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Covalent Coupling of Ribonucleic Acid to Agarose*

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ABSTRACT: A procedure for coupling RNA to a modified agarose resin is described. The amino group of $NH_2(CH_2)_5CO_2CH_3$ is coupled to the agarose by an alkaline CNBr procedure. The ester group is converted to a hydrazide by reaction with hydrazine. RNA is oxidized with IO_4^- to give a 3′-terminal aldehyde and then coupled to the resin by hydrazone formation. This coupling reaction is very slow unless negatively charged carboxyl groups on the resin surface are blocked by amide-bond formation with glycinamide as directed by a water soluble carbodiimide. The carbodiimide–glycinamide step seems to introduce some unidentified basic positively charged groups onto the resin surface, thus causing nonspecific ionic binding of RNA to the resin. The nonspecific binding accelerates the rate of hydrazone-bond formation. Excess nonspecifically bound RNA is released by raising the pH above 9. Poly(U) on the resin surface can hybridize with poly(A) in solution, and the poly(A) can be eluted in a denaturing Me_2SO solvent. With *Escherichia coli* 16S rRNA coupled to the resin, a 20-fold enrichment of rDNA from sheared denatured *E. coli* DNA of single-strand molecular weight about 7×10^4 was achieved by hybridization, followed by elution with NaOH. The procedure was not successful for enriching rDNA in a preparation of high molecular weight DNA.

In the development of techniques for the isolation of molecules which interact specifically with a given biological macromolecule, it is often useful to attach the latter to a solid support. A number of methods for attaching nucleic acids to solid supports have been developed and applied recently (Gilham, 1968, 1971; Nelidova and Kiselev, 1968; Alberts *et al.*, 1968; Litman, 1968; Alberts and Herrick, 1971; Jovin and Kornberg, 1968; Bonavida *et al.*, 1970; Poonian *et al.*, 1971). We describe here a procedure for covalent coupling of RNA molecules *via* their 3′ terminus to an agarose gel. Our final objective, the isolation of high molecular weight single strands of DNA containing rDNA genes, has not yet been achieved, but the coupling method may be useful for other applications and we accordingly describe it here.

The procedure involves the following steps: (1) coupling of $NH_2(CH_2)_5CO_2CH_3$ (ε-aminocaproic acid methyl ester) to agarose which has been activated by treatment with alkaline CNBr. The use of an ε-aminocaprolyl derivative to provide a functional group sterically separated from the agarose and therefore more accessible to reagents in solution was suggested by Cuatrecasas *et al.* (1968). The activation of a polysaccharide support by alkaline CNBr for coupling to a nucleophilic reagent was described by Axen *et al.* (1967); (2) conversion of the caprolyl ester function to a hydrazide agarose, $NH(CH_2)_5CONHNH_2$. This we call "hydrazide agarose." (4) The next step in the procedure as originally planned was the oxidation of RNA at its 3′ terminus with IO_4^- and reaction of the oxidized RNA with the hydrazide function to give a hydrazone. Low yields were observed in this step and were attributed to electrostatic repulsion between carboxyl groups on the surface of the resin and the negative polyelectrolyte RNA. Therefore step (4) was inserted into the sequence of reactions. (The coupling of RNA to polyacrylhydrazine–agar has been described by Nelidova and Kiselev (1968).) (3) In order to neutralize these putative carboxyl groups, we have treated the hydrazide agarose with glycinamide and a water-soluble carbodiimide, thus forming amide bonds to the carboxyl groups (Hoare and Koshland, 1967). The product is called "blocked hydrazide agarose." The resulting product is coupled to RNA according to step 4 above. In addition to amide-bond formation, the carbodiimide–glycinamide treatment evidently introduces some positive charges onto the resin. We find that the product is capable of binding unoxidized RNA at pH's below 7. The polynucleotide so bound is released by treatment at pH 8 or greater, leaving only oxidized RNA covalently attached by the hydrazone bond to the agarose.

Materials and Methods

Activation of Agarose. Agarose (100 ml; Sepharose 4B-200 from the Sigma Chemical Co.) was washed by filtration on a coarse sintered-glass funnel with several changes of water and suspended to a total volume of 200 ml in water. Cyanogen bromide (10 g), dissolved in 200 ml of H_2O, was added to the 200-ml suspension of agarose in H_2O. The reaction flask was immersed in an ice bath and the agarose was kept in suspension by magnetic stirring. The suspension was immediately adjusted to pH 11 and maintained at this pH for 9 min by add-

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glass funnel with 2 l. of ice-cold 0.1 M $NaHCO_3$ buffer (pH 9). The activated agarose was suspended to a volume of 170 ml in 0.1 M $NaHCO_3$ buffer (pH 9). A solution of 9 g of ε-aminocaproic acid methyl ester hydrochloride (Cyclo Chemical Co.) in 30 ml of 0.1 M $NaHCO_3$ buffer was readjusted to pH 9 by the addition of 4 N NaOH. The ice-cold solution of the amino ester was added to the activated agarose suspension; gentle stirring at 4° was continued for 24 hr.

Hydrazinoloysis. The resin from the above treatment was washed with 2 l. of ice-cold water and suspended to a total volume of 200 ml. A 20-ml aliquot was removed for control assays and the remainder of the suspension in a 500-ml erlenmeyer flask was cooled on ice and 140 ml of hydrazine hydrate, 99–100% (Matheson Coleman & Bell) was added slowly. The solution was then incubated at 70° for 15 min. The resin was maintained in suspension by gentle manual swirling. The reaction temperature was observed with a thermometer. The mixture was then cooled to 25–30° and washed onto a sintered-glass funnel with 2 l. of water. A resin suspension should now be at pH ~7. The resin was suspended in water to a volume approximately equal to twice the resin volume for convenience in pipetting. It could be stored at 4° for several weeks without significant loss of aldehyde coupling capacity.

Blocking of the Carboxyl Groups. The carbodiimide-coupling procedure is basically that of Hoare and Koshland (1967). Approximately one-third of the hydrazide agarose prepared as described above (~30 ml of settled resin) was washed with 500 ml of water and resuspended in water to a total volume of 67 ml. Glycinamide hydrochloride (5.3 g; Cyclo Chemical Co.) was added with magnetic stirring and the pH adjusted to 4.75 with 1 N NaOH after which 0.67 g of 1-ethyl-3-dimethylaminipropylcarbodiimide·HCl was added. The pH was maintained at 4.75 by addition of 1 N HCl for 2 hr. This product, the blocked hydrazide resin, was washed at room temperature with several liters of distilled water and suspended in a convenient pipetting volume in 5×10^{-4} M EDTA (pH 7.2) for storage at 4°.

RNA. tRNA and synthetic polyribonucleotides were purchased from Schwarz–Mann. Some of the 16S and 23S *E.coli* rRNA used in this study was prepared from ribosomal subunits by centrifugation of the latter in sucrose gradients containing SDS (Jeanteur *et al.*, 1968). Another portion of 16S rRNA was a generous gift of Dr. T. Yamane; it had been isolated by phenol extraction of subunits of *E. coli* B ribosomes.

Alkaline Phosphatase Treatment. A stock solution of alkaline phosphatase was prepared by diluting 20 μl of concentrated enzyme suspension (Worthington Biochemical Corp.) into 5 ml of 0.2 M Tris-acetate–0.01 M magnesium acetate (pH 7.7). This solution (1 ml), after heating at 90° for 10 min, released *ca.* 20 μmoles of P_i/hr at 37° from *p*-nitrophenyl phosphate. (The enzyme is stable for 30 min at 85° in 10^{-2} M Mg^{2+} (Torriani, 1966); it was our hope that the heat treatment might inactivate contaminating RNases.) To one volume of RNA (0.5–3 mg/ml) in 0.2 M Tris-acetate–0.01 M magnesium acetate (pH 7.7) was added an equal volume of the diluted alkaline phosphatase solution. The solution was incubated at 37° for 1 hr, chilled in ice, and treated with two volumes of ethanol. The RNA precipitate was collected by centrifugation for 5 min at 8000 rpm in the SS-34 rotor of a Sorvall centrifuge. The RNA pellet was redissolved in an amount of 0.1 M sodium acetate buffer (pH 5) equal to its initial volume and stored at −20°. This treatment was used for all RNA samples except tRNA.

by Hunt (1965) and McIlreavey and Midgley (1967). To 1 ml of alkaline phosphatase treated RNA or tRNA (0.5–3 mg/ml) was added 0.14 ml of a fresh solution of 0.2 M $NaIO_4$. The solution was allowed to stand for 1 hr at room temperature in the dark. The reaction was then stopped by addition of 0.08 ml of ethylene glycol, followed by incubation in the dark for an additional 15 min at room temperature. The oxidized RNA was then either precipitated with ethanol, as described above, or dialyzed against several changes of 0.1 M sodium acetate buffer (pH 5) at 4° over a 24-hr period. Such a step for the removal of formaldehyde, produced by the reaction between excess $NaIO_4$ and ethylene glycol, is essential before treatment with the hydrazide resin. The oxidized RNA samples were stored at −20° and used over a 3-day period. Control samples of unoxidized RNA were carried through all the above steps except for the addition of $NaIO_4$, for which distilled water was substituted.

Coupling of RNA to Resins. These reactions were conveniently carried out in 13 × 100 mm Kimax screw-cap glass culture tubes. The resins can then be separated from reactants and washed by centrifugation for several minutes in a clinical centrifuge. Small portions of the resin suspension, prepared as described above, were washed several times in 0.1 M sodium acetate (pH 5) prior to the addition of RNA. The RNA solution was added. After appropriate incubation time at 25° the supernatant solution was separated. The absorbance of the supernatant solutions at 260 nm minus its absorbance at 330 nm was measured to determine RNA uptake. After taking absorbance readings, the supernatant was returned to the reaction tube and the resin resuspended. Reaction mixtures were kept in suspension by gentle rocking on a wrist-action shaker. Generally speaking, resin and RNA concentrations were adjusted so that the reactions would be complete within several hours.

With the unblocked resins, the resins, after reaction, were washed exhaustively with 0.1 M sodium acetate buffer (pH 5.0). Bound RNA was measured by digestion with pancreatic RNase at 50 μg/ml in standard saline citrate (pH 7.0) for one hour at 25°. The supernatant absorbance at 260 nm was measured to determine the amount of coupled RNA released. In all cases, there was good agreement between the amount of RNA coupled as estimated from the supernatant absorbance as a function of time during reaction and from the amount of coupled RNA released by RNase digestion.

For experiments with the blocked hydrazide agarose, the uncoupled, nonspecifically bound RNA was released from the surface of the resin by treatment with $NaHCO_3$ buffer (pH 9). Nonspecifically bound RNA was estimated from the absorbance of the supernatant solutions from this treatment. Covalently bound RNA was assayed by washing exhaustively in 0.1 M $NaHCO_3$ buffer (pH 9) and subsequent digestion with RNase as described above. In all cases, the data from RNase digestion were corrected for hyperchromicity due to hydrolysis. These hyperchromicities at 260 nm in standard saline citrate at 25° were found to be 21.6% (tRNA), 6.4% (poly(U)), 46.0% (poly (C)), and 11% (16S rRNA).

Coupling of Small Molecular Weight Aldehydes. The hydrazide content of resins was checked by coupling to benzaldehyde dissolved in 0.1 M sodium acetate (pH 5). The supernatant absorbance at 248 nm was determined as a function of time. The molar extinction coefficient of benzaldehyde was measured as 12×10^3.

Solutions of the mononucleotide (5′-UMP) were oxidized with equivalent amounts of $NaIO_4$ as previously described

TABLE I: Properties of the Coupling Reaction of Various Compounds to Hydrazide-Derived Sepharoses.[a]

Compound	Initial Concn (M)	Resin	Vol of Settled Resin (ml)	Reaction Vol (ml)	$t_{1/2}$[b] (min)	mmoles Coupled per ml of Settled Resin at Satn
Benzaldehyde	1×10^{-4}	Agarose (untreated)	0.13	2.00		0
Benzaldehyde	1×10^{-4}	Hydrazide agarose	0.13	2.00	65	1.1×10^{-3}
Unoxidized UMP	1×10^{-4}	Hydrazide agarose	0.26	2.00		0
Oxidized	1.04×10^{-4}	Hydrazide agarose	0.26	2.00	60	0.6×10^{-3}
Unoxidized tRNA	2.64×10^{-6}	Hydrazide agarose	0.26	1.52		0
Oxidized tRNA	2.52×10^{-6}	Hydrazide agarose	0.26	1.52	65	8.0×10^{-6}
Unoxidized 16S rRNA	1.09×10^{-7}	Hydrazide agarose	0.26	1.52		0
Oxidized 16S rRNA	1.00×10^{-7}	Hydrazide agarose	0.26	1.52	70	1.0×10^{-7}
Benzaldehyde	1×10^{-4}	Agarose (untreated)	0.13	2.00		0
Benzaldehyde	1×10^{-4}	"Blocked" hydrazide agarose	0.13	2.00	120	0.8×10^{-3}
Unoxidized UMP	1×10^{-4}	"Blocked" hydrazide agarose	0.26	2.00		0
Oxidized UMP	1×10^{-4}	"Blocked" hydrazide agarose	0.26	2.00	120	1.1×10^{-3}
Unoxidized tRNA	6.8×10^{-6}	"Blocked" hydrazide agarose	0.026	0.52		0
Oxidized tRNA	6.8×10^{-6}	"Blocked" hydrazide agarose	0.026	0.52	18	87×10^{-6}
Unoxidized 16S rRNA	2.8×10^{-7}	"Blocked" hydrazide agarose	0.021	0.52	*c*	0
Oxidized 16S rRNA	2.8×10^{-7}	"Blocked" hydrazide agarose	0.021	0.52	9	3.5×10^{-6}

[a] All reactions were carried out at ambient temperature (25 ± 1°). [b] Reaction time required to attain one-half the maximum amount of RNA coupled for the reaction conditions indicated. [c] A low level of unoxidized rRNA (<10% of the input rRNA) bound to the surface of this resin was not released by washing at pH 9 (see Text).

by observing the absorbance at 262 nm of the resin supernatant as a function of time. In all cases, spectra were taken throughout the course of the reaction to verify that the uv absorbance was associated with the compound of interest. The molar extinction coefficient of UMP was taken as 1.0×10^4 at 262 mμ.

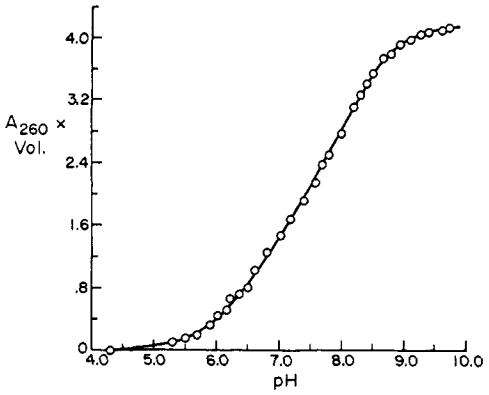

FIGURE 1: Release of tRNA nonspecifically bound to blocked hydrazide agarose as a function of pH. A sample of unoxidized tRNA (250 μg) was incubated with 0.53 ml of (volume of settled resin) blocked hydrazide agarose in 0.1 M sodium acetate buffer (pH 5.0) in a total volume of 2.0 ml at 25°. The amount of tRNA bound reached saturation after 20 min. The resin was then washed with carbonate free water and the pH adjusted to 4.0 in a total volume of 2.0 ml. The pH was increased by adding microliter quantities of 0.015 M NaOH solution. After each addition of base the pH was recorded and the solution centrifuged to permit the A_{260} of the supernatant to be determined. Each point then represents the observed pH and observed A_{260} of the supernatant multiplied by the volume of the solution at that point. The midpoint for release of tRNA from the resin corresponds to the titration of a monobasic acid with a pK_a of

Results

The results are presented in Table I. The hydrazide agarose is capable of binding approximately 10^{-3} mmoles of C_6H_5CHO or oxidized 5′-UMP per ml of resin. Only about 0.01 as many of the hydrazide groups react with oxidized tRNA, and the coupling of 16S RNA is lower by an additional factor of 100. The blocking treatment with carbodiimide and glycinamide does not significantly affect the binding capability for small molecules, but the coupling of tRNA is increased by a factor of 10, and that of 16S RNA by a factor of 35. Furthermore, the time to reach the plateau level of binding was decreased by the blocking reaction. The control studies show that in all cases oxidation of the 3′ terminus of the RNA is necessary for reaction.

Amino acid analysis, determined in a Beckman amino acid analyzer after hydrolysis of the blocked resin in 1 N HCl at 100° for 24 hr, indicated 2.43×10^{-4} mmoles of glycine/ml of settled resin resulting from the blocking reaction. (We are

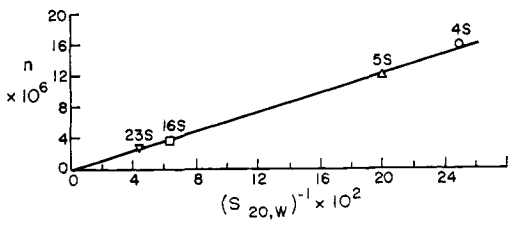

FIGURE 2: Effect of RNA size on the amount nonspecifically bound to blocked hydrazide agarose. Samples of unoxidized RNA were nonspecifically bound to blocked hydrazide agarose in 0.1 M sodium acetate buffer at pH 5 and 25°. For each RNA, the amount that is bound is plotted against the reciprocal of sedimentation coefficient for that RNA, which is a measure of the hydrodynamic size of the

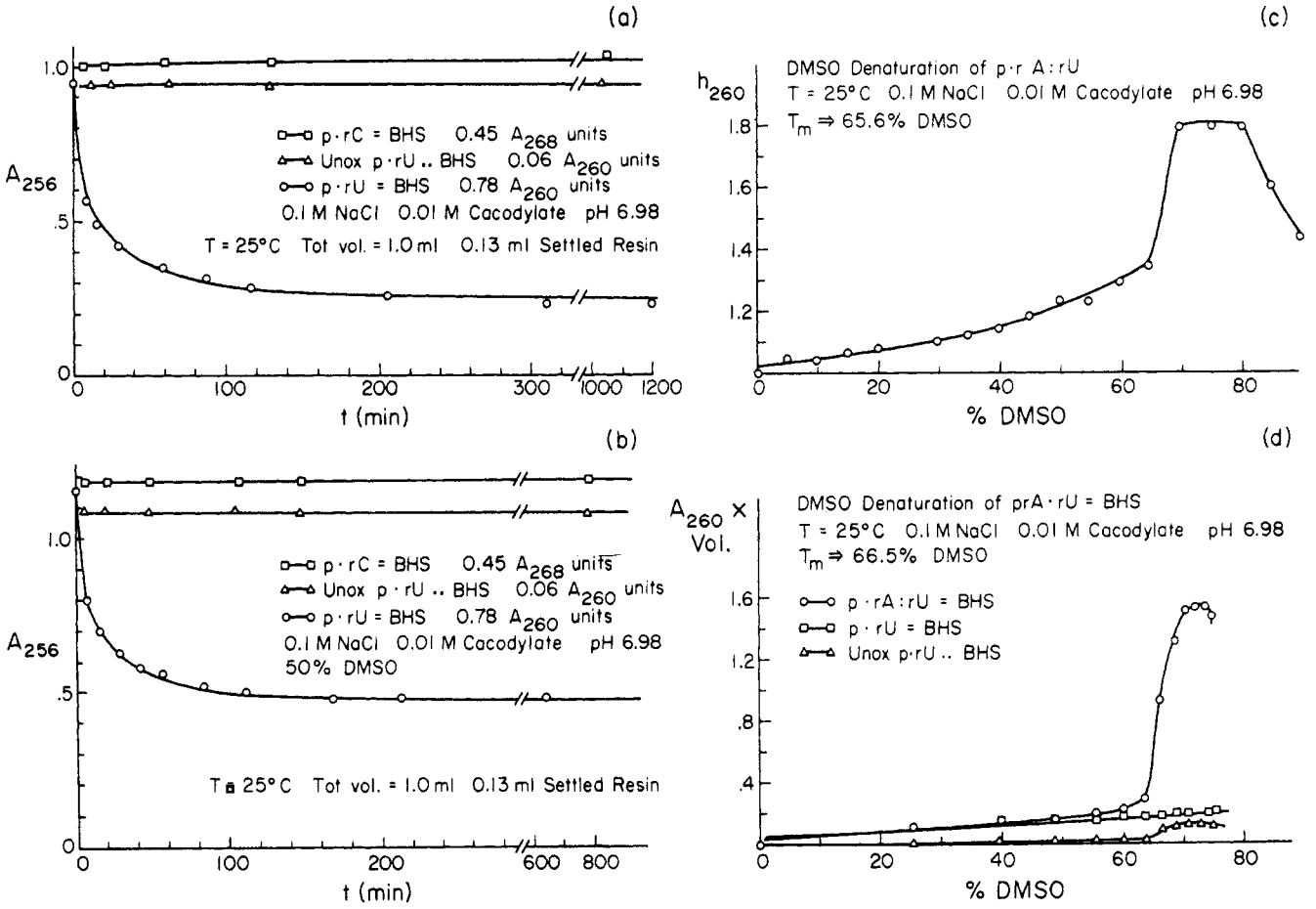

FIGURE 3: Hybridization of poly(rA) to resin-coupled poly(rU). (a) Hybridization of poly(rA) to poly(rU) which had been coupled to the blocked hydrazide agarose (BHS). The concentration of poly(rA), indicated by the absorbance at 256 nm in the supernatant, was determined as a function of time (○). The buffer used was 0.1 M NaCl–0.01 M sodium cacodylate (pH 6.98). The hybridization was carried out at 25° in a total volume of 1.0 ml of which 0.13 ml represented the settled resin to which polynucleotide has been coupled. At the indicated times after addition of p·rA, the suspension was centrifuged and the supernatant absorbance at the indicated wavelength was determined. The supernatant was then returned to the reaction tube and the resin resuspended. The resin was kept in suspension by a wrist-action shaker. The symbols p·rC = BHS and p·rU = BHS mean oxidized polynucleotides covalently coupled to the resin. The symbol unox p·rU··BHS means unoxidized polynucleotide noncovalently bound to the resin. One control sample consisted of p·rC coupled to BHS (a total of 0.45 A_{268} absorbance unit) (□). The other control sample consisted of BHS resin incubated with unoxidized p·rU which was subsequently washed as described in Materials and Methods (△). This resin contained <0.06 A_{260} absorbance unit of p·rU. The BHS sample to which p·rU had been coupled contained 0.78 A_{260} absorbance unit of p·rU. (b) Same as for (a) except that the hybridization buffer is 0.1 M NaCl–0.01 M sodium cacodylate (pH 6.98), 50 vol % Me_2SO. (c) Solution denaturation as evidenced by the hyperchromicity (h_{260}) of a 1:1 complex of p·rA and p·rU by increasing concentrations of Me_2SO (volume per cent) at constant ionic strength (0.1 M NaCl–0.01 M sodium cacodylate, pH 6.98) at 25°. The decrease in hyperchromicity at concentrations of Me_2SO greater than 80% probably indicate precipitation of the polynucleotide(s). The midpoint of the denaturation corresponds to 65.6% Me_2SO. (d) Me_2SO denaturation at constant ionic strength (0.1 M NaCl–0.01 M sodium cacodylate, pH 6.98) of the complex formed between p·rA and BHS coupled p·rU, resulting in release of p·rA to the supernatant. The samples used here were derived from part b after exhaustive washing with the hybridization buffer. The amount released was measured after centrifugation. The midpoint for release of p·rA to the supernatant corresponds to 66.5% Me_2SO.

grateful to Mr. Jon Racs for this analysis.) The blocked hydrazide agarose exhibits binding of unoxidized polynucleotides at pH 5. The "nonspecifically" bound polynucleotide can be released from the surface of the resin by raising the pH of the washing medium. Figure 1 displays a plot of the amount released as a function of pH. The curve has the same shape as a titration curve of a monobasic acid with a pK_a of 7.5. More than 95% of the nonspecifically bound tRNA is released at pH 9. (The blocked hydrazide resin loses binding capacity at a rate of about 10%/month at 4° in 5×10^{-4} M EDTA (pH 7.2).)

The larger the RNA, the smaller the number of millimoles of RNA nonspecifically bound per milliliter of settled resin (Figure 2). The data fit the relation (millimoles bound) $\approx 6 \times 10^{-5}/s_{20,w}$, where $s_{20,w}$ is the sedimentation coefficient of

The observations are qualitatively consistent with a picture of binding to some positively charged basic sites that titrate to uncharged sites with a pK_a below 7.5. However, we have not been able to identify the positively charged basic groups introduced by the carbodiimide–glycinamide treatment.

RNA and polynucleotides coupled to the blocked resin are available, to some extent, for hybridization with complementary polynucleotides. Figure 3a,b shows that 0.5–0.7 A_{260} unit of poly(A) will bind to a resin containing 0.78 A_{260} unit of covalently coupled poly(U), but there is no significant binding to a poly(C) resin. The hybridization can be carried out in aqueous solution or in 50% Me_2SO.

A 1:1 poly(A)·poly(U) complex in 0.1 M NaCl at 25° denatures at 65–70% Me_2SO; the same Me_2SO concentration will cause the hybridized poly(A) to be dissociated from the

A sample of ^{32}P-labeled *E. coli* DNA was sheared to a double-strand molecular weight of 1.4×10^5. Four milliliters of solution, containing 50 μg of DNA in 50% Me_2SO, 0.1 M NaCl, 0.01 M Tris, and 0.001 M EDTA (pH 8), was incubated for 12 hr with 0.26 ml of resin containing 48 μg of coupled 16S RNA. The resin was washed and incubated a second and a third time with 4 ml of fresh DNA solution. After further washing, bound DNA was extracted from the resin with 0.1 N NaOH. A quantity of 0.09 μg of DNA was recovered. By membrane filter hybridization, 7.2% of this product, as compared to 0.34% of the starting DNA, was complementary to ^{14}C-labeled 16S rRNA. Thus, an enrichment of ribosomal genes by a factor of 21 with a 15% yield was achieved. (In a similar previous experiment with only one incubation with DNA, an enrichment by a factor of 21 with a 40% yield was achieved.)

It should be noted that several very successful procedures for isolating rDNA or tDNA from short DNA strands by hybridization with rRNA or tRNA and separation of RNA–DNA hybrids from the remaining single-strand DNA have already been described (Colli *et al.*, 1971; Kohne, 1968; Marks and Spencer, 1970; Brenner *et al.*, 1970). Our final objective was the isolation of rDNA sequence in long DNA strands. Several hybridization experiments with DNA with an average single-strand molecular weight of 19×10^6 were tried, but very little enrichment was achieved.

Discussion

We have described a procedure for the formation of hydrazone bonds between terminally oxidized RNA and hydrazide groups on a resin. For high molecular weight RNA, the rate of the coupling reaction is greatly accelerated if the RNA is nonspecifically bound to the resin. In the present instance, the nonspecific binding seems to be due to an electrostatic interaction between the RNA and some unidentified basic groups introduced into the resin by the carbodiimide–glycinamide step.

It is quite likely that there are steric barriers to reaction of a 3′-terminal aldehyde group on an RNA with a hydrazide on the surface of a resin. In general, for a random coil molecule, the topological end is buried on the physical inside of the random coil particle, with only a low probability of being available for reaction with a reagent that cannot penetrate the coil. If the nucleic acid is adsorbed as a mobile two-dimensional random coil on the surface of the resin, the reaction rate of the 3′-terminal aldehyde wtih a hydrazide would be accelerated by the reduction in dimensionality effect discussed by Adam and Delbrück (1968).

We do not know the nature of the putative positively charged basic group introduced by the carbodiimide–glycinamide step. The titration curve of Figure 1 suggests that the pK_a is less than 7.5, since the dissociation of a complex between the BH^+ group (BH^+ signifies the protonated basic group) and the negative RNA site should occur at a higher pH than that at which BH^+ dissociates to B and H^+. An obvious first guess as to the identity of the basic group would be the dimethylaminopropyl group of the carbodiimide, but this should have a pK_a greater than 8.

Because of other commitments, we were unable to pursue the study of the enrichment of rDNA genes by hybridization to resin coupled RNA beyond the few experiments reported here. One point that we were unaware of at the time should be mentioned. Hunt (1969) reports that the RNA hydrazone bond is sensitive to cleavage by β elimination in the presence of amine buffers. Therefore, the Tris used in our DNA experiments should not have been used.

Our few experiments suggest that the complementary rDNA sequence in a high molecular weight DNA strand are less available for reaction with rRNA on the resin surface than are the same sequences on a shorter DNA strand. This we believe is an example of the excluded volume effect in nucleic acid renaturation (Wetmur and Davidson, 1968; Wetmur, 1971).

The coupling procedure, or some improved modification, is potentially useful for many other applications, so we have accordingly described it here.

Acknowledgment

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