Improvements of the membrane filter method for DNA:rRNA hybridization

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We describe and recommend the following improvements of DNA:rRNA membrane filter hybridization methods. One of our aims was to avoid DNA release from filter discs during hybridization.

1. Our hybridization conditions are 2 SSC in aq. dest., with 20% formamide, 50 C, overnight for 16 hr.
2. Duplexing is over in 8–10 hr.
3. Formamide has to be very pure (O.D. $\leqslant$ 0.2/cm light path at 270 nm).
4. RNAase treatment: 250 μg/5 ml 2 SSC/filter at 37 C for 1 hr.
5. Our conditions for stepwise thermal denaturation are: 5 °C steps from 50 C to 90 C in 1.5 SSC in 20% formamide.
6. Single-stranded DNA, fixed on membrane filters, and stored in vacuo at 4 C, can be used reliably for hybridization for up to 20 months.
7. Concentrated DNA in 0.1 SSC, quick-frozen at –50 C and stored at –90 C for up to 2 years can be used for hybridization without much change.
8. A CsCl gradient purification step yields much purer DNA, but increases the release of DNA from filters by about 20%. Filters with 20% more DNA is a compensation.
9. rRNA can be stored for 20 months in SSC or 2 SSC at –12 C without changing the hybridization results.

INTRODUCTION

In previous papers from this laboratory, one of us examined critically technical aspects of DNA: DNA hybridization techniques: the DNA agar method (De Ley, 1971), the membrane filter methods (De Ley and Tytgat, 1970) and the new initial renaturation rate method (De Ley, Cattoir and Reynaerts, 1970). The present contribution in this series deals with the membrane filter DNA:rRNA hybridization method.

In 1963, Nygaard and Hall described that nitrocellulose membrane filters retain bound RNA, not free RNA. DNA:RNA duplexing was effected in solution and the filters were used only for hybrid detection (Nygaard and Hall, 1964). Gillespie and Spiegelman (1965) fixed DNA on filters, which were now used for both hybrid formation and detection. Bonner, Kung and Bekhor (1967) used formamide to suppress the loss of DNA from filters at high temperatures during DNA:RNA hybridization. The dependence of DNA release on hybridization temperature was established by De Ley and Tytgat (1970). McConaughy, Laird and McCarthy (1969) studied the effect of temperature, salt and formamide concentration on the rate and specificity of the duplexing. The DNA:RNA hybridization methods of the pre-formamide period were reviewed by Gillespie (1968). Although some other methods have been developed to determine the degree and the rate of DNA:RNA hybridizations (see below), no method so far is suitable for, e.g., systematic comparative investigations requiring periods of many months or even years.

In the present paper we investigate the limits of the existing filter DNA: rRNA hybridization method and we propose several improvements.

MATERIALS AND METHODS

Organisms and media. Agrobacterium rhizogenes ICPB TR7, *Agrobacterium tumefaciens* B6, T37, 3/1, 176, 9P Zinca, ATCC 15955, ATCC 17805, and ICPB TT111, *Rhizobium leguminosarum* 4.1 and USDA 316C10a, *Rhizobium japonicum* USDA 3I1b59 and *Chromobacterium lividum* DA were grown on media described by Heberlein, De Ley and Tytgat (1967). *Erwinia herbicola* ("*Agrobacterium gypsophilae*") ATCC 13329, *Erwinia amylovora* NCPPB 683, *Bordetella bronchiseptica* NCIB 8761, *Bacillus pumilus* B12 and *Bacillus megaterium* 899 thy$^-$ were grown on a medium containing (all in w/v) 1% peptone (Oxoid), 1% beef extract (Oxoid), 0.5% NaCl and 2.5% agar.

Arthrobacter oxydans CBRI 21010, CBRI 21011 and *Arthrobacter* species CBRI ER 39 were grown on the medium described by Lochhead and Burton (1954). *Aeromonas salmonicida* NCMB 833 and *Aeromonas hydrophila* AB 833 were grown on the medium described by De Ley and Friedman (1964) and *Escherichia coli* B was grown in (w/v) 0.5% glucose, 0.8% yeast extract (Nederlandse Gist- en Spiritusfabriek, Brugge, Belgium), 1.1% K_2HPO_4, 0.85% KH_2PO_4 and 2.5% agar. All strains except those used for the preparation of ^{14}C-labeled rRNA, were grown in Roux-flasks for 2 to 3 days at 30 C (*Aeromonas salmonicida* at 25 C, and *E. coli* at 37 C). *Zymomonas mobilis* Z1 was grown at 30 C in liquid medium containing 2% glucose and 0.5% yeast extract. All

necessary measures were taken to ensure that bacteriologically pure cultures were always used.

Preparation of ^{14}C-labeled ribosomal RNA. The cells were grown in 1-liter Erlenmeyer-flasks with a Klett-tube as side-arm. They contained 100 ml of the appropriate liquid medium (see above) and 0.1 mCi of filter-sterilized uracil-2-^{14}C (The Radiochemical Center, Amersham, Buckinghamshire, England). Growth was followed turbidimetrically. At the end of the log phase the cells were harvested and rRNA was prepared according to Moore and McCarthy (1967). In the final purification approximately 1 mg of RNA in 0.5 ml SSC (SSC contains 0.15 M NaCl and 0.015 M trisodium citrate at pH 7.0) was carefully layered on 28 ml of a 15 to 30% (w/v) linear sucrose gradient in 0.1M sodium acetate pH 5.1 in 0.05 M NaCl (McConkey, 1968) and centrifuged for 18 hr at 27000 rpm and 4 C in the SW 27 rotor of a preparative ultracentrifuge (Beckman L2–65B). After centrifugation 1-ml fractions were collected and the radioactivity of each one was measured to detect the peaks. The 23S and 16S peaks were dialyzed against 2 SSC at 4 C. Residual DNA was at most 0.2% and residual protein less than 1%. The rRNA solution was stored at —12 C. Radioactive 16S and 23S rRNA were prepared from *Agrobacterium tumefaciens* ICPB TT111, *A. rhizogenes* ICPB TR7, *Arthrobacter oxydans* CBRI 21010 and *Escherichia coli* B with a specific activity of 3000, 2400, 3800 and 4800 cpm/μg respectively.

Preparation of DNA. DNA was prepared by a combination of the methods of Marmur (1961) and Kirby (Kirby, 1957; Kirby, Fox-Carter and Guest, 1967) as described by De Ley, Cattoir and Reynaerts (1970). *Arthrobacter* lyzed with difficulty or not at all in 0.15 M NaCl–0.1 M EDTA buffer, even when the lysozyme concentration was increased. Lysis occurred more readily in 0.033 M Tris–0.001 M EDTA pH 8 with 5 mg of lysozyme/g wet cells (Crombach, 1972).

Purification of DNA by centrifugation in preparative CsCl gradients (De Ley, unpublished). To decrease the remaining impurities a number of DNA preparations were purified by equilibrium density centrifugation in CsCl. Centrifugation was carried out in a 50 titanium rotor with 1.6 × 7.6 cm tubes in the Beckman preparative ultracentrifuge L2–65B for 20 hr at 40000 rpm and 20 C. Each tube contained approximately 500 μg DNA in 11 ml 0.1 SSC and the appropriate CsCl concentration, such that the mean density of the solution was the same as the buoyant density of the DNA. The tubes were completely filled by loading *n*-heptane (UCB, p.a.) on top of the DNA-CsCl solution. After centrifugation the gradient was fractionated with an ISCO 640 density gradient fractionator with bromoform as the heavy bottom layer. The DNA fraction was then dialyzed against 0.1 SSC solution (3 times 1 liter) at 4 C for 40 hr.

Fixation of denatured, high-molecular DNA on membrane filters. This was carried out according to De Ley and Tytgat (1970). Originally we used HAWP Millipore filters. Some lots, however, did not retain DNA at room temperature for reasons unknown. SM 11309 Sartorius membrane filters, diameter either 90 or 47 mm, were commonly used. The amount of DNA fixed was calculated from the difference in absorbance of the DNA solution before and after filtration. These filters were stored at 4 C in vacuo (see below). For hybridizations, small discs were punched from the main filters. The diameter was selected so that each disc contained approximately 50 μg of high-molecular single-stranded DNA.

Chemical determination of the DNA content of the filter discs. After incubation of filter discs in hybridization conditions (see below), the DNA content was measured with the method of Meijs and Schilperoort (1971). The filters were treated with 1 ml 1.6 N perchloric acid at 70 C during 30 min. After cooling, 2 ml of diphenylamine reagent (Burton, 1956) was added. Then the filter discs were removed and the mixture was incubated at 30 C for 17 hr. The optical density was measured at 600 nm.

DNA:rRNA hybridizations. In our standard procedure a filter disc loaded with 50 μg high-molecular single-stranded DNA was incubated in 1 ml of SSC solution made up in 20% formamide, 80% water (to be called hereafter 2SSC–20FA) and 10 μg of ^{14}C-labeled rRNA in a small air-tight, screw-capped vial. Eight vials were simultaneously completely immersed in a water bath at 50 C and rotated slowly at 50 rpm for 16 hr. When DNA was previously purified with a CsCl step we recommend using 55–60 μg DNA on the filter disc. When other conditions were used, they will be specified below. After hybridization each filter disc was removed, incubated for 5 min at 50 C in 2 ml 2SSC–20FA to remove non-specifically bound rRNA, rinsed twice in 50 ml 2 SSC solutions at room temperture, and incubated in 5 ml 2 SSC solution containing 250 μg RNAase (crystalline, bovine pancreas, Worthington) at 37 C. Originally, 20 min incubation was used (Biship, 1970). Later we used 1 hr of treatment which gave better results (see below).

Determination of thermal stability of DNA:rRNA hybrids. After RNAase treatment each filter disc was removed and rinsed twice in 50 ml of 2 SSC solutions at room temperature. They were incubated separately for 15 min in each one of a series of small vials, containing 2 ml of 1.5SSC – 20FA, in a series of water baths of increasing temperatures in 5 °C steps from the hybridization temperature up to 90 C. Negligible traces of radioactive material remained on the filter. When other conditions were used, they will be given in Results. The radioactivity in each vial was measured after addition of 2 ml water and 10 ml of scintillation solution (500 ml Triton X 100, 500 mg dimethyl-POPOP and

5 g PPO in 1 liter of toluol). Radioactivity on filter discs was determined in the same scintillation solution. All measurements were carried out in a Tricarb 3310 Liquid Scintillation Spectrometer at 2 C during 50 min. $T_{m(e)}$ is the temperature at which 50% of the bound rRNA is eluted. We call "% rRNA binding" the amount of rRNA in μg bound to 100 μg of DNA.

RESULTS

When rRNA binds with single-stranded DNA of the same organism we call the result a "homologous duplex" or "homologous DNA:rRNA duplex." When rRNA binds to single-stranded heterologous DNA, the result is a "DNA:rRNA hybrid." The term "DNA-rRNA duples" or "duplex" is used here in its broadest sense, irrespective whether both strands are homologous or heterologous.

The nature of the ^{14}C-rRNA preparations. In this and other research projects in our laboratory (De Ley et al., to be published) several ^{14}C-rRNA preparations were separated on sucrose gradients. It turned out that it is not always as easy to prepare 23S rRNA as with *E. coli*. *Chromobacterium lividum* NCTC 9796 (Fig. 1A) and *Pseudomonas fluorescens* ATCC 13525 ribosomes liberate a similar excess of 23S rRNA over 16S rRNA as *E. coli* B. On the other hand, *Agrobacterium tumefaciens* ICPB TT111 (Fig. 1B), *A. rhizogenes* ICPB TR7 and *Pseudomonas acidovorans* ATCC 15668 ribosomes liberate much more 16S than 23S rRNA. *Arthrobacter oxydans* CBRI 21010 produces about equal amounts of both rRNA types (Fig. 1C). That our *Agrobacterium* strains gave low yields of 23S rRNA is in agreement with the observations of Le Goff (1968) and Grienenberger and Simon (1972). Whether rRNA was prepared in the presence of bentonite according to Midgley (1965), or with 2% sodium dodecyl sulfate (SDS) instead of 1% (Noll and Stutz, 1968), the yield of 23S rRNA remained unchanged. It cannot be excluded that the 16S peak of *Agrobacterium* rRNA contains subunit components from the 23S rRNA.

The effect of the incubation temperature on the hybridization of rRNA with filter-fixed DNA. During homologous duplexing in 2 SSC less rRNA is bound to DNA at higher temperatures (Table 1). Ninety percent of the homologous duplex is stable in 0.5 SSC after RNAase treatment and its $T_{m(e)}$ remains unchanged (83.5 C). This decrease is due to DNA release from the filter at higher temperature (De Ley and Tytgat, 1970) and perhaps to thermal degradation of RNA (Gillespie and Gillespie, 1971). One of our aims was to avoid the annoying loss of DNA. By addition of dimethylsulfoxide (DMSO) or formamide the hybridization temperature can be lowered. In formamide

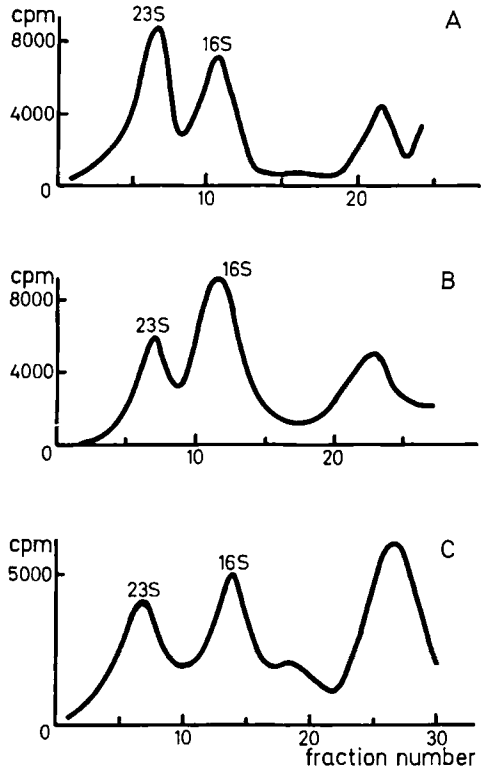


Fig. 1. Types of ^{14}C-rRNA distribution on a sucrose gradient (see text).
Curve A: *Chromobacterium lividum* NCTC 9796;
Curve B: *Agrobacterium tumefaciens* ICPB TT111;
Curve C: *Arthrobacter oxydans* CBRI 21010.

Table 1. Amount and thermal stability of homologous DNA:rRNA duplexes formed in different conditions.

Duplexing was carried out between 16S ^{14}C-rRNA and homologous DNA from *Agrobacterium rhizogenes* TR7. Stability measurements were carried out in 0.5 SSC after RNAase treatment. RNA "noise" was measured by incubating a filter disc without DNA in the same conditions. Stable rRNA is the amount of radioactivity released upon melting in 0.5 SSC between 65 C and 95 C. All results on rRNA binding are given in counts per 50 min, cp50m.

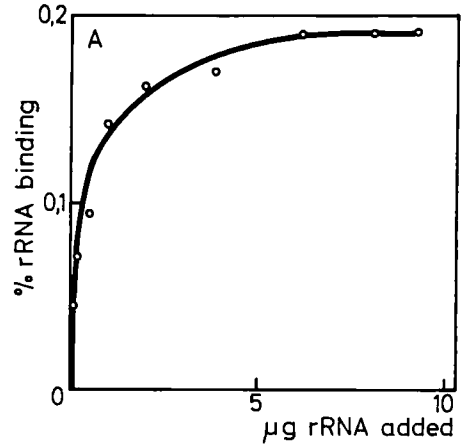
Incubation conditions	Total rRNA bound	rRNA "noise"	Stable rRNA	$T_{m(c)}$ in °C
2 SSC, 55 C	6545		5702	83.5
2 SSC, 65 C	5972	472	5496	83.5
2 SSC, 70 C	4412		4041	83.5
2SSC–20FA, 50 C	7816	39	6586	83
2 SSC in 30% DMSO, 46 C	6894		5654	80

$T_{m(e)}$ decreases with 0.7 C/1% FA (McConaughy et al., 1969) and in DMSO with 0.6 C/1% DMSO (De Ley and Tijtgat, 1970). The optimal renaturation temperature decreases in the same fashion. Therefore we effected duplexing in 2SSC – 20FA at 50 C and in 2 SSC in 30% DMSO at 46 C, both comparable with the hybridizations in 2 SSC at 65 C carried out by Moore and McCarthy (1967). The thermal stability of both duplex types was measured in 0.5 SSC. The duplexes formed in 2SSC – 20FA and in 2 SSC are equally stable with the same $T_{m(e)}$, but the duplex formed in the DMSO mixture is 3 C less stable. For all further hybridizations we used the formamide mixture.

Saturation of filter-fixed DNA by rRNA in 2SSC – 20FA at 50 C. The results are shown in Fig. 2. With homologous DNA the reaction is complete after 5 hr and with heterologous DNA after 8 hr. There is no change on further incubation up to 16 hr. For practical reasons all hybridizations were carried out for 16 hr overnight. On incubation of rRNA with a filter disc without DNA in the above conditions, there was nearly no noise level of nonspecifically bound rRNA; in the case of hybridization in 2 SSC the noise level was much higher (Table 1).

DNA retained on filter discs in our standard hybridization conditions. The effect of a CsCl purification step on DNA retention (Table 2). De Ley and Tytgat (1970) showed that hybridization at higher temperatures releases considerable amounts of DNA from membrane filter discs. We attempted to keep this loss as low as possible in our hybridization conditions. We report here on 138 membrane filter discs from 26 genera and 2 unnamed groups of bacteria, containing DNA not purified with a CsCl step. The average loss is 1 μg or 2%. Eighty-two percent of the filter discs lost less than 10% of their DNA content. The losses occurred mainly with DNA from gram-positive strains. Five filter discs are outside the 2 s limits (1/36 *Agrobacterium*; 1/2 *Azomonas*; 1/2 *Azotobacter*; 1/3 *Microbacterium*; 1/1 *Mycobacterium*) all possibly due to poor quality (fragmented?) DNA.

The purity of DNA used for hybridization is very important for meaningful results (De Ley and Tytgat, 1970). A CsCl preparative purification step (see Methods) is used as a routine procedure in DNA preparation in this laboratory. It detects and removes remaining protein and RNA impurities in our samples. However, DNA is less firmly fixed on the filters. A possible reason is increased DNA fragmentation during the manipulations. The 156 filter discs used lost on the average 9 μg or 18% DNA. Statistical treatment showed that this is significantly less (($\nu = 292$; $t = 9{,}85$; $P \leqslant 0.001$) than with DNA not purified with the CsCl step. The loss was nearly always contained between 2 and 15 μg. In 17 out of 156 cases the release is outside the 2 s range (1/13 *Alcaligenes*; 4/7 *Beneckea*: 1/11 *Chromobacterium*; 1 *Comamomonas*; 1/5



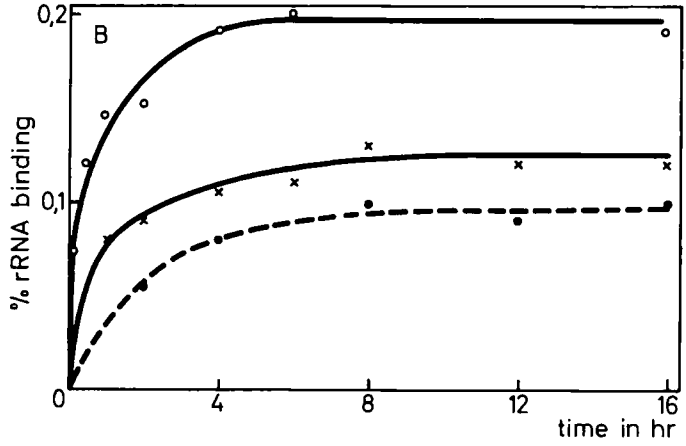


Fig. 2. The saturation of rRNA cistrons in filter-fixed DNA.

Hybridizations were carried out with 23S ^{14}C-rRNA from *Agrobacterium tumefaciens* ICPB TT111 as described under Methods, in 2SSC–20FA at 50 C. % rRNA binding on DNA was measured after 20 min RNAase action.

(A) Filter discs with 53 µg of homologous DNA were incubated with different amounts of radioactive rRNA for 16 hr.

(B) Incubation for several time intervals with filter discs carrying

○———○ DNA from *Agrobacterium tumefaciens* TT111;

×———× DNA from *Bacillus pumilus* B12;

●———● DNA from *Aeromonas salmonicida* NCMB 833.

Table 2. The release of high-molecular, single-stranded DNA of several genera from membrane filter discs in our standard hybridization conditions. The effect of a CsCl purification step during DNA preparation on DNA release.

The first and second numbers are the average μg DNA released/filter disc ± standard deviation. The amount of DNA fixed before, and the remainder after hybridization was determined as reported in Methods. The number of filters used is shown in parenthesis. Most filters were less than 1 year old, no filter was over 2 years old. Many filter data were available from other research projects in this laboratory (J. De Ley, P. De Vos, R. Tytgat, P. Segers, unpublished).

Genus or group name, as received	CsCl purification step during DNA preparation	
	not included	included
Acetobacter		7 ± 3 (4)
Achromobacter	0 (2)	8 (2)
Aeromonas	1 ± 6 (4)	8 (1)
Agarbacterium		12 (1)
Agrobacterium	2 ± 4 (36)	8 ± 6 (8)
Alcaligenes	0 (2)	11 ± 8 (13)
Arthrobacter	2 ± 5 (19)	
Azomonas	12 ± 10 (2)	
Azotobacter	12 ± 9 (2)	
Azotococcus		4 (1)
Azotomonas		10 ± 4 (2)
Bacillus	0 ± 4 (4)	
Beneckea		19 ± 4 (7)
Bordetella	0 (3)	8 (1)
Brevibacterium	0 (2)	
Campylobacter	0 (3)	
Chromobacterium	0 (2)	8 ± 9 (11)
Comamonas		22 (1)
Corynebacterium	1 ± 7 (11)	
Cytophaga		9 (1)
Edwardsiella		18 (1)
Enterobacter	0 (1)	
Erwinia	0 ± 5 (7)	3 (1)
Escherichia	4 (1)	0 (1)
Flavobacterium		12 ± 5 (7)
Gluconobacter		2 ± 1 (2)
Hafnia		14 ± 6 (5)
Jensenia	8 (1)	
Klebsiella	3 ± 4 (3)	
Lucibacterium		7 ± 10 (3)
Microbacterium	4 ± 12 (3)	
Mycobacterium	11 ± 8 (2)	
Mycoplana	0 (2)	
Nocardia	0 ± 6 (6)	
Paracoccus		5 ± 2 (2)
Photobacterium		7 ± 10 (6)
Phyllobacterium		7 ± 5 (6)
Plesiomonas		15 (1)
Pseudomonas	0 ± 4 (3)	7 ± 7 (38)
Proteus		15 ± 2 (3)

Table 2 (continued)

Genus or group name, as received	CsCl purification step during DNA preparation	
	not included	included
Rhizobium	4 ± 4 (11)	
Rhodopseudomonas		8 ± 7 (3)
Ruiter's strains	0 (2)	6 ± 3 (4)
Salmonella	0 (1)	
Serratia	0 (1)	
Spirillum		11 (1)
Sundman's "agrobacteria"	2 ± 1 (2)	
Vibrio		14 ± 10 (14)
Xanthomonas		13 ± 5 (2)
Yersinia		3 (1)
Zymomonas		0 (2)

Hafnia; 1/6 *Photobacterium*; 3/38 *Pseudomonas*; 5/14 *Vibrio*). We suspect that the easy fragmentation or decomposition of DNA from *Vibrio* and *Beneckea* is an inherent feature. It is interesting to point out that both taxa are genetically related (De Ley et al., to be published). The 8 other cases of high DNA loss are possibly due to preparative difficulties. In the majority of cases the CsCl fractionation step is a necessity for the preparation of pure DNA. In these cases we recommend the use of a filter disc with 60 μg of DNA as compensation.

Thermal stability of DNA:rRNA duplexes in different salt and formamide concentrations. The stability of a DNA:rRNA duplex or hybrid depends on the concentration of cathions and formamide: e.g. more cathions and less formamide increase the stability. Table 3 shows the lowering of $T_{m(e)}$ with decreasing salt concentration in 20% FA. Between 0.75SSC – 20FA and 0.5 SSC – 20FA the duplex loses its stability completely. In the latter solvent more

Table 3. Thermal stability of homologous DNA:rRNA duplexes in different salt and formamide concentrations. Duplexing was carried out between 10 μg 16S ^{14}C-rRNA from *Agrobacterium rhizogenes* TR7 and 50 μg of homologous DNA on a filter disc in standard conditions (see Methods). After RNAase treatment the stability curve was determined in several solutions.

Stability in	% of the duplex melting between 55 and 90 C	$T_{m(e)}$ in °C
0.5 SSC in 50% FA	< 5	< 52
0.5 SSC in 30% FA	< 5	< 52
0.5 SSC in 20% FA	< 5	< 52
0.75 SSC in 20% FA	82	73
1 SSC in 20% FA	85	77
1.5 SSC in 20% FA	88	80
2 SSC in 20% FA	93	82

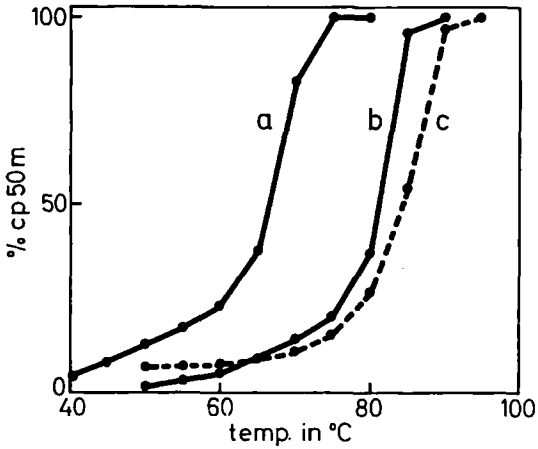


Fig. 3. Denaturation curves of DNA:rRNA duplexes in different conditions.
Duplexing was carried out between 16S ^{14}C-rRNA from *Agrobacterium rhizogenes* TR7 and homologous DNA. Curve (a) duplexing in 2SSC–50FA at 40 C and stability in 2SSC–50FA; curve (b) duplexing in 2SSC–20FA at 50 C and stability in 1.5SSC–20FA; curve (c) duplexing in 2 SSC at 65 C and stability in 0.5 SSC.

than 95% of the duplex cannot exist above 55 C. Fig. 3 shows the thermal stability of the duplex in different conditions. In 1.5SSC – 20FA, $T_{m(e)}$ is 81 C and approximately 80% of the hybrid melts between 75 C and 85 C; in the other conditions this happens in a broader temperature interval of 15 to 20 C. All further duplexing was carried out in 2SSC – 20FA as a compromise solvent between sufficient reaction rate and limited filter-DNA release. All thermal denaturation curves were measured in 1.5SSC – 20FA.

It is important to check the purity of the formamide. The compound used in these studies was purchased from the Union Chimique Belge and showed an absorbance of 0.2 at 270 nm in a 1 cm path-length cuvette. Unknown impurities in formamide decrease the thermal stability of duplexes. In formamide with an absorbance of more than 3 at 270 nm, the $T_{m(e)}$ of the duplexes is 3 C lower.

Ribonuclease treatment of DNA:rRNA duplexes. Nygaard and Hall (1964) reported that the resistance of homologous DNA:RNA duplexes to RNAase increased with increasing salt concentrations up to 0.2 M KCl. The stable fraction varied from 50 to 80%. We carried out RNAase treatment in 2 SSC as recommended by Gillespie (1968).

Fig. 4 shows the action of RNAase in function of time. The action on homologous duplexes on filters with up to 52 μg DNA is complete after 20 min. With filters carrying more DNA and duplex, the hydrolysis is complete after 40 min to 1 hr. Most of the RNAase-sensitive rRNA from heterologous hybrids is removed in 40 min to 1 hr. Afterwards the reaction proceeds only slowly.

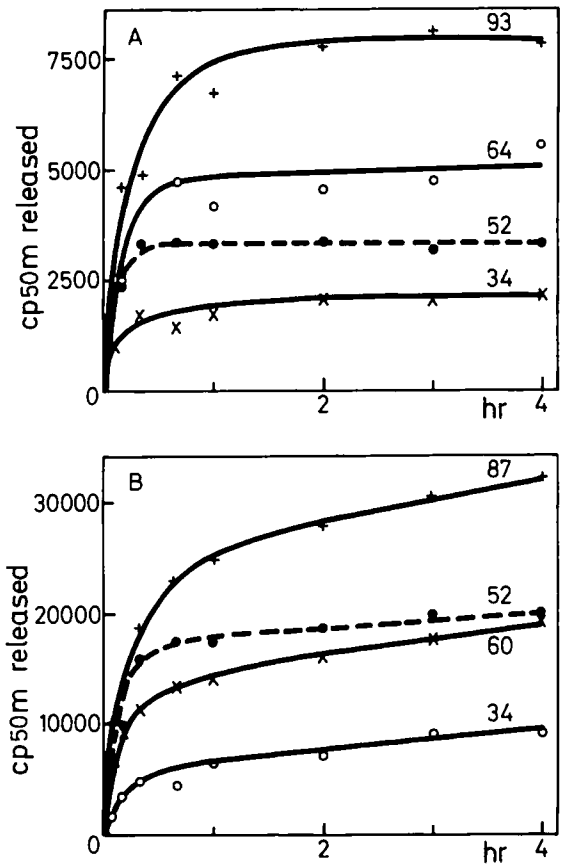


Fig. 4. Ribonuclease action on DNA:rRNA duplexes.

Filter discs with different amounts of DNA were incubated with 23S ^{14}C-rRNA in 2SSC–20FA at 50 C for 16 hr. After rRNA-binding the filter discs were incubated in 10 ml of 2 SSC containing 500 μg RNAase at 37 C. 1 ml samples were pipetted and the rRNA released was counted. The amount of μg DNA on each disc is shown at right.

(A) Action on homologous duplexes

——— *Arthrobacter oxydans* 21010;

- - - - - *Agrobacterium tumefaciens* TT111.

(B) Action on heterologous hybrids

——— hybrid between rRNA from *Arthrobacter oxydans* 21010 and DNA from *Bacillus pumilus* B12;

- - - - - hybrid between rRNA from *Agrobacterium tumefaciens* TT111 and DNA from *Bacillus megaterium* 899 thy$^-$.

Hayashi and Spiegelman (1961) and Gillespie (1968) warned that exhaustive treatment with RNAase can result in partial or complete loss of perfectly bound RNA. Therefore, we used a 1 hr treatment of 250 μg RNAase in 5 ml 2 SSC at 37 C in our standard procedure.

Effect of RNAase treatment on the thermal stability of DNA:rRNA duplexes. Fig. 5 gives an example of the thermal stability of duplexes before and after RNAase treatment. About 22% of bound rRNA is removed from the homologous duplex, but $T_{m(e)}$ remains unchanged at 81 C. This fraction is associated

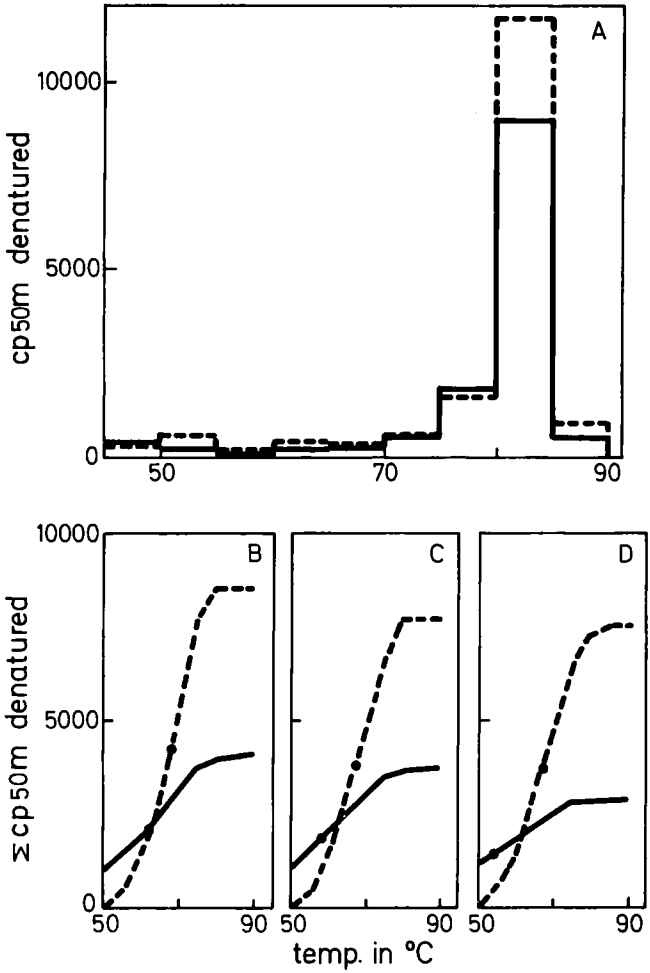


Fig. 5. Effect of RNAase treatment on the amount and the thermal stability of DNA:rRNA duplexes.

Duplexing was carried out with 23S ^{14}C-rRNA from *Agrobacterium tumefaciens* TT111, in 2SSC–20FA at 50 C for 16 hr, followed by 20 min of RNAase action. The origin of the filter DNA: Fig. 5A: *Agrobacterium tumefaciens* TT111; Fig. 5B: *Erwinia amylovora* NCPPB 683; Fig. 5C: *Chromobacterium lividum* DA; Fig. 5D: *Arthrobacter oxydans* CBRI 21011.

Fig. 5A: differential denaturation profiles of homologous duplex. Fig. 5B-D: integrated denaturation curves. The dots represent $T_{m(e)}$.

– – – – – before and ——— after RNAase treatment.

mainly with the perfectly bound rRNA. The explanation of this phenomenon is not entirely clear. Duplexing is carried out in a 100-fold excess of rRNA. All available rDNA is saturated (Fig. 2). In the preparation of DNA, quite a number of rRNA cistrons are probably broken. The RNAase-sensitive part of the duplex could represent the free ends, because the bound rRNA molecules do not have the same length as the rRNA cistron fragments. If this is so, about 22% of the length of the tightly bound strands might be free. Another possibility is the suggestion of Bishop et al. (1969) that up to 20% of perfectly bound homologous RNA can be attacked by RNAase.

RNAase action on heterologous hybrids is more drastic. The $T_{m(e)}$ of the three hybrids in Fig. 5B–D is 67 to 68 C without RNAase treatment. Fifty to 60% of each hybrid is removed by RNAase; the $T_{m(e)}$ of the RNAase-resistant hybrids are now considerably different: 54, 58 and 62 C. This points to a different nature of the RNAase-resistant hybrids. Pancreas RNAase hydrolyses single-stranded RNA after pyrimidines. Gillespie and Gillespie (1971) assumed that one mispaired base can be hydrolyzed. Mispaired loops with pyrimidines are attacked so that the hybridized RNA molecule is broken into shorter fragments. Their thermal stability depends not only on mispairings but also on their length and % GC. Determining the thermal stability after RNAase treatment is therefore a more sensitive measure to detect differences between hybrids. There is no further difference in $T_{m(e)}$ values, when RNAase acts for 1 hr instead of 20 min.

Storage of DNA. DNA solutions are rather labile. It is common experience in this laboratory (e.g. De Ley, Park, Tijtgat, Van Ermengem, 1966) that concentrated solutions containing 1 mg or more DNA/ml SSC with one drop of chloroform, are stable for several months at 4 C; solutions with about 100 μg or less DNA/ml 0.1 SSC are stable for less than two weeks at 4 C. When DNA has to be prepared from a large number of strains, e.g. for research in bacterial systematics, the rapid deterioration of the older samples is a heavy handicap. We therefore attempted to find ways to preserve DNA over longer periods of time. We examined two procedures over a period of 3 years.

Our first method consists in preservation of concentrated DNA solutions in 0.1 SSC at —90 C. Crombach (1973) showed that quick freezing of DNA at —70 C and storing at —21 C for 6 months or probably one year affected neither the molecular weight of the fragments, nor the degree of DNA:DNA hybridizations. Table 4 shows that DNA fragments from 5 different *Agrobacterium* strains still had the same high molecular weight of about 10^7 after quick freezing and preservation for up to 2 years at —90 C in 0.1 SSC.

An aliquot of freshly prepared DNA from several bacteria was fixed on a filter. After hybridization in our standard conditions (see above) the % rRNA

Table 4. The effect of preservation at –90 C on the fragment size of DNA.

After certain time intervals, samples of DNA solutions in 0.1 SSC were thawed and examined in the analytical ultracentrifuge Spinco, model E in UV light at 37020 rpm. Sedimentation coefficients were calculated from densitometric tracings of photographic plates (De Ley and Tytgat, unpublished).

Agrobacterium tumefaciens strain used	Svedberg units S and mol. weight M $\times 10^{-6}$ of the DNA fragments	Age of frozen DNA sample in months						
		0.1	2	3	6	12	21	23
ATCC 15955	S	23.4	25.5		24.4	24.6		25.0
	M	9	11		10	10		10
ATCC 17805	S		24.7		23.7	23.4		24.1
	M		10		9	9		10
Zinca 9P	S	28.7		27.7	27.5		27.7	
	M	15		14	14		14	
ICPB TT111	S	25.6	26		24.4	25		25
	M	11	11		10	10		10
176	S	24.3		23.6	24			
	M	10		9	10			

binding and the $T_{m(e)}$ of the hybrid were measured. Another aliquot in 0.1 SSC was quickly cooled at —50 C and stored at —90 C. After definite time intervals a sample was thawed, fixed on a filter, and the same hybridizations were carried out in the same conditions. The results are shown in Table 5. $T_{m(e)}$ of the duplexes is the same or almost the same in all cases. The % rRNA binding varies less than 0.05%. DNA can thus be preserved in concentrated solutions in the deep-freeze at —90 C in 0.1 SSC for two years without much change.

Our second method consists in storage of DNA on filter discs in vacuo at 4 C for up to 3 years. For each strain a filter disc was used right away, for hybridization and $T_{m(e)}$ determination. Another filter disc was stored for about, 1, 2 or 3 years and hybridized in the same conditions with freshly prepared ^{14}C-rRNA (Table 6). The thermal stability of the hybrids remained the same when the DNA was up to one year, and in some cases, up to 32 months old. However, in most cases, filter-fixed DNA of over 20 months old gave less stable hybrids. With filter discs of 2 years and older, we observed two other anomalies. Firstly, the amount of rRNA hybridized/100 μg DNA on the filter had apparently increased, and secondly, the amount of DNA fixed on the filter had apparently decreased, when determined chemically. Both anomalies add up, giving a false picture of the % rRNA binding, about 100% too high. Therefore, hybridizations with filter-fixed DNA, stored as described above, yield only reliable results when filters are less than 20 months old.

Table 5. The stability of DNA, quick-frozen at –50 C and stored at –90 C.
Freshly prepared DNA was fixed on filters and hybridized with 16S ^{14}C-rRNA from *Agrobacterium tumefaciens* ICPB TT111 as described under Methods. Aliquots of DNA were stored frozen, thawed after definite times, filter-fixed and used for hybridization as above.

Strain	Age of DNA preparation in months	Fixation on filter μg DNA/ cm² added	μg DNA/ cm² fixed	% rRNA binding	$T_{m(e)}$ in °C
Rhizobium	0.1	48.0	47.2	0.14	81
leguminosarum	0.5	48.6	46.3	0.14	81
4.1	3	46.7	44.9	0.14	81
	7	48.4	48.4	0.11	81
Agrobacterium tumefaciens	0.1	45.7	43.3	0.19	81
ICPB TT111	10	48.8	42.3	0.14	81
Bordetella	0.1	47.9	46.7	0.04	58.5
bronchiseptica	12	45.8	42.3	0.06	60
NCIB 8761	23	46.4	46.4	0.05	59
Erwinia	0.1	48.7	47.4	0.12	61.5
amylovora	13	47.5	46.5	0.13	63.5
NCPPB 683	24	50.5	49.8	0.12	63
Arthrobacter	0.1	51.5	51.5	0.09	55
spec.	14	45.8	42.3	0.07	55.5
CBRI ER 39	25	45.4	45.4	0.09	55

Storage of rRNA. Storage of rRNA solutions requires special care because glass-ware is easily contaminated by traces of RNAase. Therefore it is necessary to sterilize all glassware used and to store RNA solutions at —12 C. We examined the storage of bulk RNA (2 mg/ml SSC solution before sucrose gradient centrifugation) and pure 16S or 23S rRNA (50 to 100 μg/ml 2 SSC solution after sucrose gradient centrifugation) at —12 C in 1-ml fractions in glass tubes sterilized by dry heat.

After twenty months of storage, bulk RNA of *Agrobacterium tumefaciens* ICPB TT111 was purified by sucrose gradient centrifugation as described in Methods. The pattern on sucrose gradient was still exactly the same as with freshly prepared RNA; storage has thus no effect on the molecular weight of the RNA molecules. This purified rRNA gave the same hybridization results as freshly prepared RNA. Freshly prepared pure RNA was stored for about one year and hybridized with freshly prepared DNA. Hybridization and thermal stability of the DNA:rRNA duplexes were the same as with freshly prepared rRNA (Table 7). These results show clearly that 20 months of storage of RNA solutions at —12 C has no effect on the hybridizations.

Table 6. Effect of storage of DNA on filter discs in vacuo at 4 C, on the degree of hybridization and thermal stability of the hybrids.

Filter discs with DNA from *Escherichia coli* B, *Aeromonas hydrophila* AB 833, *Erwinia herbicola* ("*Agrobacterium gypsophilae*") ATCC 13329 and *Erwinia amylovora* NCPPB 683 were hybridized with freshly prepared 23S ^{14}C-rRNA from *E. coli* B. The other filter discs were hybridized with freshly prepared 16S ^{14}C-rRNA from *Agrobacterium rhizogenes* ICPB TR7. Some aged filter discs do not contain the same amount of DNA as fresh ones; they were from the same DNA preparation but from a different mother filter.

Strain	Age of DNA filter disc in months	DNA content from				$T_{m(e)}$ in °C
		absorbancy measurement during fixation		chemical determination after incubation		
		µg DNA on filter disc	% rRNA binding	µg DNA on filter disc	% rRNA binding	
Escherichia	0.1	51	0.15	51	0.15	81
coli B	10	52	0.15	52	0.15	81
Aeromonas	0.1	55	0.25	64	0.22	72.5
hydrophila AB 833	11	55	0.23	64	0.20	71.5
Erwinia herbicola	0.1	54	0.21	54	0.21	77.5
("*Agrobacterium gypsophilae*") ATCC 13329	12	54	0.16	54	0.16	77.5
Erwinia amylovora	0.1	54	0.23	52	0.23	77.5
NCPPB 683	13	54	0.21	48	0.24	78.5
Rhizobium japonicum	0.1	54	0.04	51	0.05	73
USDA 3I1b59	20	42	0.07	36	0.08	71
R. leguminosarum	0.1	50	0.15	48	0.15	81
USDA 316C10A	20	43	0.13	37	0.15	77
Agrobacterium tumefa-	0.1	50	0.20	43	0.17	78.5
ciens 3/1	24	41	0.29	38	0.32	77
A. tumefaciens	0.1	51	0.15	47	0.16	78
T37	25	41	0.26	29	0.36	72
A. rhizogenes	0.1	53	0.16	55	0.15	81
ICPB TR7	30	52	0.24	43	0.29	81
A. tumefaciens	0.1	51	0.21	55	0.19	78
ICPB TT111	32	49	0.25	32	0.38	77
A. tumefaciens	0.1	37	0.17	37	0.17	77
B6	33	50	0.24	38	0.32	75

Table 7. The stability of pure rRNA stored at −12 C.
Fifty to 100 μg rRNA/ml 2 SSC solution was stored in 1 ml fractions in glass tubes at —12 C. After definite time intervals, rRNA-samples were hybridized with either freshly prepared or aged DNA as described under Methods. The rRNA types used are: 23S from *Escherichia coli* B, 23S from *Arthrobacter oxydans* CBRI 21010, 16S from *Agrobacterium tumefaciens* ICPB TT111 and 16S from *A. rhizogenes* ICPB TR7.

DNA-rRNA duplex	Age of pure rRNA in months	Age of DNA preparations in months	% rRNA binding	$T_{m(e)}$ in °C
Arthrobacter oxydans CBRI 21010	0.1	0.5	0.17	81.5
homologous	8	10	0.16	81
Agrobacterium rhizogenes ICPB TR7	0.1	0.5	0.15	81
homologous	11	0.5	0.15	81
Escherichia coli B	0.1	0.5	0.15	81
homologous	12	0.5	0.16	80.5
Agrobacterium tumefaciens ICPB TT111	0.1	0.5	0.19	81
homologous	12	0.5	0.17	81
DNA from *Zymomonas mobilis* Z1 and rRNA from	0.1	0.5	0.16	68
A. tumefaciens TT111	12	15	0.16	67.5

DISCUSSION

The temperature of hybridizations involving DNA on membrane filters, should on one hand be low enough to prevent severe DNA release (De Ley and Tytgat, 1970), and on the other hand be high enough to allow sufficiently high duplexing rates to shorten the experiments. Fry and Artman (1969) reported that rRNA heated at 66 C for 20 hr, became extensively degraded and sedimented at about 8S. The average base composition of rRNA cistrons is about 53% GC. T_m of the DNA:rRNA duplex will be about 91 C in SSC. It can be calculated that in 2SSC – 20FA (Gillis, De Ley and De Cleene, 1970; McConaughy et al., 1969) T_m will be about 80 C. This is confirmed experimentally (Table 2, Fig. 2). The temperature of optimal hybridization rate T_{OR} (Gillis et al., 1970) is about 55 C. We preferred to work at 50 C. There is only very little unspecific duplexing between homologous DNA and rRNA, and some heterologous mispairing of taxonomic interest is detected. Theoretically at this temperature one has a choice between e.g. 0.5SSC – 10FA, SSC – 15FA, 2SSC – 20FA, 4SSC – 26FA or 6SSC – 29FA. We selected 2SSC – 20FA. Most authors still use the hybridization conditions of Gillespie and Spiegelman (1965) in 2 SSC or 6 SSC at 66 C. This can no longer be recommended because

of the reasons mentioned above. In the conditions we described, duplexing is completed in about 10 hr. It represents a considerable saving of time over other conditions — certainly when large numbers of determinations have to be carried out. Daniel et al. (1970) used 2SSC – 30FA at 25 C for 50 hr. Gillespie and Gillespie (1971) used 3SSC – 50FA at 35 C: after two days duplexing was not yet complete; not infrequently they carried out hybridizations for one week. McConaughy et al. (1969) pointed out that the best reaction conditions proposed by Bonner et al. (1967) for DNA:RNA hybridization in 2SSC – 30FA at 24 C, or SSC – 30FA at 0 C are of quite low specificity and are not advisable.

For long-term systematic investigations using rRNA, we recommend labeling with stable isotopes. The use of ^{32}P-rRNA is not advisable because of its short half life.

For the type of experiments we use, we prefer the filter technique as described here, over some other methods. Melli et al. (1971) presented a new method for DNA:RNA hybridization in vast DNA excess. The preparation of large amounts of DNA is less practical to achieve, and not always possible when hundreds of hybridizations have to be carried out. The preparation of DNA:RNA hybrids in solution, its collection on hydroxyapatite columns and release by heat, are also less practical than the filter method when very many samples are involved. Alternatively, it is possible that urea (Kourilsky et al., 1970) may be substituted for formamide. This remains to be investigated.

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REFERENCES

BISHOP, J. O. 1970. Examination of an equilibrium interpretation of deoxyribonucleic acid – ribonucleic acid hybridization data. – Biochem. J. **116**: 223–228.

BISHOP, J. O., ROBERTSON, F. W., BURNS, J. A. and MELLI, M. 1969. Methods for the analysis of deoxyribonucleic acid – ribonucleic acid hybridization data. – Biochem. J. **115**: 361–370.

BONNER, J., KUNG, G. and BEKHOR, I. 1967. A method for the hybridization of nucleic acid molecules at low temperature. — Biochemistry **6**: 3650–3653.

BURTON, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. — Biochem. J. **62**: 315–323.

CROMBACH, W. H. J. 1972. DNA base composition of soil arthrobacters and other coryneforms from cheese and sea fish. — Antonie van Leeuwenhoek **38**: 105–120.

CROMBACH, W. H. J. 1973. Deep-freezing of bacterial DNA for thermal denaturation and hybridization experiments. — Antonie van Leeuwenhoek **39**: 249–255.

DANIEL, J. C., GREENE, R. F., MITCHELL, R. A. and FLICKINGER, R. A. 1970. An improved method of detecting DNA-RNA hybrids bound to nitrocellulose filters. — Anal. Biochem. **37**: 330–332.

DE LEY, J. 1971. Hybridization of DNA, p. 311–329. *In* Norris, J. R. and Ribbons, D. W., (eds.), Methods in Microbiology Vol. 5A. — Academic Press, London and New York.

DE LEY, J., CATTOIR, H. and REYNAERTS, A. 1970. The quantitative measurement of DNA hybridization from renaturation rates. — Eur. J. Biochem. **12**: 133–142.

DE LEY, J. and FRIEDMAN, S. 1964. Deoxyribonucleic acid hybrids of acetic acid bacteria. — J. Bacteriol. **88**: 937–945.

DE LEY, J., PARK, I. W., TIJTGAT, R. and VAN ERMENGEM, J. 1966. DNA homology and taxonomy of *Pseudomonas* and *Xanthomonas*. — J. Gen. Microbiol. **42**: 43–56.

DE LEY, J. and TIJTGAT, R. 1970. Evaluation of membrane filter methods for DNA-DNA hybridizations. — Antonie van Leeuwenhoek **36**: 461–474.

FRY, M. and ARTMAN, M. 1969. Deoxyribonucleic acid – ribonucleic acid hybridizations. — Biochem. J. **115**: 287–294.

GILLESPIE, D. 1968. The formation and detection of DNA:RNA hybrids, p. 641–668. *In* Colowick, S.P. and Kaplan, N. O., (Eds.), Methods in Enzymology, Vol. XII B. — Academic Press, New York and London.

GILLESPIE, S. and GILLESPIE, D. 1971. Ribonucleic acid – deoxyribonucleic acid hybridization in aqueous solutions and in solutions containing formamide. — Biochem. J. **125**: 481–487.

GILLESPIE, D. and SPIEGELMAN, S. 1965. A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. — J. Mol. Biol. **12**: 829–842.

GILLIS, M., DE LEY, J. and DE CLEENE, M. 1970. The determination of molecular weight of bacterial genome DNA from renaturation rates. — Eur. J. Biochem. **12**: 143–153.

GRIENENBERGER, J. M. et SIMON, D. 1972. Etude de la structure et de la dégradation des acides ribonucléiques ribosomaux de quelques bactéries du genre *Agrobacterium*. — C.R. Acad. Sci. (Paris) **274**: 1399–1402.

HAYASHI, M. and SPIEGELMAN, S. 1961. The selective synthesis of informational RNA in bacteria. — Proc. Nat. Acad. Sci. U.S.A. **47**: 1564–1580.

HEBERLEIN, G. T., DE LEY, J. and TYTGAT, R. 1967. Deoxyribonucleic acid homology and taxonomy of *Agrobacterium*, *Rhizobium* and *Chromobacterium*. — J. Bacteriol. **94**: 116–124.

KIRBY, K. S. 1957. A new method for the isolation of deoxyribonucleic acids: evidence on the nature of bonds between deoxyribonucleic acid and protein. — Biochem. J. **66**: 495–504.

KIRBY, K. S., FOX-CARTER, E. and GUEST, M. 1967. Isolation of deoxyribonucleic acid and ribosomal ribonucleic acid from bacteria. — Biochem. J. **104**: 258–262.

KOURILSKY, PH., MANTEUIL, S., ZAMANSKY, M. H. and GROS, F. 1970. DNA-RNA hybridization at low temperature in the presence of urea. — Biochem. Biophys. Res. Comm. **41**: 1080–1087.

LE GOFF, L. 1968. Acides ribonucléiques des bactéries du crown-gall *Agrobacterium tumefaciens* (Smith et Town) Conn. exposées au borate de sodium. — Ann. Inst. Pasteur **115**: 232–248.

LOCHHEAD, A. G. and BURTON, M. O. 1954. Qualitative studies of soil microorganisms. XII. Characteristics of vitamin-B12-requiring bacteria. — Can. J. Microbiol. **1**: 319–330.

MARMUR, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. — J. Mol. Biol. **3**: 208–218.

MCCONAUGHY, B. L., LAIRD, C. D. and MCCARTHY, B. J. 1969. Nucleic acid reassociation in formamide. — Biochemistry **8**: 3289–3295.

MCCONKEY, E. H. 1968. The fractionation of RNA's by sucrose gradient centrifugation, p. 620–634. *In* S. P. Colowick and N. O. Kaplan, (Eds), Methods in Enzymology, Vol. XII. — Academic Press, New York and London.

MELLI, M., WHITFIELD, C., RAO, K. V., RICHARDSON, M. and BISHOP, J. O. 1971. DNA-RNA hybridization in vast DNA excess. — Nature New Biol. **231**: 8–12.

MEIJS, W. H. and SCHILPEROORT, R. A. 1971. Determination of the amount of DNA on nitrocellulose membrane filters. — FEBS Letter **12**: 166–168.

MIDGLEY, J. E. M. 1965. Effects of different extraction procedures on the molecular characteristics of bacterial ribosomal ribonucleic acid. — Biochim. Biophys. Acta **95**: 232–243.

MOORE, R. L. and MCCARTHY, B. J. 1967. Comparative study of ribosomal ribonucleic acid cistrons in Enterobacteria and Myxobacteria. — J. Bacteriol. **94**: 1066–1074.

NOLL, H. and STUTZ, E. 1968. The use of sodium and lithium dodecyl sulfate in nucleic acid isolation, p. 129–155. *In* S. P. Colowick and N. O. Kaplan, (eds.), Methods in Enzymology, Vol. XIIB. — Academic Press, New York and London.

NYGAARD, A. P. and HALL, B. D. 1963. A method for the detection of RNA-DNA complexes. — Biochem. Biophys. Res. Comm. **12**: 98–104.

NYGAARD, A. P. and HALL, B. D. 1964. Formation and properties of RNA-DNA complexes. — J. Mol. Biol. **9**: 125–142.