# DNA-RNA HYBRIDIZATION AT LOW TEMPERATURE IN THE PRESENCE OF UREA

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SUMMARY

The effect of temperature and urea concentration upon DNA-RNA hybridization kinetics has been studied. Filter hybridization at 40°C in 8 M urea 2xSSC can be used as a reliable routine technique. Relatively large amounts of presumably intact specific RNA molecules can be purified from DNA-RNA hybrids formed in these conditions.

## INTRODUCTION

While protein fractionation is a fairly advanced technical field, purification of messenger RNA (mRNA) species, a problem of fundamental interest, has received no general solution. In principle, DNA-RNA hybridization techniques should provide a basis for purifying the RNAs which are complementary to a given DNA sequence : once purified, DNA-RNA hybrids can be dissociated by various treatments, yielding specific RNAs. The main drawback to such an approach is that the high temperature (60°C to 70°C) required for optimal hybridization for both the Nygaard and Hall liquid (1,2) and the Gillespie and Spiegelman filter techniques (3) causes degradation of the RNAs (4,5,6). It is therefore necessary to perform the annealing reaction at a lower temperature, for instance by the addition of formamide (5). In this report, it is shown that conditions can be found where DNA-RNA hybridization takes place at relatively low temperature in the presence of urea (7), and that hybrids formed in this way yield a rather high proportion of undegraded RNA molecules.

## MATERIALS AND METHODS

Mann extra pure urea was used in all experiments. The dissolution of 717 mg of urea into 1 ml of 3xSSC yields 1,5 ml of 8M urea 2xSSC. SSC is 0.15M

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NaCl 0.015 M Na citrate pH 7.0.

Strains $W_{3350}$ ($\lambda C_{I\ 857}$ sus $R_5$) and $W_{3102}$ ($\lambda C_{I\ 857}\ x_{13}$) (the latter kindly provided by Dr. M. Gottesman) served as a source of late phage RNAs and $1_3$ molecules respectively (see text).

Bacteriophage λ DNA and "early" and "late" pulse labeled RNAs from induced lysogenic cells were prepared as previously described (8). Because λ growth does not cause a complete shut-off of host functions, both the host and phage RNAs are labeled. The maximum percents of counts hybridized are about 7% and 25% for "early" and "late" RNAs respectively. The radioactive precursor used was $^3H$ 5 uracil (C.E.A. 24 c/mM). E. coli DNA (from strain $C_{600}$) was extracted by the pronase procedure (9). 23 S ribosomal RNA, extracted from $C_{600}$ cells labeled for 3 generations, was freed of 16 S RNA by sucrose gradient sedimentation.

Filters (Sartorius MF50, diameter 25 mm) were loaded with alkali denatured DNA as previously reported (8). Microfilters with a diameter of 5 mm were often used (10). 7 or 8 such microfilters can be cut in a 25 mm filter previously loaded with DNA. Regardless of their size, all filters were numbered with a pencil before use. After incubation, 50 to 100 filters were washed altogether in a pierced beaker, by repeated immersion into 2xSSC, then treated together with RNAse (10 μg/ml in 2xSSC, 1 hour at 20°C), washed 3 times as above, dried and counted. When hybridization was carried out in liquid, the hybridization mixture was diluted at least 10 fold, and generally 100 fold prior to filtration, otherwise concentrated urea prevents retention of the hybrids by the membranes. The hybrids were collected on numbered filters which were treated as described above.

When the size of the eluted RNAs was to be studied, urea was dissolved extemporaneously into the reaction mixture. After incubation, the RNAse treatment was omitted. Instead, all buffers and glassware were sterilized. The filters were washed by filtration with 100 ml prefiltered 2xSSC on each side, rinsed in 2xSSC in order to wash the edges, then rinsed in $10^{-3}$ M EDTA, pH 7.0, in order to dilute the salt. Filters were then incubated in 8 M urea $10^{-3}$ M EDTA pH 7.0, at 45°C for 30 minutes; this treatment was repeated 3 times. 80 to 85% of the hybridized counts were usually found in the first eluate, about 10% in the second one. Little DNA is released from the filters during the the elution, except if the hybrid has been made in liquid. After a 100 fold dilution in 2xSSC, no RNA is retained by a filter : the hybrid is, therefore, dissociated. The eluates were then concentrated and freed of urea by alcoholic precipitation with unlabeled carrier RNA and 0.4 M NaCl, then resuspended in Tris $10^{-2}$ M pH 7.2, and subjected to acrylamide (2.7%) gel electrophoresis (11,8). The gels were sliced; the slices digested overnight at 20°C in 0.5 ml ammonia, then counted in 10 ml of Bray's solution (12).

RESULTS

In order to determine optimal conditions for DNA-RNA hybridization at low temperature in the presence of urea, we have used the following approach :

- let H(t) be the amount of DNA-RNA hybrid formed as a function of time t (in hours).
- let Hp be the theoretical plateau value (obtained for t infinite), and $v_o$ = (dH/dt) for t = 0 the initial speed of the reaction.

On the basis of simple theoretical considerations, we assume that, in large DNA excess : (1) the function H(t) is determined by two parameters only : Hp and $v_o$. (2) Hp does not vary much with urea concentration and temperature (otherwise hybridization at low temperature would hardly be possible). Finally, we define the initial rate $V_o = v_o/Hp$ as a normalized initial speed, so that we can compare different labeled RNA preparations, independently of their specific activities.

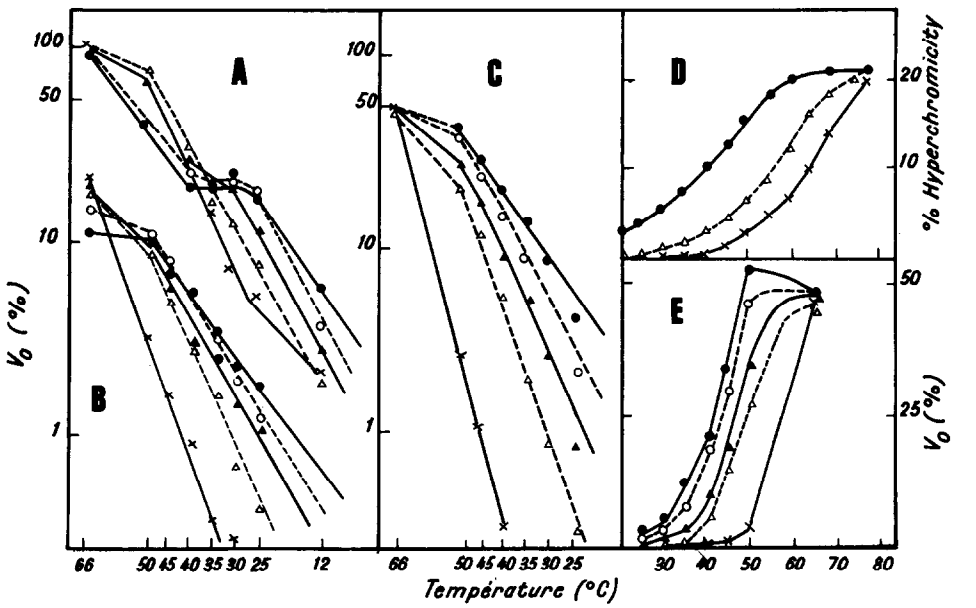


Figure 1 : Initial kinetics of DNA-RNA hybridization as a function of temperature and urea concentration. In A, B and C, Log $V_o$ is plotted versus the reciprocal of the absolute temperature. A. Liquid reaction ; λ DNA and $^3H$ "late" λ mRNA ($10^7$ cpm/mg) concentration : 20 μg/ml. B. Filter reaction ; 2 μg λ DNA per microfilter; $^3H$ "late" λ mRNA ($10^7$ cpm/mg) : 20 μg/ml. Final volume : 100 μl. C. Filter reaction; 100 μg E. coli DNA per filter; $^3H$ 23S ribosomal RNA ($10^8$ cpm/mg) 1 μg/ml. Final volume : 0.5 ml. D. Same as C, but the plot is linear. E. Melting curves of the 23S RNA recorded on a Cary 15. The % hyperchromicity is plotted as a function of temperature. The renaturation curves were almost superimposable to the melting curves.

In all cases the buffer was 2xSSC. —x—x— 0 M urea; ---Δ---Δ--- 2 M urea; —▲—▲— 4 M urea; ---o---o--- 6 M urea; —●—●— 8 M urea. In experiments A and B, DNA was in large excess. In experiments C and D, it was not (% 23S RNA hybridized under these conditions : 15%).

The effect of temperature and urea concentration on the initial rate in both the liquid and filter reaction is shown on Fig. 1. The initial speed $v_o$ was computed from the initial slope, during the first hours of the reaction, of curves such as these shown on Fig. 2, but with an expanded time scale. As an estimate of Hp, we measured the plateau value obtained in 2xSSC at 66°C by the filter technique. Following assumption (2), all $v_o$ measurements were normalized with this value. Finally, we plotted log $V_o$ versus the reciprocal of the absolute temperature. This plot is convenient, because the graphs show straight segments. Whether or not their slope is related to the activation energy of the reaction is unknown.

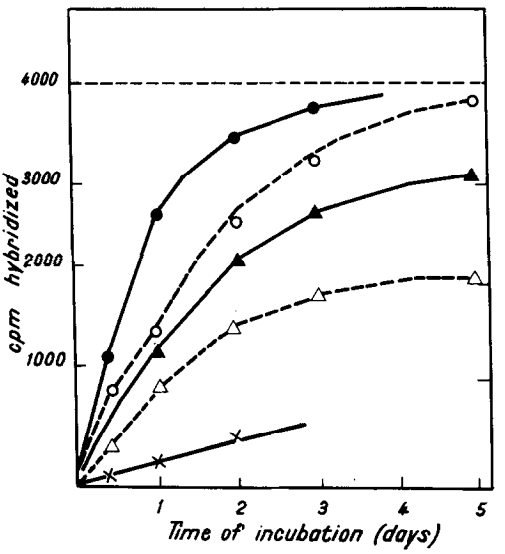


Figure 2 : Kinetics of filter hybridization in 8M urea-2xSSC at various temperatures. 2 µg λ DNA per microfilter; "early" λ mRNA ($20 \cdot 10^6$ cpm/mg) : 20 µg/ml. Final volume : 100 µl. λ DNA was in large excess. ---Δ---Δ--- 25°C; —▲—▲— 30°C; ---o---o--- 35°C; —●—●— 40°C. Controls in 2xSSC without urea : —x—x— 40°C;-------plateau value at 66°C (3 determinations).

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The following comments are relevant to the results shown in Fig. 1 :

(1) The liquid reaction is 10 to 20 times faster than the filter reaction carried out in the same conditions, at any temperature and urea concentration (Fig. 1A and B).

(2) At certain temperatures, the addition of urea to a liquid hybridization mixture slows down the reaction. This does not occur if the reaction is performed on filters, which probably means that, in liquid, the addition of urea favours DNA renaturation with respect to DNA-RNA hybridization (Fig. 1A and B).

(3) Addition of urea to a filter reaction mixture considerably speeds up the reaction at all temperatures below 50°C. At 40°C, the addition of 8 M urea increases the initial rate by a factor of 5 to 10 when pulse-labeled messenger RNAs transcribed in vivo from the bacteriophage λ chromosome are hybridized to λ DNA (Fig. 1B). A 100 or 200 fold increased initial rate is observed when labeled 23S ribosomal RNA (or transfer RNA) is hybridized to homologous E. coli DNA (Fig. 1C). In the latter case, one function of urea may be to destroy the secondary structure of the 23S RNA : this is suggested by the strong correlation existing between hybridization kinetics and hyperchromicity measurements (Fig. 1D and E). It seems, then, that the RNAs do not hybridize unless they are partially melt.

These data also show that, in all cases studied, the addition of 8 M urea makes the filter reaction at 40°C initially as fast as if it were carried out without urea at 55°C or 60°C, in which case we know, from previous experience, that the reaction is reasonably rapid. From assumption (1), we expect the whole kinetics to be similar, in which case the plateau value, in 8 M urea 2xSSC at 40°C, should be approached in a reasonable amount of time. It is shown in Fig. 2 that these expectations are essentially correct, and that the plateau values are unlikely to differ widely, which a posteriori justifies assumption (2) and our approach to the problem. There was no detectable loss of DNA from the filters during incubation at 40°C in 8M urea 2xSSC after as long as 6 days.

One may question the specificity of the hybrids formed,with respect to both template recognition and hybrid structure. Hybrids formed in any of the conditions used are resistant to RNAse. E. coli labeled RNAs do not hybridize to λ DNA (less than 0.01 % of input cpm). In some instances, the RNAs eluted from urea hybrids were shown to rehybridize with a 80% efficiency to their own template. Therefore, we conclude that the hybrids formed at 40°C in 8M urea 2xSSC retain the specificity of their template. We do not know if the hybrid structure is perfect, as it can only be studied by estimating the size of RNA fragments eluted from RNAse treated DNA-RNA hybrids (4). However, it is our experience that hybrids made in urea on filters lose less radioactivity during RNAse treatment (0-20%) than hybrids formed on filters at 66°C in 2xSSC (30 to 50%). This observation suggests a better structural specificiy of urea hybrids.

We then studied the molecular weight (M.W.) of the RNAs eluted from hybrids made on filters, either at 66°C in 2xSSC, or in 8M urea 2xSSC at 40°C. RNAse treatment was avoided. Instead, the filters were carefully washed. The hybridized RNAs were eluted and concentrated as described in the material and methods section, then subjected to electrophoresis on polyacrylamide gels. Two

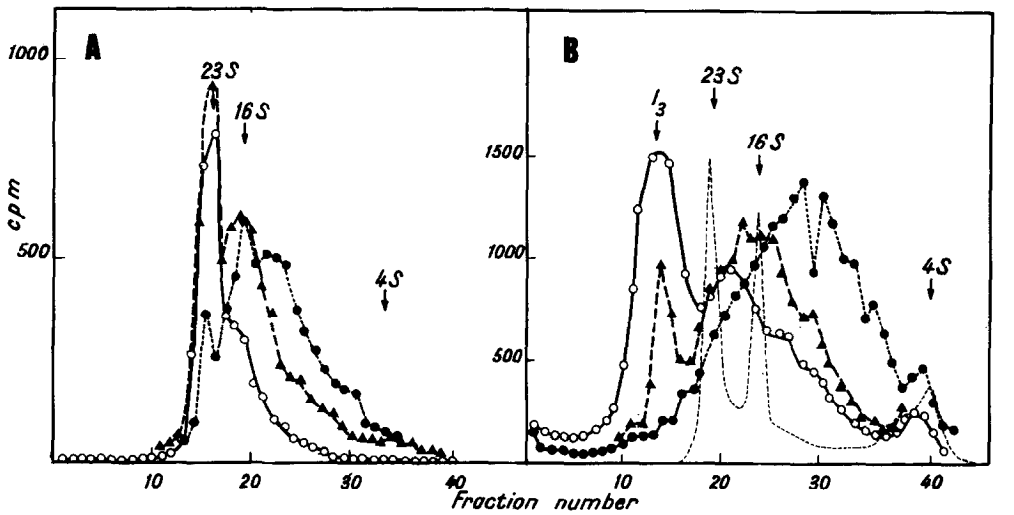


Figure 3 : Size of RNA molecules eluted from DNA-RNA hybrids. Various $^{3}H$ labeled RNA samples were run on different acrylamide gels, with $^{32}P$ E.coli stable RNAs as mobility markers. Several patterns have been superimposed on the same graphs. A. $^{3}H$ 23S ribosomal RNA. B. $^{3}H$ RNAs from induced W3102 (λ CI 857 x13), a strain which produces mainly $l_3$ molecules as phage transcription products (13). —o—o— untreated RNAs; - - ▲ - ▲ - - recovered from urea hybrids; ---●---●--- RNAs recovered from hybrids made at 66°C in 2xSSC; ····· $^{32}P$ labeled mobility markers. It must be realized that the pattern of the untreated λ mRNAs is revealed by the hybridization of each slice eluate with excess λ DNA. In contrast the slices containing RNAs recovered from hybrids are counted directly. In several experiments these RNAs were eluted. They hybridized with an efficiency of 80% to their own template, and little or not to E. coli DNA. Estimates of intact material recovered were computed from the fractions of the pattern area which migrate with the mobility of initially intact 23S or $l_3$ molecules.

types of RNAs were used : purified labeled 23S ribosomal RNA, and pulse-labeled RNA preparations rich in one mRNA species, called $l_3$, which is hyperproduced by certain mutants of bacteriophage λ (13). Species $l_3$ has a very high M.W. of about $1.8\ 10^6$ daltons (see Fig. 3B). Hybridization was carried out long enough to approach the plateau. The time of incubation required was 8 hours when 23S RNA was hybridized to E. coli DNA at either 66°C without urea or 40°C with 8M urea. It was 2 days when $l_3$ containing RNAs were hybridized to λ DNA at 66°C without urea, and 3 days at 40°C when 8M urea was added.

In all cases, it is obvious that the RNAs eluted from urea hybrids are less degraded than those eluted from hybrids made at 66°C in the absence of urea. Rough estimates, based on the assumption that there is no selective loss of high or low M.W. material during the hybridization and elution procedures, indicate that 65% of the 23S RNA hybridized in urea is recovered intact (versus 20% at 66°C). 25% of presumably intact $l_3$ molecules were recovered after hybri-

dization in urea (versus 5% at 66°C). Attempts have been made to minimize the time of incubation. After a 4 hours liquid hybridization in 2xSSC at 66°C without urea, about 30% of $1_3$ molecules were recovered intact. After having been hybridized for 18 hours in liquid 8M urea 2xSSC at 40°C, between 60 and 95% of intact $1_3$ molecules were recovered.

## DISCUSSION

In this publication, we have reported the effect of urea upon both liquid and filter DNA-RNA hybridization. The rationale used to set up the technique may be of relevance in other studies. It consists in analyzing the initial kinetics rather than looking for plateau values, the assumption being that, if two reactions have similar initial rates, they obey similar long range kinetics.

Hybridization on filters in 8M urea 2xSSC at 40°C is a reasonably fast reaction, and can be used as a routine technique. It seems to be as reliable as the Gillespie Spiegelman technique, and may even be recommended for several reasons. Although somewhat slower, the urea technique provides the same "plateau" values. The backgrounds are extremely low, the hybrids formed may be more specific in terms of hybrid structure. No DNA at all seems to be released from the membrane during incubation. This is also true for certain animal cell DNAs (14,15) and SV 40 DNA (16) which are partially released from the filters at 66°C. Finally, the urea technique allows a higher recovery of presumably intact RNA molecules purified through hybridization and elution procedures. It may also allow sufficient material to be obtained for sequence analysis, especially since RNA competition techniques would permit the purification of one labeled uncompeted RNA species, together with a mixture of other unlabeled species.

The performances of the urea and formamide techniques have not been compared yet. As far as recovery of intact ribosomal RNA is concerned, the urea technique seems to be at least equivalent to the formamide technique (6). Further work is in progress to try and improve the recovery of intact RNA molecules purified through hybridization procedures.

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