Biochem. J. (1971) **125**, 481–487
Printed in Great Britain

Ribonucleic Acid–Deoxyribonucleic Acid Hybridization in Aqueous Solutions and in Solutions Containing Formamide

By S. GILLESPIE* and D. GILLESPIE*
Department of Biology, Brandeis University, Waltham, Mass. 02154, *U.S.A.*

(*Received* 4 *June* 1971)

Hybridization in 6×SSC (SSC, 0.15M-sodium chloride–0.015M-sodium citrate) at 66°C was compared with hybridization in formamide–6×SSC (1:1, v/v) at 35°C. As expected, the RNA hybridization potential was labile in the former system and stable in the latter. DNA retention by filters was poor in the formamide system, but could be improved. Several other properties of the hybridization reaction were explored and it was concluded that the formamide system is generally superior.

RNA–DNA hybridization on membrane filters has been extensively used to measure nucleotide-sequence complementarity of RNA and DNA. To maximize hybridization specificity, the reaction is performed as near the hybrid 'melting point' as possible, i.e. at high temperatures (Gillespie & Spiegelman, 1965) or in formamide at moderate temperatures (Bonner, Kung & Bekhor, 1967). Hybridization experiments at 66°C are limited to short reaction times (24–48h), because of thermal scission of RNA (Gillespie, 1966, 1968). It has been generally accepted that because hybridization takes place at lower temperatures, long-term reactions can be performed in formamide (Bonner *et al.* 1967; McCarthy & Duerskin, 1970; McCarthy, Shearer & Church, 1970), even though the stability of RNA hybridization potential in formamide has not been directly measured.

There has been a general reluctance to use the formamide system, even though increased RNA stability in formamide is likely, because the formamide system has not been well characterized. Attempts to assay DNA retention, RNA 'noise' (the extent to which RNA is bound to the filter in the absence of DNA), and the specificity and rate of a standardized hybridization reaction have either been done under formamide conditions that do not yield maximum specificity (Bonner *et al.* 1967) or under unusual high-temperature conditions (McConaughy, Laird & McCarthy, 1969).

We have measured these parameters and explored properties of saturation and competition experiments in the formamide system, with the following results. 1. Under conditions that generate maximum specificity, the retention of DNA is poor, but losses are complete in 12h or less. Retention can be improved by extensive heating of DNA filters at 80°C *in vacuo* or by using freshly prepared DNA. 2. The RNA 'noise' level is virtually undetectable. 3. The rate of hybrid formation is equivalent to that observed in 6×SSC at 66°C. 4. The RNA hybridization potential is stable for at least 5 weeks. 5. RNA–DNA hybrids are stable for at least 4 weeks (less than 5% decomposition). 6. With homologous RNA species 90% competition can be attained. 7. Saturation values for both the ribonuclease-resistant hybrid and the ribonuclease-sensitive hybrid are the same as in 6×SSC. 8. RNA–DNA hybrids are ribonuclease-resistant over stretches involving at least 50 consecutive nucleotides.

MATERIALS AND METHODS

Preparation of nucleic acids. DNA from *Escherichia coli* A2325 (Jacobson & Gillespie, 1968) was prepared as described by Gillespie & Spiegelman (1965). RNA from *E. coli* A2325 was prepared by extraction with diethyl pyrocarbonate (Summers, 1970). Cells were broken by freezing and thawing in the presence of lysozyme and electrophoretically pure deoxyribonuclease (Gillespie & Spiegelman, 1965) and made 1.5% in recrystallized sodium dodecyl sulphate. Diethyl pyrocarbonate (Eastman Organic Chemicals, Rochester, N.Y., U.S.A.) was added (0.03ml/ml of broken cells) and the system was incubated at 37°C for 5min. Saturated NaCl (0.5 vol.) was added to the mixture, which was then chilled to 0°C, clarified twice by centrifugation for 20min at 12000*g*, and the final supernatant containing the RNA was kept. The RNA was precipitated by ethanol and dissolved in 6×SSC*.

* Present address: National Institutes of Health, Gerontology Research Center, Baltimore City Hospitals, Baltimore, Md. 21224, U.S.A.

* Abbreviation: SSC, 0.15M-sodium chloride–0.015M-sodium citrate.

Pulse-labelled RNA was used for hybridization at this stage of purification. The mRNA-free rRNA, isolated from cells treated for 30 min at 37°C with 200 μg of rifampicin/ml, was further purified by sucrose-density-gradient centrifugation in sodium dodecyl sulphate (Jacobson & Gillespie, 1968) and used for competitive hybridization experiments. Chase-^{32}P-labelled rRNA (Gillespie & Spiegelman, 1965) was additionally dialysed against gradient buffer and treated at 30°C for 20 min with 20 μg of electrophoretically pure deoxyribonuclease/ml after the first sucrose-density-gradient centrifugation and then repurified on sucrose-density gradients. Excess of sodium dodecyl sulphate was removed from relevant RNA preparations before hybridization by chilling them to 0°C and removing the precipitate by centrifugation. Finally, the ^{32}P-labelled RNA preparation was passed three times through nitrocellulose membrane filters (Schleicher and Schuell, type B-6; from Matheson and Higgins, Cambridge, Mass., U.S.A.).

RNA–DNA hybridization. DNA was denatured in 1 ml of 0.3 M-NaOH at 22°C for 10 min. The solution was adjusted to approx. pH 7 with 0.29 ml of 1 M-HCl and diluted to 25–50 ml with 6 × SSC. Portions were immediately loaded on 25 mm nitrocellulose filters. One DNA preparation was stored denatured in 0.01 × SSC at pH 7.0 for 1 year at 0°C and is referred to as 'aged' DNA. The DNA filters were dried at room temperature for 2 h and then at 80°C under vacuum for 2 h. In some cases 18 mm circles were punched out of the larger filters for use in small-volume hybridization experiments.

Hybridization was performed by immersing the DNA filters, DNA-side down, in 2.0 ml of solution (for 25 mm filters) or in 0.2 ml of solution (for 18 mm filters). In the present paper, a solution referred to as 0.2 ml of formamide–6 × SSC (1:1, v/v) is 0.1 ml of formamide and 0.1 ml of 6 × SSC, pH 7.0. Annealing was performed at the temperatures indicated in each experiment and the hybrid-containing filters were in general processed by: 1, soaking the filters for at least 15 min in cold 2 × SSC, 2, washing the filter on each side with 30 ml of 2 × SSC, 3, incubating the filter in 5 ml of 2 × SSC containing 100 μg of boiled pancreatic ribonuclease (Sigma Chemical Co., St Louis, Mo., U.S.A.), and 4, rinsing the filters by immersion in a beaker of 2 × SSC. In exhaustive hybridization experiments, filters were simply removed from the hybridization mixture and rinsed in a beaker of 2 × SSC.

Chemical determination of filter-bound DNA. The method of Meijs & Schilperoort (1971) was used. DNA filters were incubated in 1 ml of 1.6 M-$HClO_4$ at 70°C for 30 min to elute DNA. Then 0.5 ml of the cooled eluate was added to 1 ml of a freshly prepared mixture containing 0.5 g of diphenylamine (Fisher Scientific Co., Fair Lawn, N.J., U.S.A.), 50 ml of acetic acid, 0.5 ml of conc. H_2SO_4 and 0.25 ml of a 16 mg/ml solution of acetaldehyde in

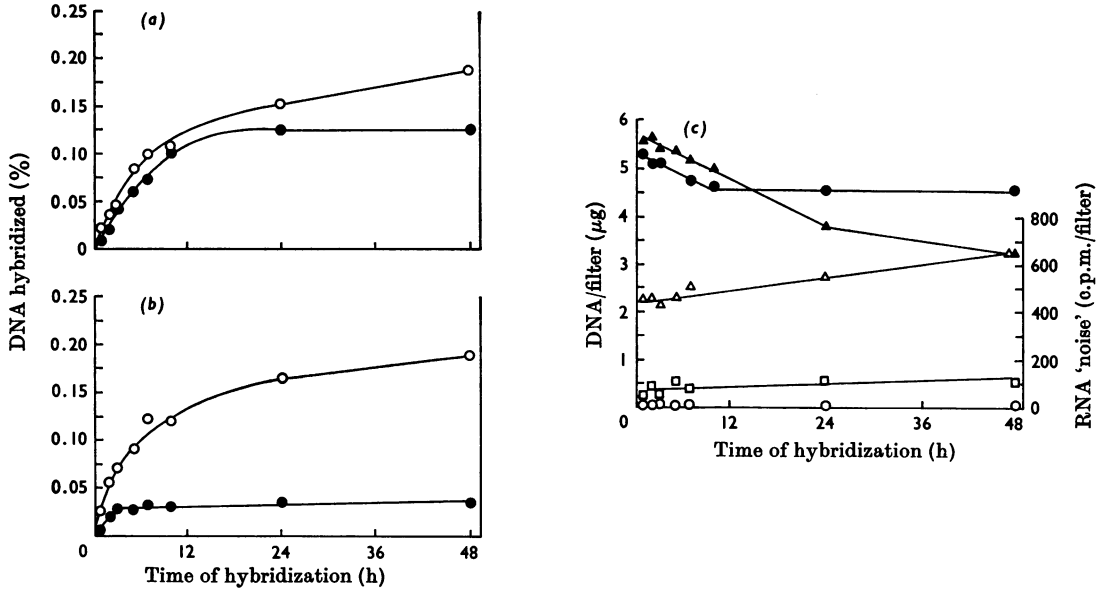

Fig. 1. Hybridization kinetics in 6 × SSC or in formamide–6 × SSC. (*a*) Hybridization of 0.3 μg of ^{32}P-labelled rRNA (100000 c.p.m./μg) to 5 μg of ^{3}H-labelled DNA (822 c.p.m./μg) were performed in 2 ml of 6 × SSC at 66°C or in 2 ml of formamide–6 × SSC (1:1, v/v) at 35°C. ●, In 6 × SSC; ○, in formamide–6 × SSC. At intervals, filters were removed, washed, treated with ribonuclease and rinsed as described in the Materials and Methods section. (*b*) The procedure and symbols were the same as in Fig. 1(*a*), except that the RNA solutions were preincubated for 24 h under hybridization conditions before addition of the filter. (*c*) Additional results obtained from the experiments described in Figs. 1(*a*) and 1(*b*). ● and ▲, DNA remaining on the filter; △ and ○, RNA 'noise' in the experiment of Fig. 1(*a*); □, RNA 'noise' in the experiment of Fig. 1(*b*); □, △, ▲, in 6 × SSC; ○, ●, in formamide–6 × SSC.

water (Burton, 1968). After incubation for 18–20h at 30°C, the E_{600} of the solution was determined and corrected for control filters containing 75μg of DNA over a blank reading of approx. 0.05. Comparison with dissolved DNA standards indicated that over 90% of the DNA was released from the filter and was as available for assay as the dissolved DNA. Further, DNA filters freshly prepared and untreated gave the same results on assay as filters incubated in formamide under hybridization conditions or as filters that had been stored in toluene–Liquifluor (Pilot Chemicals, Boston, Mass., U.S.A.) for determination of radioactivity, then dried and assayed. No change in the blank values was observed with any of these treatments. Consequently, hybridization was performed on DNA filters and the radioactivity on the filter was determined (Gillespie & Spiegelman, 1965); then the filters were removed, dried and incubated in $HClO_4$, and the eluate was assayed for DNA.

RESULTS AND DISCUSSION

Retention of DNA by membrane filters during hybridization. Fig. 1(*c*) shows that DNA is not completely retained by membrane filters in either the 6×SSC or the formamide–6×SSC system. The DNA loss from filters in formamide–6×SSC is complete in 12h, whereas the loss in 6×SSC continues for at least 48h. Some 90% of the DNA is retained by the filter in the formamide–6×SSC system and this DNA remains on the filter for at least 5 weeks under hybridization conditions (Fig. 5, curve C).

Several laboratories have found that DNA preparations vary in their capacity to be retained by filters during hybridization in formamide–6×SSC. We have found that at least two types of DNA preparations are poorly retained when DNA filters are prepared in the usual manner (Table 1). DNA preparations that have been stored for a long time in 0.01×SSC ('aged' DNA) or that have been sonified to about 10^6 daltons are not effectively retained during incubation in formamide–6×SSC, whereas freshly prepared DNA ('new' DNA) is retained (sample 2). Aged or sonified DNA is also lost from the filter during incubation in 2×SSC under hybridization conditions (sample 5), but is not lost during incubation in 6×SSC (sample 4). Prolonged heating of DNA filters before incubation increases the retention in formamide–6×SSC markedly (sample 3), but the heat treatment cannot be carried out for too long or DNA is once again lost from the filter (results not shown). Our experience suggests that the ability of DNA to be retained by nitrocellulose membrane filters declines rather rapidly on storage at 0°C in 0.01×SSC. The retention capacity of DNA preparations may best be preserved by storing DNA in the undenatured state, frozen at −20°C (A. Levine, personal communication). When DNA loss occurs, however, it seems to be random, since filters containing 50 or 90% of the original DNA show the same saturation values for rRNA.

RNA degradation and RNA noise. Fig. 1(*a*) shows the kinetics of hybridization of 0.3μg of ^{32}P-labelled rRNA to 5μg of DNA in formamide–6×SSC. The early kinetics in the two systems are similar. Neither system reaches saturation (Gillespie & Spiegelman, 1965) by the end of the experiment, but hybridization is still taking place in formamide–6×SSC while an unusually low plateau is reached in 6×SSC. The input RNA was somewhat degraded (average *s* value 16S). Based on the previously determined degradation rate of

Table 1. *Retention of DNA by membrane filters*

Denatured DNA (5μg) was loaded on 25mm membrane filters in 6×SSC and the filters were washed with 10ml of 6×SSC. The efficiency of 'sticking' new DNA or sonified DNA ('sonified' DNA was sonified at 40μg/ml in 0.01×SSC at 0°C in a Branson Sonifier; the solution was pulsed five times, with 10s duration per pulse, at 3.5A) to the filter was 100% in either 6×SSC or 2×SSC. The efficiency of 'sticking' aged DNA to the filter was 80% in 6×SSC and 40% in 2×SSC. All filters were dried at 25°C for 2h; those so designated were additionally dried at 80°C for the specified lengths of time. Dried filters were placed in 25mm scintillation vials containing 5ml of 2×SSC or 6×SSC, or 2ml of formamide–6×SSC (1:1, v/v). The vials were incubated at the specified temperature for 5h, then rinsed with 2×SSC at 25°C. Subsequent washing and ribonuclease-treatment procedures usually used to purify hybrids (Gillespie & Spiegelman, 1965) did not release additional DNA. All measurements were done in duplicate.

Sample no.	Drying conditions	Incubation conditions	Percentage retained: New DNA	Aged DNA	Sonified DNA
1	25°C, 2h	Formamide–6×SSC, 35°C	86	4	8
2	80°C, 2h	Formamide–6×SSC, 35°C	101	54	59
3	80°C, 18h	Formamide–6×SSC, 35°C	96	86	92
4	80°C, 2h	6×SSC	101	90	103
5	80°C, 2h	2×SSC	97	70	67

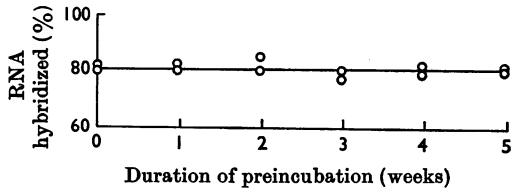

Fig. 2. Stability of pulse-labelled RNA in formamide–6×SSC. Non-radioactive DNA (100 μg) was loaded on 25 mm nitrocellulose filters and dried as described in the Materials and Methods section. Discs (18 mm) containing 75 μg of DNA were cut out of these filters and incubated at room temperature in formamide–6×SSC (1:1, v/v) for 24 h. Hybridization vials containing 0.2 ml of formamide–6×SSC (1:1, v/v) and 0.1 μg of pulse-labelled RNA, isolated from cells labelled for 1 min with 10 μCi of [^{3}H]-uridine (10 μCi/μg)/ml, were prepared and incubated at 35°C without DNA filters. At the times indicated on the abscissa of the Figure, DNA filters were introduced. Hybridization was performed for 8 days. Filters were removed and rinsed in a beaker of 2×SSC at room temperature. The amount of DNA on the filter was assayed chemically as described in the Materials and Methods section.

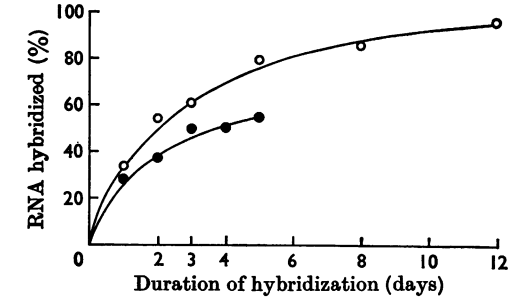

Fig. 3. Kinetics of hybridization of pulse-labelled RNA. Conditions are the same as those described for Fig. 2, except for the duration of the hybridization reaction. ○, DNA filters preincubated in formamide–6×SSC (1:1, v/v) for 24 h at 25°C before use; ●, DNA filters not preincubated.

ribo-oligonucleotides in 6×SSC at 66°C of 1 chain-break/6 h (Gillespie, 1966), one would expect the hybridization reaction to stop at about 24 h in this system with this RNA preparation.

If the cause of the early plateau in the experiment with 6×SSC is RNA degradation, preincubation of the RNA under hybridization conditions for 24 h in the absence of the filter should destroy the hybridization capacity of the RNA. Fig. 1(*b*) shows that this is the case. This assay shows that RNA can be preincubated in formamide–6×SSC for at least 5 weeks without affecting the hybridization potential of the RNA (Fig. 2).

The apparent stability of RNA in the formamide system makes it possible to perform experiments that require long-term hybridizations. Fig. 3 shows the kinetics of hybridization of pulse-labelled RNA (0.1 μg) to DNA (75 μg). Under these conditions, DNA should be in excess of all the RNA species (rRNA, 0.085 μg; DNA complementary to rRNA, 0.225 μg), and the reaction goes nearly to completion in 12 days. Hybridizations of this duration are not possible in 6×SSC. Losses of DNA from filters make hybridizations of this type (exhaustive hybridizations) impossible, since this dissolved DNA will compete with immobilized DNA and hybrids involving dissolved DNA will not be recovered on the filter (Fig. 3). For this reason, the DNA-containing filters were preincubated for 20 h in formamide–6×SSC lacking RNA before use in exhaustive hybridizations. Exhaustive hybridizations make it possible to directly measure the fraction of labelled RNA that is tRNA, rRNA or mRNA.

Fig. 1(*c*) compares the attachment of RNA to filters lacking DNA (RNA 'noise') in the 6×SSC and formamide–6×SSC systems. It is apparent that the RNA isolation procedure we have used is not suitable for hybridizations in 6×SSC, since about half of the radioactivity complexed in the presence of DNA is RNA 'noise' (compare Figs. 1*a* and 1*c*). The degree of 'noise' in formamide–6×SSC is not detectable, even in less pure preparations. In fact, every RNA preparation we have obtained shows considerably more 'noise' in 6×SSC than in formamide–6×SSC, including rRNA, tRNA, pulse-labelled RNA and RNA synthesized *in vitro*.

In experiments where the hybridization values are extremely low, e.g. with a purified species of tRNA, the 'noise' can be decreased in the following manner. After hybridization, filters are washed extensively on both sides with 2×SSC. Ribonuclease treatment may or may not be included, depending on the nature of the experiment. Filters are then placed in fresh formamide–6×SSC (1:1, v/v) under hybridization conditions for 15–60 min, removed, rinsed in 2×SSC at 22°C, dried, and counted for radioactivity. The hybrid is stable during the final incubation in formamide–6×SSC, but the 'noise' decreases by a factor of 2–3 (S. Reich, unpublished work).

Saturation and competition experiments. Fig. 4(*a*) presents saturation curves in the formamide–6×SSC system and shows the effect of ribonuclease on the hybrid structures. Both the ribonuclease-resistant hybrid and the ribonuclease-sensitive complex occupy about 0.3% of the DNA at saturation. This is in excellent agreement with the results in 6×SSC (Gillespie, 1968).

Fig. 4(*b*) presents pre-hybridization competition experiments with ^{32}P-labelled rRNA and non-

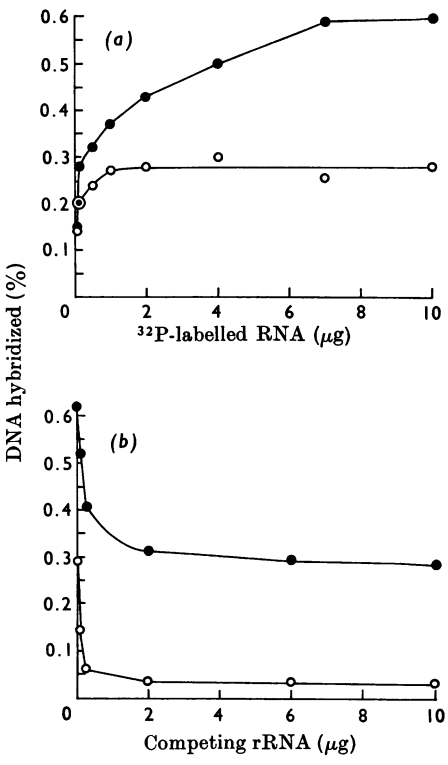

Fig. 4. Competitive and saturation hybridization in formamide–6×SSC. (*a*) Hybridizations were performed as described for the formamide–6×SSC system in Fig. 2, except that different amounts of ^{32}P-labelled rRNA were used and the hybridization reaction was performed for 20h. ●, Without ribonuclease; ○, with ribonuclease. (*b*) Designated amounts of mRNA-free rRNA (non-radioactive) were hybridized in 0.2ml of formamide–6×SSC (1:1, v/v) to 18mm DNA filters containing 75μg of DNA for 20h at 35°C. Filters were removed, rinsed in 2×SSC and placed in vials containing 7.0μg of ^{32}P-labelled rRNA in 0.2ml of formamide–6×SSC (1:1, v/v). The second period of hybridization was performed at 35°C for 20h; then the filters were removed and processed as described in the Materials and Methods section with and without ribonuclease. DNA was measured chemically as described in the Materials and Methods section. ●, Without ribonuclease; ○, with ribonuclease.

radioactive rRNA. This experiment is performed by hybridizing different amounts of non-radioactive mRNA-free rRNA (see the Materials and Methods section) to 5μg of DNA, removing and rinsing the filter, then challenging the RNA–DNA complex with ^{32}P-labelled rRNA under hybridization conditions. After the second hybridization the filters are removed and processed with or without ribonuclease. When ribonuclease is included in the hybrid purification, about 90% of the ^{32}P-labelled RNA can be competed with and complete competition occurs at a non-radioactive RNA input where saturation is just reached (compare Figs. 4*a* and 4*b*). The competition value of 90% is less than the 95% observed in the 6×SSC system (Gillespie, 1966, 1968) and is reproducible. When ribonuclease is included after both periods of hybridization, low competition values (70%) are obtained (results not shown). We consider that the ribonuclease used to treat the 'pre-hybrid' remains bound to the filter and increases the RNA 'noise' during the subsequent hybridization. Experiments with blank filters show the expected high 'noise' value.

When ribonuclease is not included in the final hybrid purification procedure, only 50% competition is observed and the residual uncompeted ^{32}P-labelled RNA occupies the same amount of DNA as does the ribonuclease-sensitive complex. It has been shown in the 6×SSC system that the ribonuclease-sensitive complex is dilution-sensitive (Gillespie, 1968). It is therefore likely that the preformed non-radioactive ribonuclease-sensitive complex falls off during the second hybridization, permitting formation of a radioactive complex. If this were the case, one would expect that at very low RNA inputs, the ratio of ribonuclease-sensitive to ribonuclease-resistant complex would be low, and this is observed (Fig. 4*a*). In experiments where it is necessary to remove the ribonuclease-sensitive fraction of the prehybrid but where ribonuclease cannot be used, the prehybrid can be incubated in an excess of formamide–6×SSC under hybridization conditions (but lacking radioactive RNA) for about 60min, then transferred to a new vial for a second period of hybridization.

Fig. 5 (curve B) shows that the preformed non-radioactive RNA–DNA hybrid is stable, since incubation of this hybrid for several weeks in formamide–6×SSC containing radioactive RNA decreases the competition by only 4% from the value observed at 1 or 2 days.

Finally, the hybrid formed in formamide appears to be composed of perfect complementary base pairs over a stretch of at least 50 consecutive nucleotides. It has been reported that this number of uninterrupted base pairs is required for maintenance of a stable hybrid at 66°C in 6×SSC (Gillespie, 1966). It is assumed that ribonuclease will excise a single mispaired base. Treatment with ribonuclease at 66°C of a preformed hybrid will cause release of the hybrid from the DNA if RNA fragments of less than 50 nucleotides in length are generated. Table 2 shows that hybrids formed in 6×SSC or formamide–6×SSC are quantitatively resistant to this treatment. Since the *E. coli* genome is comprised of 10^7 nucleotides and random chances of finding a unique sequence of 50 nucleotides in

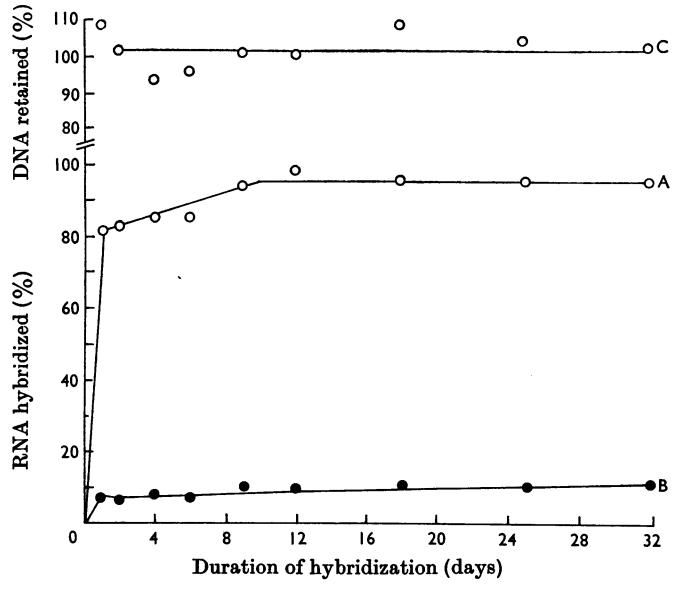

Fig. 5. Stability of RNA–DNA hybrids. Curve B, mRNA-free rRNA (7 μg) was annealed to 75 μg of DNA as described in the legend of Fig. 4(*b*). The filters were rinsed and placed in 0.2 ml of fresh formamide–6 × SSC (1:1, v/v) containing 0.15 μg of ^{32}P-labelled rRNA. After the indicated times filters were removed, rinsed in 2 × SSC (no ribonuclease), dried and counted for radioactivity. Curve A, as for curve B, but the first period of hybridization was omitted. Curve C presents the amount of DNA per filter, determined chemically (see the Materials and Methods section).

Table 2. *Ribonuclease resistance of hybrids*

A sample (7 μg) of ^{32}P-labelled rRNA was annealed to 5 μg of ^{3}H-labelled DNA for 20 h in 6 × SSC or formamide–6 × SSC (1:1, v/v) as described for Fig. 1(*a*). Hybrids were purified as described in the Materials and Methods section.

Sample	Incubation conditions	Ribonuclease treatment	DNA hybridized%
1	6 × SSC, 60°C	None	0.60
2		Room temperature, 60 min	0.30
3		66°C, 60 min	0.29
4	Formamide–6 × SSC, 35°C	None	0.69
5		Room temperature, 60 min	0.28
6		66°C, 60 min	0.28

length are about 1 in 10^{11} (1 in 4^{50}), ribonuclease-resistant RNA–DNA hybrids should therefore consist predominantly of RNA molecules hybridized to their gene of origin. Hybridization of an RNA molecule to a gene other than its gene of origin should therefore reflect an evolutionary or metabolic constraint, rather than a lack of specificity of the hybridization reaction.

This research was supported by Public Health Service Grant no. GM 14368 from the National Institute of General Medical Sciences and by Grant IN-29 from the American Cancer Society.

REFERENCES

Bonner, J., Kung, G. & Bekhor, I. (1967). *Biochemistry, Easton*, **6**, 3650.

Burton, K. (1968). In *Methods in Enzymology*, vol. 12B, p. 163. Ed. by Grossman, L. & Moldave, K. New York: Academic Press Inc.

Gillespie, D. (1966). Ph.D. Thesis: University of Illinois, Urbana, Ill.

Gillespie, D. (1968). In *Methods in Enzymology*, vol. 12B, p. 641. Ed. by Grossman, L. & Moldave, K. New York: Academic Press Inc.

Gillespie, D. & Spiegelman, S. (1965). *J. molec. Biol.* **12**, 829.

Jacobson, A. & Gillespie, D. (1968). *J. Bact.* **95**, 1030.
McCarthy, B. J. & Duerskin, J. D. (1970). *Cold Spring Harb. Symp. quant. Biol.* **35**, 621.
McCarthy, B. J., Shearer, R. N. & Church, R. B. (1970). *RNA in Development*. University of Utah Press.
McConaughy, B. L., Laird, C. D. & McCarthy, B. J. (1969). *Biochemistry, Easton*, **8**, 3289.
Meijs, W. H. & Schilperoort, R. A. (1971). *FEBS Lett.* **12**, 166.
Summers, W. (1970). *Analyt. Biochem.* **33**, 459.