect of a hybrid specific DNase in DNA–RNA hybridization is to decrease the fraction of DNA hybridized. The amount of hybrid (if there is hybrid formation) increases and then decreases with increasing time of incubation as shown in fig. 2c. This type of nuclease shifts the $C_{r,0}t$ curve to the right; the size of the shift increasing with increasing nuclease concentration, time of incubation (fig. 2d) or decreasing initial RNA concentration. It is independent of the initial DNA concentration as can be seen from the formulae:

$$C/C_0 = e^{-K_1 C_{r,0} t}$$

and

$$\frac{H}{C_0} = \frac{K_1 C_0}{K_2 - K_1}\left(e^{-K_1 C_{r,0} t} - e^{-K_2 t}\right),$$

which are a minor modification of the equations of a simple chain reaction consisting of two irreversible first order reactions [15].

4.3. Hybrid specific RNase (RNase H)

RNase H degrades the RNA in a DNA–RNA hybrid. As RNA is initially in excess, a "steady state" prevails with hybrid formation being almost equal to hybrid degradation. First, hybrid forms (if possible); the amount of hybrid then appears to be constant and finally, as the RNA concentration falls so that it is no longer significantly in excess, the hybrid concentration falls. This is shown in fig. 3. The shape of the $C_{r,0}t$ curve is affected by the initial concentrations of RNA, DNA and nuclease (figs. 3b and c). Incubation for constant time will cause the curve to shift to the right (data not shown). The differential equations for this reaction do not have a simple solution but a good approximation can be obtained by dividing the reaction into two components, an initial fast reaction (during which $R = R_0$) and a later slow reaction (during which $dH/dt = 0$). For the fast reaction

$$dH/dt = K_1 C_{r,0}(C_0 - H) - K_2 H,$$

so

$$H = \frac{K_1 C_{r,0} C_0}{K_1 C_{r,0} + K_2}\left(1 - e^{-(K_1 C_{r,0} + K_2)t}\right),$$

for $0 \leqslant t \leqslant t_1$ where $t_1 = \ln(100)/(K_1 C_{r,0} + K_2)$.

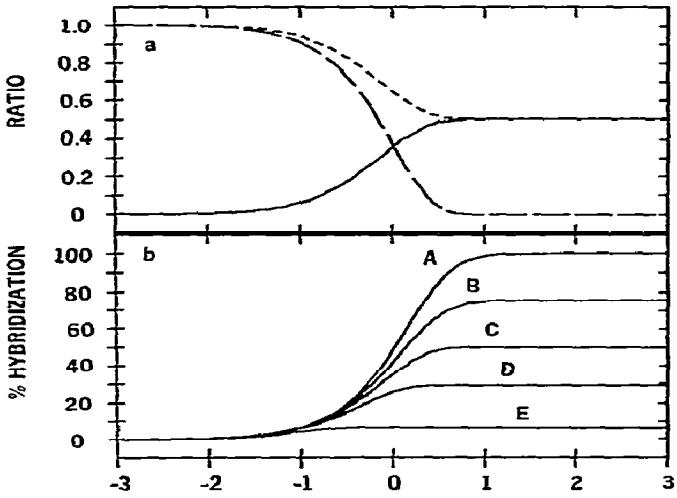

Fig. 4. The effect of a single strand specific RNase on DNA–RNA hybridiaztion. (a) In this example, $C_0 = 10^{-9}$ M, $R_0 = 10^{-5}$ M and $K_2 = 10^{-5}$ s^{-1}. - - - represents C/C_0, —·— represents $C_r/C_{r,0}$ and —— represents H/C_0. (b) The effect of varying the concentration of single strand specific RNase on DNA–RNA hybridization. In this case, $C_{r,0}$ has been chosen as 10^{-5} M and K_2 is less than 10^{-6} s^{-1} for curve A, 5×10^{-6} for curve B, 10^{-5} for curve C, 2×10^{-5} for curve D and 10^{-4} for curve E. The curves represent H/C_T.

For the slow reaction with $t > t_1$

$$dH/dt = K_1 C_r(C_0 - H) - K_2 H = 0,$$

or

$$H = K_1 C_r C_0/(K_1 C_r + K_2)$$

and therefore

$$dC_r/dt = -K_1 C_r(C_0 - H) = -K_1 C_r\left[C_0 - \frac{K_1 C_r C_0}{K_1 C_r + K_2}\right].$$

C can be approximated by numerical analysis using a computer and the values of C_r then used to calculate H.

The back reaction can be considered as a special case of RNase H. The back reaction converts hybrid into DNA and (intact) RNA while the digestion reaction converts hybrid into DNA and ribonucleotides. As the RNA is not digested, it remains in excess throughout the reaction and the steady state that is reached is the equilibrium position. Usually, this back reaction can be ignored. However, with low DNA and/or low RNA input, significantly less than 100% of

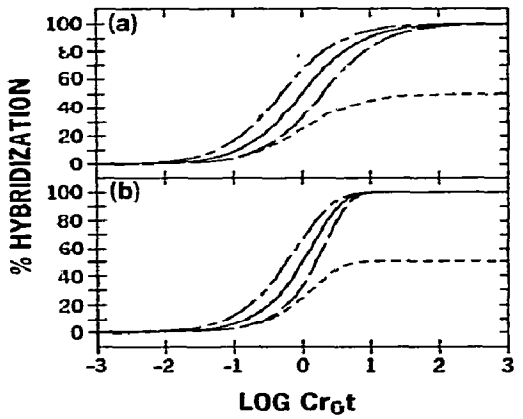

Fig. 5. The effect of the addition of nucleases after hybridization. Top: The effect on DNA–DNA hybridization. In this figure, it has been assumed that the nuclease has destroyed half of its potential substrate (either the hybridized or unhybridized DNA). —— represents H/C_0 in the presence of no nuclease or a single strand specific nuclease, - - - represents H/C_0 in the presence of a hybrid specific nuclease, — · — represents H/C_T in the presence of a single strand specific nuclease and ——— represents H/C_T in the presence of a hybrid specific nuclease. Bottom: The effect on DNA–RNA hybridization. —— represents H/C_0 in the presence of no nuclease or a single strand specific nuclease, - - - represents H/C_0 in the presence of RNase H or a hybrid specific DNase, — · — represents H/C_T in the presence of a single strand specific DNase and ——— represents H/C_T in the presence of a hybrid specific DNase.

the DNA can hybridize. The effect of concentration on hybridization has been discussed [16].

4.4. Single strand specific RNase

A single strand specific RNase will destroy RNA and lower the effective RNA concentration available to form hybrid. This will shift the $C_{r,0}t$ curve to the right as well as decrease the final amount of hybrid formed. The effect of this nuclease on DNA, RNA and hybrid concentration are shown in fig. 4a. The effect of varying the nuclease concentration or the initial RNA concentration is shown in fig. 4b. Incubation for constant time will cause the curve to shift to the right (data not shown). As long as the initial RNA concentration is ten-fold greater than the DNA concentration, the initial DNA concentration has no appreciable effect.

5. Introduction of nuclease after hybridization

Nuclease can be added (experimentally as well as theoretically) at the start of the hybridization reaction as already described (in which case there is competition between hybridization and degradation) or after the hybridization reaction has been stopped but prior to determining the fraction of DNA hybridized. If C_0 is known or determined before nuclease is introduced, only a hybrid specific nuclease will affect the results and it will cause a decrease (by a constant fraction) in the amount of hybrid without altering the position of the curve (fig. 5). If instead of C_0, C_T is used, the presence of any DNase will cause a shift in the curve. A single strand specific DNase will shift the curve to the right by log z where z is the fraction of DNA degraded, while a hybrid specific DNase will shift the curve to the left by log z. The effects of the various nucleases added after hybridization on C_0t and $C_{r,0}t$ curves are summarized in fig. 5. The difference in shape between C_0t (fig. 5a) and $C_{r,0}t$ (fig. 5b) curves can also be seen; the $C_{r,0}t$ curve has a more rapid transition from 0 to 100% hybridization. This is related to DNA–DNA hybridization being a second order reaction while DNA–RNA hybridization being a pseudo-first order reaction (when RNA is in excess).

6. DNA–RNA titration

In addition to $C_{r,0}t$ analysis, DNA–RNA hybridization can be studied by titration (or saturation) analysis [17]. In this method, a constant amount of DNA is titrated with increasing amounts of RNA, under conditions that allow completion of hybridization reactions (constant time). The titration curve (plot of the fraction of DNA hybridized versus RNA concentration) consists of a line through the origin for DNA excess and a horizontal line for RNA excess (assuming DNA size homogeneity). For DNase the equations are independent of DNA concentration so that the titration curves will be identical (except for the use of a linear rather than a log plot) to $C_{r,0}t$ curves obtained with constant times of incubation (figs. 1f and 2d). The single strand specific DNase will increase the slope of the line while the hybrid specific DNase will decrease the slope of the line. The analysis will appear normal although the slope of the line has changed. The presence of RNases

produces more complications since the equations are dependent on the initial DNA and RNA concentrations as well as on the time of incubation and the amount of nuclease. A single strand specific RNase will decrease the slope of the line. RNase H alters the shape of the curve by causing the line to intersect to the right of the origin (as with DNA excess and long incubations, all the hybrid will be degraded). This nuclease also decreases the slope of the line. Therefore, neither $C_{r,0}t$ analysis nor titration analysis is safe from the artifacts produced by nuclease contamination.

7. Discussion

Nucleic acid hybridization has been used in a wide range of biologic studies. However, very few studies have determined that no nucleases were present [5,9]. Figs. 1–5 show for the ideal case how the presence of each type of nuclease can alter a C_0t (or $C_{r,0}t$) curve. The type of alteration is determined by the specificity of the nuclease while the magnitude of the alteration is determined by the amount of nuclease (as well as length of incubation and concentration of substrates). Only in the cases of a hybrid specific DNase (on C_0t curves) and RNase H (on $C_{r,0}t$ curves) does the fraction of DNA hybridized (H/C_T) decrease with increasing C_0t (or $C_{r,0}t$). With all other nucleases, the curves have approximately the same shape as in the absence of nuclease. The maximum amount of DNA hybridized can be less than 100% in the presence of ribonucleases and in plots of H/C_0. However, hybridization in the absence of nuclease is rarely 100% and it may be impossible to distinguish between imperfect hybridization and the presence of a nuclease. When DNA is not a single species but a complex mixture of DNA's or when the nucleic acids to be hybridized are not good complements, the amount hybridized can be very low [18]. In the other cases, the sole effect of the nuclease is to shift the position of the curve. A single strand specific DNase shifts the curve to the left while an RNase or a double strand specific DNase shifts it to the right. As most experiments are performed to determine the $C_0t_{1/2}$, the presence of a nuclease will change the experimental $C_0t_{1/2}$.

Under optimal conditions for a nuclease (for example T1 RNase [19]) a low concentration of nuclease (i.e. 0.02 μg/ml) will give effects similar to those presented. Fortunately, hybridization conditions (at high temperatures or in the presence of formamide) are far from optimal for nucleases; however, nucleases can cause degradation under hybridization assay conditions [20]. While Harrison et al. [4] found only negligible DNA degradation at a C_0t of 8000, some degradation occurred after incubation to a C_0t value of 80 000. If this degradation was due to nuclease, longer incubation times or lower DNA concentrations could have led to spurious results.

As the presence of a nuclease cannot always be determined by inspection of the experimental data (since the curves can be changed in regard to position rather than shape), the best way to handle the problem of nucleases is to avoid them by scrupulous attention to technique (i.e. autoclaved solutions, rubber gloves, etc. [21]). Simultaneously, the presence of nuclease should be searched for, either using standard nuclease assays or by taking advantage of the fact that the use of different initial RNA (or DNA) concentrations will give different curves in the presence of nuclease. If an experiment measures different fractions hybridized at a C_0t (or $C_{r,0}t$) value obtained with different values of C_0 or $C_{r,0}$, this can be taken as evidence for the presence of a nuclease. Repeating nucleic acid hybridizations under different conditions (i.e., constant time of incubation versus constant nucleic acid concentration) provides a control for the presence of nuclease as well as determining the reproducibility of the results.

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