[2] As is well known, the phosphate linkages of RNA are at the 3′ and 5′ positions. The 2′ isomers arise as artifacts of alkaline hydrolysis by migration of the phosphate to the 2′ position. This migration does not occur in the case of a terminal monoesterified phosphate in the 3′ position, which yields only 3′ isomers on alkaline hydrolysis.[3]

[3] Brown, D. M., and A. R. Todd, in *The Nucleic Acids*, E. Chargaff and J. N. Davidson, eds. (New York: Academic Press, 1955), vol. 1, p. 409.

[4] Sinsheimer, R. L., and J. F. Koerner, *J. Biol. Chem.*, **198**, 293 (1952).

[5] Cohn, W. E., and Khym, J. X., in *Biochemical Preparations*, ed. D. Shemin (New York: John Wiley and Sons, 1957), vol. 5, p. 40.

[6] Smith, J. D., in *The Nucleic Acids*, E. Chargaff and J. N. Davidson, eds. (New York: Academic Press, 1955), vol. 1, p. 267.

[7] King, E. J., *Biochem. J.*, **26**, 292 (1932).

[8] Beaven, G. H., E. R. Holiday, and E. A. Johnson in *The Nucleic Acids*, E. Chargaff and J. N. Davidson, eds. (New York: Academic Press, 1955), vol. 1, p. 493.

[9] In initial experiments, not presented here, we obtained considerably higher weighted means from diesterase hydrolysis than from alkaline hydrolysis. This effect was associated with very high specific activities of adenosine—5′-phosphate. Our enzyme preparation was evidently acting on some impurity present in RNA to give AMP of high specific activity. We found that repeated precipitation of RNA with ethanol removed this impurity, which clings quite tenaciously.

[10] Wyatt, G. R., in *The Nucleic Acids*, E. Chargaff and J. N. Davidson, eds. (New York: Academic Press, 1955), vol. 1, p. 243.

[11] Chargaff, E., in *The Nucleic Acids*, E. Chargaff and J. N. Davidson, eds. (New York: Academic Press, 1955), vol. 1, p. 307.

[12] Miura, K., T. Kitamura, and Y. Kawade, *Biochem. Biophys. Acta*, **27**, 420 (1958).

[13] We wish to stress that our usage of the term "statistically random" does not imply that the sequence is necessarily undefined. A "statistically random" sequence could be replicated as an exact copy during cell multiplication. See ref. 14.

[14] Yčas, M., in *Symposium on Information Theory in Biology*, H. P. Yockey, A. L. Platzman, and H. Quastler, eds. (New York: Pergamon Press, 1958), p. 70.

[15] Staehelin, M., *Biochem. Biophys. Acta*, **29**, 43 (1958).

[16] Shigeura, H. T., and E. Chargaff (*J. Biol. Chem.*, **233**, 198 (1958)) have presented data of experiments similar to our own, comparing the specific activities of 3′ and 5′ nucleotides of RNA isolated from liver. They tentatively concluded that a nonrandom sequence of nucleotides exists in RNA. We feel that since no calculation of the degree of nearest neighbor preferences was made, this conclusion should be accepted with reservations.

[17] Astrachan, L., and E. Volkin, *Biochem. Biophys. Acta*, **29**, 536 (1958).

[18] Spirin, A. S., A. N. Belozersky, V. Shugaeva, and U. F. Vanyushin, *Biokhimia*, **22**, 744 (1957).

[19] Stent, G. J., *J. General Physiology*, **38**, 853 (1955).

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*PARTIAL PURIFICATION OF SOLUBLE RNA**

BY PAUL C. ZAMECNIK, MARY L. STEPHENSON, AND JESSE F. SCOTT

THE JOHN COLLINS WARREN LABORATORIES OF THE HUNTINGTON MEMORIAL HOSPITAL OF HARVARD UNIVERSITY, AT THE MASSACHUSETTS GENERAL HOSPITAL, BOSTON

Communicated by Joseph C. Aub, April 22, 1960

Evidence from a number of laboratories indicates that a low molecular weight ribonucleic acid fraction (S-RNA) serves as an intermediate carrier of activated amino acids on the pathway to protein synthesis. The amino acids are bound in ester linkage to the ribose moiety of the terminal adenosyl unit of the S-RNA.[1-4] Lack of competition of amino acids for attachment to this S-RNA fraction,[5] the specificity of activating enzymes in catalyzing the esterification of individual amino

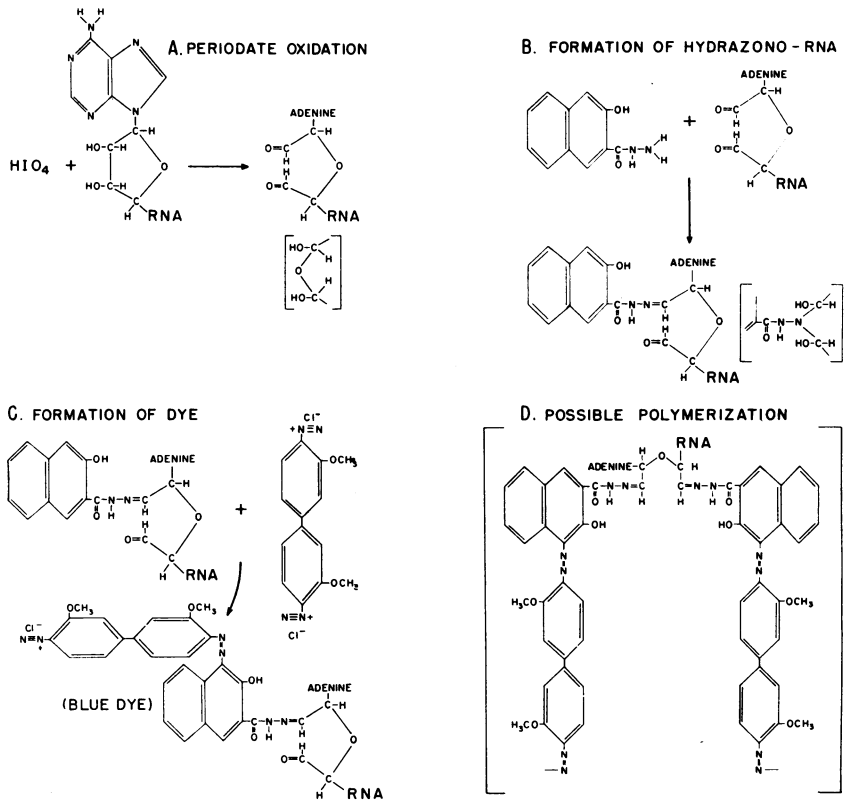

FIG. 1.—Steps in the formation of dye-RNA.

acids to S-RNA,[3, 6] and partial separation of S-RNA into fractions with enhanced abilities to accept particular amino acids[6–10] all suggest that separate S-RNA molecules may exist, each coded for a specific amino acid.

It has been observed[2–4] that the presence of an amino acid in ester linkage on the 3′ or 2′ hydroxyl group of the ribosyl moiety of the terminal adenosyl unit of S-RNA prevents oxidation of these positions to aldehydes by periodate. A possible separation of S-RNA bearing a particular amino acid from the remaining nonesterified S-RNA has been based on this observation.

It had been found that dinitrophenylhydrazine can be attached to periodate-oxidized S-RNA, but not to S-RNA which is not so oxidized,[11] and the dinitrophenylhydrazono-RNA gave some promise of separability from untreated RNA of this type, in countercurrent distribution systems such as that of Warner and Vaimberg.[12] It appeared desirable, however, to look for a substituent larger than dinitrophenylhydrazine to attach to the aldehyde groups produced by periodate oxidation of the terminal ribosyl unit of the RNA. 2-Hydroxy-3-naphthoic acid hydrazide has been found also to form a hydrazone with the aldehyde groups, and when tetrazotized *o*-dianisidine[13] is added to this latter hydrazono-RNA, a blue dye is formed, bound firmly to the RNA (Fig. 1*A*, *B*, and *C*). The dye-bound RNA can readily be separated from the untreated RNA by precipitation from a system composed of potassium phosphate buffer at pH 7.5 and *n*-propanol.

Materials and Methods.—Preparation of RNA: The RNA is prepared by direct phenol extraction of whole yeast cells as described by Monier *et al.*[11] This type of RNA is similar in physical characteristics and in ability to accept amino acids to the S-RNA prepared by the conventional homogenization and centrifugation techniques. The phenol extraction is a slight modification of the methods of Kirby[14] and of Gierer and Schramm.[15] Briefly, the RNA is extracted from the yeast cell suspension with an equal volume of 90 per cent phenol, precipitated by addition of alcohol, and then treated with charcoal. After a second precipitation by alcohol, the RNA is separated from polysaccharides by extraction from phosphate buffer into ethylene glycol monomethyl ether, and is again precipitated from alcohol. The latter extraction step is repeated, and the RNA is dialyzed against distilled water. The RNA is stripped of its amino acids by incubation for 60 min at 37° with 0.1 *M* Na_2CO_3, previously adjusted to pH 10 by means of NaOH. The solution is neutralized and dialyzed against distilled water. 1.4 μmoles of $MgCl_2$ are finally added per mg of RNA.

Preparation of enzyme, free of amino acid and nucleic acid: Enzymes from Ehrlich ascites tumor cells are used.[4] The 100,000 × *g* supernatant fraction of ascites cells from which the pH 5 fraction has been removed is freed of amino acids and nucleic acid by fractionation on an N,N-diethylaminoethylcellulose (DEAE) column, as follows:

The column (2 cm × 19 cm) is equilibrated with 0.02 *M* potassium phosphate at pH 7.7. About 100 ml of the crude enzyme fraction is dialyzed against the same buffer and then poured on the column. The phosphate buffer is washed through at a rate of 1.5 ml per min until the absorbancy at 260 mμ and 280 mμ is almost down to the background level. This procedure removes nucleotides and free amino acids. The crude enzyme fraction is eluted by means of 0.02 *M* potassium phosphate buffer at pH 7.7 containing 0.25 *M* NaCl. 10 ml fractions are collected. The elution is continued until the A_{260} and A_{280} of the effluent approaches the background values. At this salt concentration the small amount of S-RNA present in the original fraction remains on the column. The fractions are pooled, lyophilized and dialyzed for 4 hr against a medium containing 0.25 *M* sucrose, 0.025 *M* KCl, 0.005 *M* $MgCl_2$, and 0.05 *M* tris(hydroxymethyl)aminomethane buffer at pH 7.5. The final preparations contain approximately 1 mg of protein per ml. Although a substantial fraction of the activating enzymes is removed in the pH 5 precipitate, this preparation still contains valine activating enzyme, now almost free of amino acids and S-RNA.

Incubation with C^{14}-amino acid: The incubation mixture, as previously described,[4] contains the following in each ml: 10 μmoles of adenosine triphosphate, 0.5 μmoles of cytidine triphosphate, 10 μmoles of phosphopyruvate, 10 μg of pyruvate kinase, 0.6 ml of ascites enzyme fraction, 2-3 mg of RNA containing 1.4 μmoles of $MgCl_2$/mg RNA and 0.1 μmole of C^{14}-L-valine containing 450,000 counts per min. Incubation is for 15 min at 37°.

After incubation the C^{14}-valine-RNA is reisolated from the incubation mixture using a phenol extraction procedure. The incubation mixture is stirred for 30 min at room temperature with an equal volume of 90 per cent phenol. This is followed by centrifugation for 15 min at 10,000 × *g*. The upper phase containing the RNA is removed. The lower phase is stirred with about one-fourth its volume

of water and is centrifuged. The aqueous supernatants are combined and the RNA precipitated by the addition of one-tenth the volume of 2 *M* potassium acetate and 2.5 volumes of cold alcohol. After standing for 4 hr at $-10°$ the tube is centrifuged and the RNA pellets dissolved in a minimum amount of water. The RNA solution is stirred with acid-washed charcoal (Norit A, Fisher Scientific Company) for 15 min at 0°, using about 1.5 gm. charcoal per 100 mg RNA. The charcoal is removed by centrifugation at 12,000 $\times$ *g* for 15 min, and the charcoal pellet washed with a small amount of water. The RNA of the combined supernatants is precipitated by the addition of cold acetate and alcohol as previously described. A minimum amount of water is used to dissolve the RNA pellet and the solution is dialyzed for 12 hr against 0.001 *M* Na acetate at pH 5.

Preparation of dye-RNA: 2-Hydroxy-3-naphthoic acid hydrazide and tetrazotized *o*-dianisidine were obtained from the Dajac Laboratories, Borden Chemical Company, Philadelphia, Pennsylvania. It is reported that the tetrazonium compound comprises approximately 25 per cent of the weight of the commercial material, stabilizing reagents making up the remainder.

(i) *Oxidation of RNA containing no terminal amino acid*: Free 2′ and 3′ hydroxyl groups on the ribose of the terminal adenylic acid moiety of S-RNA may be oxidized to the dialdehyde by treatment with periodate.[16, 17] RNA (5 mg/ml) is incubated for 1 hr at room temperature in the dark, with 0.05 *M* sodium acetate at pH 5.0 and 0.1 *M* $NaIO_4$. Following incubation, most of the excess periodate is precipitated as the potassium salt by the addition of 0.2 *M* KCl. After standing 10 min at 0°, the precipitate is removed by centrifugation and the oxidized RNA dialyzed for 2–3 hr against distilled water.

(ii) *Addition of 2-hydroxy-3-naphthoic acid hydrazide to oxidized RNA, to form a hydrazone:* The incubation mixture contains the following in each ml: 5 mg of oxidized RNA, 50 μmoles of sodium acetate pH 5, and 0.5 ml of redistilled ethylene glycol monomethyl ether, containing 2.5 mg of 2-hydroxy-3-naphthoic acid hydrazide. Incubation is for 30 min at 37°.

The incubation mixture is extracted twice with an equal volume of ethyl acetate to remove most of the excess of 2-hydroxy-3-naphthoic acid hydrazide. It is essential to remove this excess 2-hydroxy-3-naphthoic acid hydrazide, since tetrazotized *o*-dianisidine reacts with it to form a blue dye even in the absence of RNA. The RNA is precipitated from the incubation mixture by the addition of one-tenth volume of 20 per cent potassium acetate pH 5 and 2.5 volumes of cold ethanol. After standing at 0° for 30 min, the RNA is collected by centrifugation, redissolved in a small volume of distilled water and is reprecipitated from alcohol as described above. The final RNA pellet (2-hydroxy-3-naphthoic acid hydrazono-RNA) is washed with cold 95 per cent ethanol.

(iii) *Coupling of the hydrazono-RNA with tetrazotized o-dianisidine to form an RNA-bound dye:* About 5 mg of hydrazono-RNA is incubated in 0.1 *M* potassium acetate pH 5 containing 1 mg of the stabilized salt of tetrazotized *o*-dianisidine in a volume of 1 ml. The tetrazonium salt is freshly dissolved in water at a concentration of 10 mg/ml just prior to each experiment. After standing at 0° for 5 min, 0.5 ml of cold absolute ethanol is added. The solution turns blue almost immediately, with increase in intensity upon addition of the alcohol. After 5 more min at 0° an additional 0.5 ml of cold ethanol is added. At the end of 10 more min

at 0° the RNA is precipitated by the addition of 1.5 ml of cold alcohol and 0.05 ml of 2 *M* potassium acetate, pH 5. After standing at 0° for at least 1 hr the pellet is washed once with 2 ml of ice-cold absolute ethanol. The washed RNA pellet (dye-RNA) is dissolved in distilled water and fractionated as soon as possible. Prolonged storage results in variable loss of dye from the RNA. The absorbancy maximum for the blue dye is quite broad ranging from 545 to 560 mμ.

Fractionation of RNA: The RNA pellets are dissolved in a small volume of distilled water (about 5 mg RNA/ml). All of the following steps are carried out near 0°. Nine volumes of 2.5 *M* potassium phosphate buffer at pH 7.5 are added, giving a final concentration of 2.25 *M*. A large fraction of the dye-RNA precipitates and is removed by centrifugation. Redistilled *n*-propanol equilibrated with the 2.5 *M* phosphate buffer is added dropwise to the aqueous phosphate solution, which is stirred constantly by a magnetic stirrer. After 0.25 volumes of n-propanol are added dropwise to the phosphate solution, the mixture is poured into a tube which is centrifuged briefly. Two phases form, with a precipitate of dye-RNA at the interface. The upper, propanol phase is discarded. The lower, aqueous phase is carefully transferred to the mixing flask, leaving behind the precipitate of RNA. The propanol is added to the mixing flask in increments of 0.25 volumes, and the separation repeated as described. All of the dye-RNA is precipitated by the time 1.5 volumes of propanol solution equilibrated with phosphate have been added. Up to 3 volumes of the propanol solution are added. At this point n-propanol which has not been equilibrated with the phosphate buffer is added in increments of 0.25 volumes. RNA, free of dye, then precipitates at the interface.

The precipitates of RNA are drained as free as possible of solution by careful inversion over absorbent paper, and are dissolved in distilled water. The A_{260}, A_{280}, and A_{560} are determined using a Beckman spectrophotometer.

Determination of radioactivity present in the solutions of RNA obtained from the propanol-phosphate fractionation: Since traces of inorganic phosphate present in aliquots of RNA solution to be used for counting interfere with accurate estimations of specific activity, special precautions must be taken to free the samples of phosphate before counting. Aliquots of the RNA solution are taken, and the RNA is precipitated by the addition of an equal volume of 8 per cent trichloroacetic acid. This nucleic acid precipitate is washed twice with 0.002 *N* HCl in 80 per cent ethanol, and finally with absolute ethanol. The resulting pellet of nucleic acid, free of inorganic phosphate, is dissolved in 0.2 *M* potassium acetate at pH 5 and the potassium salt of the nucleic acid is precipitated by the addition of 2.5 volumes of cold ethanol. The pellet is dissolved in water. An aliquot is taken for determination of A_{260}. The absorbancy index used is 32.3 $cm^2\ mg^{-1}$ RNA at 260 mμ in 0.1 *N* NaOH.[18] Another aliquot is plated directly on glass planchets for counting, using a Nuclear-Chicago Gas Flow, end-window counter with a counting efficiency of approximately 35 per cent. The specific activity of the sample is determined, and the total counts calculated from the RNA present in the total interface precipitate.

Results.—The degree of completeness of the reaction of dye binding to the RNA is dependent upon a number of individual steps. Since the oxidation of the 2′ and 3′ hydroxyl groups of the ribose moiety of the terminal adenosyl unit will not

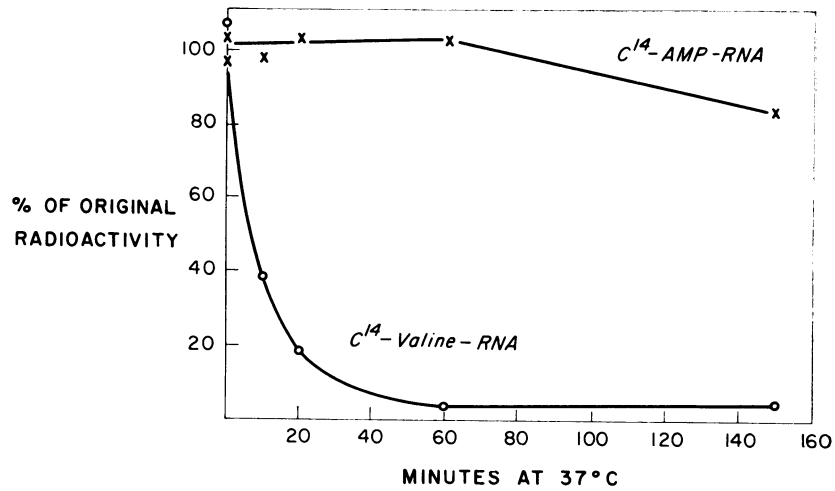

FIG. 2.—Effect of incubation at pH 10 upon C^{14}-valine-RNA and C^{14}-AMP-RNA. Incorporation of C^{14}-labeled amino acid and terminal nucleotide into S-RNA is carried out as previously described.[4, 18] After incubation, the RNA is precipitated by the addition of lysozyme 1.5 mg/mg RNA at pH 5.0. The RNA is isolated by phenol extraction.[4] C^{14}-valine-calf liver S-RNA containing 1,100 cpm/mg and C^{14}-AMP-yeast S-RNA (terminal position only) containing 13,700 cpm/mg are used. 0.6 mg of each is incubated with 0.1 *M* K_2CO_3 at pH 10 and 0.002 *M* $MgCl_2$ at 37° for the various time intervals indicated. After incubation, carrier protein is added and the RNA samples are reisolated for counting by the sodium chloride method described previously.[4] The results are expressed as (cpm/mg RNA)/(original cpm/mg RNA) × 100.

take place if amino acids are bound in ester linkage to the 2′ or 3′ position, it is important that any amino acids so situated be removed prior to the oxidation step. Figure 2 shows that treatment at pH 10 for 60 min at 37° removes 95 per cent of valine bound to S-RNA. Figure 2 indicates that although the ester linkage of the amino acid to S-RNA is labile at this pH, the phosphodiester linkage of the terminal adenylic acid moiety to the remainder of the S-RNA molecule is stable for at least 1 hr under these conditions. Such alkali treatment does not therefore harm the terminal units of the S-RNA molecules.

Figure 3 shows a time curve of the addition of 2-hydroxy-3-naphthoic acid hydrazide to oxidized RNA. The extent of the reaction has been measured by the over-all dye binding under otherwise constant conditions. The pH at which the naphthoic acid hydrazide is added is not critical, since the extent of the dye binding is the same over a pH range of 4.0 to 9.2. pH 5 has been used routinely because the amino acid ester linkage to RNA is stable at this pH.

The pH of the coupling reaction is not critical over the range of 4 to 6. At pH 7.3 and above, however, there is a small but significant binding of dye to the control sample of unoxidized RNA. For this reason pH 5 is used for the routine addition of tetrazotized o-dianisidine to the hydrazono-RNA.

Figure 4 shows that the reaction of hydrazono-RNA with tetrazotized *o*-dianisidine is virtually complete using 0.3 mg of stabilized diazonium salt per ml. There is no reaction of diazonium salt with unoxidized RNA over the entire range of concentration. In samples of dye-RNA the maximum ratio of A_{560}/A_{260} is 0.048.

Figure 5 shows the efficiency with which the dye-bound RNA separates from

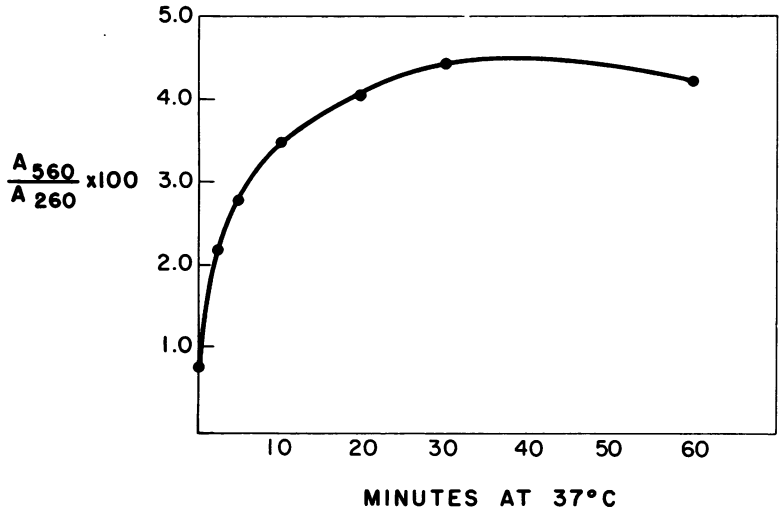

FIG. 3.—Time curve of addition of 2-hydroxy-3-naphthoic acid hydrazide to oxidized RNA. Conditions for preparing the hydrazono-RNA are described in the methods section except that 1 mg oxidized-S-RNA is used for each time point and the incubation of this oxidized RNA with 2-hydroxy-3-naphthoic acid hydrazide is carried out for the time periods indicated in the figure.

the non-dye-bound RNA. The RNA is first stripped of esterified amino acids by incubation at pH 10 for 1 hr at 37°. A single C^{14}-amino acid (L-valine) is bound by ester linkage by incubation with a crude ascites enzyme fraction freed of endogenous amino acids and RNA by passage through a DEAE column. The labeled RNA is reisolated from the incubation mixture as described in the methods section. One half is subjected to the dye addition procedures. Both samples are

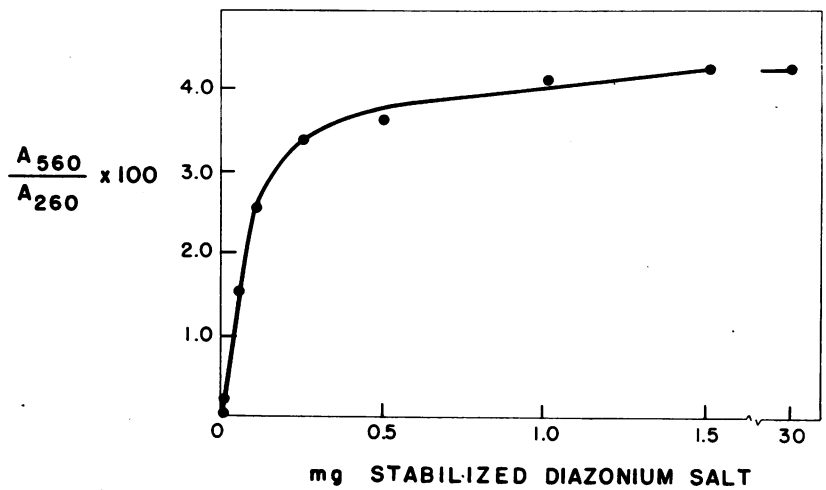

FIG. 4.—Coupling of hydrazono-RNA with tetrazotized *o*-dianisidine Conditions for the coupling reaction are the same as described in the methods section. Each aliquot contains 0.5 mg of hydrazono-RNA. The abscissa represents the approximate amount of stabilized salt of tetrazotized *o*-dianisidine added per ml of solution.

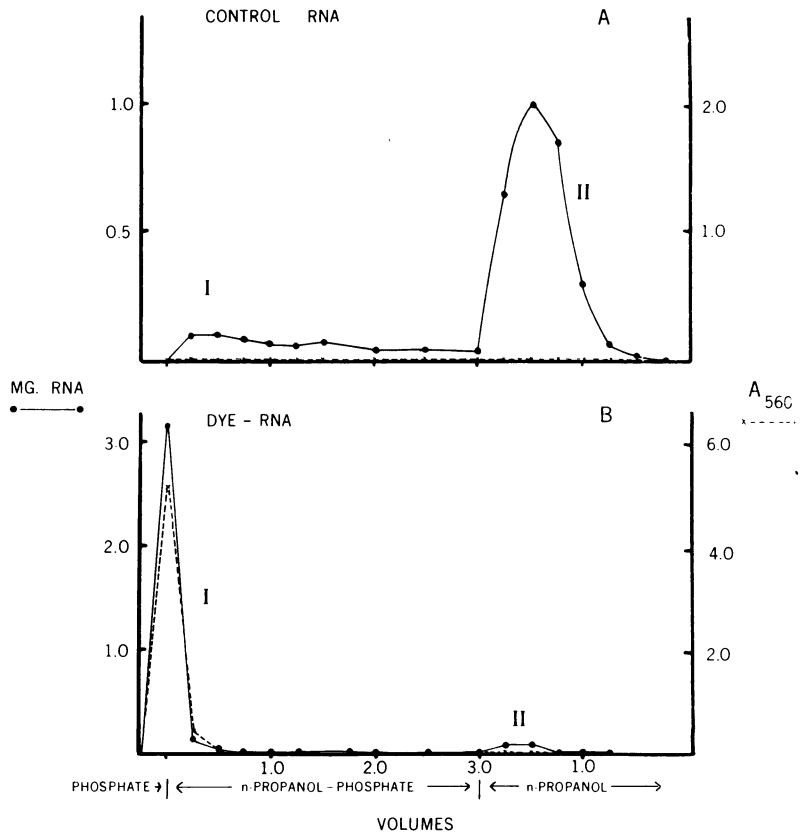

FIG. 5.—Partial fractionation of C^{14}-valine-RNA before and after addition of dye to the RNA. The details of the labeling of RNA with one amino acid and the fractionation procedure are described in the methods section. Curve A is the fractionation of a sample of C^{14}-valine-RNA. Curve B is the fractionation of a sample of the same RNA to which dye is added. The solid line indicates the mg RNA found in the interfacial precipitate and the dotted line indicates the absorbancy at 560 mμ.

then fractionated in the phosphate-propanol system. Figure 5 indicates the results of the fractionation. The top curve (A) shows the precipitation pattern of untreated RNA, whereas the lower curves (B) indicate the precipitation distribution of the dye-bound RNA. The solid line indicates the mg of RNA and the dotted line the A_{560} of the sample. There is essentially no A_{560} (blue dye) in area II in curve B, which corresponds to the point in curve A at which untreated RNA emerges. About 10 per cent of the sample of treated RNA is free of the dye.

The C^{14}-valine RNA might be expected to appear in area II of the RNA precipitation pattern, with a resultant increase of specific activity over the unfractionated "original sample." Fortunately, the amino acid ester bond to S-RNA is stable throughout the entire procedure of the addition of dye to the RNA molecules containing no amino acid. It is also quite stable during the fractionation procedure if the latter is carried out quickly and near 0°. Figure 6 indicates the location of radioactivity in the same RNA fractionation shown in Figure 5. Before dye treatment, almost all of the RNA and C^{14}-valine are found in the control area (Fig. 6, curve A, II). There is a slight enrichment of specific activity, indi-

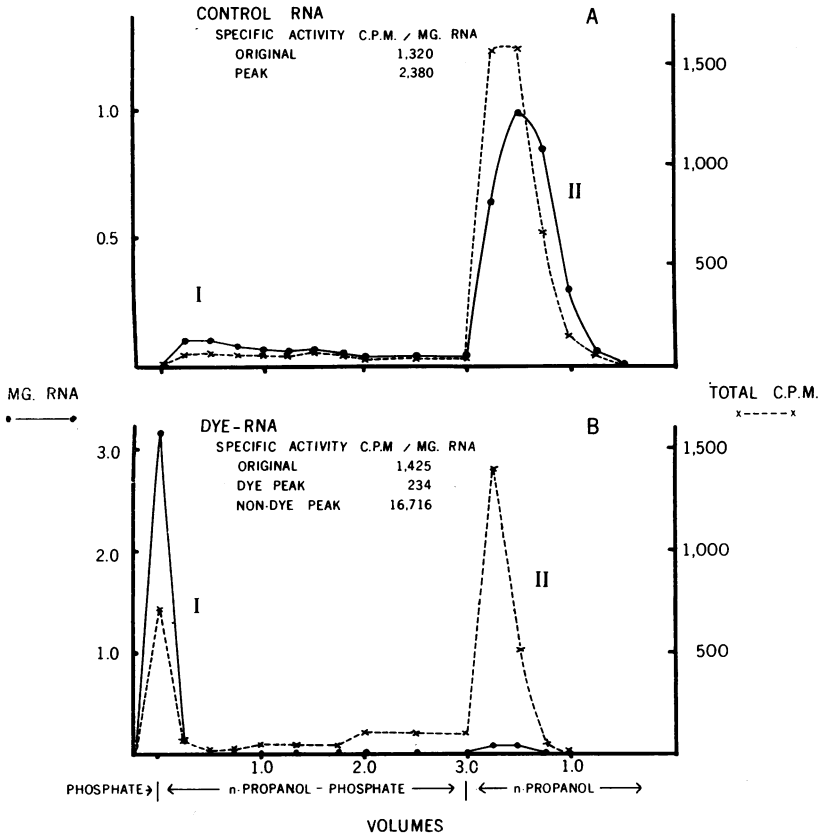

FIG. 6.—Location of radioactivity in the fractionation of C^{14}-valine-RNA before and after addition of dye. The RNA sample is labeled and fractionated as described in the methods section. The solid lines indicate the mg of RNA in the precipitate and the dotted lines show the total counts per minute found in the precipitate. The specific activity of the RNA of the unfractionated sample, the peak tube of the dye-RNA area (I), and the tube in the control area (II) containing the greatest number of counts are indicated. Curve A shows the fractionation of untreated C^{14}-valine-RNA and curve B shows the fractionation of the same sample after addition of dye.

cating a partial fractionation of valine-RNA in respect to the rest of the RNA molecules.

Fractionation of the same sample of RNA treated with dye shows that the bulk of the C^{14}-valine still appears in the nondye RNA peak (curve B, II), while 85–90 per cent of the RNA has reacted to form bound dye (curve B, I) and is well separated from the RNA containing amino acid and no dye. The figure indicates a maximum 12-fold enrichment of specific activity over the initial "mixed" sample of RNA. Some radioactivity is found in the dye-RNA peak (peak I), presumably due to coprecipitation of some RNA along with the dye-RNA molecule.

This coprecipitation has been minimized in other experiments by adding 2.5 *M* phosphate buffer, very slowly and with careful stirring, to the aqueous solution of RNA (containing both dye-RNA and non-dye-RNA) and by removing the precipitate as it forms.

It is interesting to inquire whether the lack of superimposability of the two

curves in Figure 6 A, II is due to partial separation of the valine-RNA from other amino acid-accepting RNA's, or is due to the presence of RNA unable to accept any amino acid. In an effort to distinguish these possibilities, a sample of RNA was labeled with C^{14}-AMP, using C^{14}-ATP as a precursor,[18] and was subjected to the phosphate-propanol fractionation. As shown in Figure 7, the curves of total

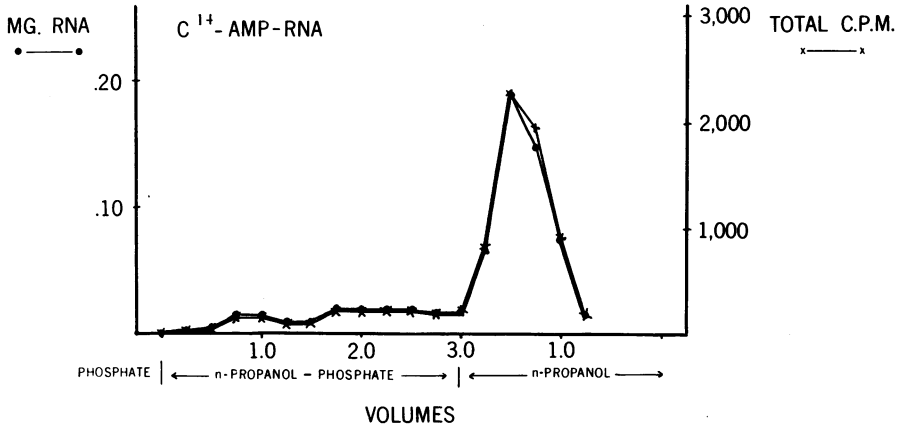

FIG. 7.—Location of radioactivity in the fractionation of C^{14}-AMP-RNA. Incorporation of the terminal nucleotide into S-RNA using C^{14}-ATP as precursor is carried out as previously described.[18] In this fractionation 0.80 mg of RNA containing 13,700 cpm/mg were used. Other conditions are described in the methods section.

radioactivity and of mg of RNA are nearly superimposable. Virtually all of the RNA is thus capable of adding a terminal AMP group and, by inference, of accepting an amino acid on that terminal AMP-group. The lack of superimposability of the two curves in Figure 6, curve A, II, therefore appears to be a fractionation of the C^{14}-valine-RNA from the rest of the family of amino acid-accepting RNA's.

In a separate labeling and isolation experiment using larger amounts of RNA, a sample similar to Fraction II of Figure 6B was subjected to further scrutiny to determine whether this RNA could be freed of its radioactive valine, then relabeled. A further question was whether this fraction could be relabeled with an amino acid other than valine. Accordingly, four separate fractions from a curve similar to 6B II were incubated at pH 10, 37°C, for 30 min to hydrolyze the C^{14}-valine-RNA bond. The RNA fractions were neutralized and dialyzed to free them of C^{14}-valine, and were then reincubated as previously described for amino acid labeling. The fractions relabeled about one half as well as before with C^{14}-L-valine, but not with C^{14}-L-leucine, although the original RNA sample prior to the fractionation procedure had labeled well with both C^{14}-amino acids. It may be concluded that a large part of this fraction of S-RNA (6B II) therefore is still capable of being relabeled, and has been freed nearly completely of leucine acceptor RNA.

A similar over-all labeling and fractionation experiment was carried out using C^{14}-leucine-RNA. The fractionation curves were quite similar to those obtained using C^{14}-valine-RNA and there was a maximum 9-fold enrichment of specific activity over the original "mixed" sample of C^{14}-leucine-RNA. The leucine peak emerged just prior to but overlapping the location of the value peak.

Discussion.—An increase in the specific activity for valine-S-RNA of the order of 15–25 times might be expected if this molecular species were freed from all others in the S-RNA fraction. Admittedly, even this estimate is open to uncertainty. The lack of superimposability of the curves for valine radioactivity and for 260 mμ absorbancy expressed as mg RNA in the enriched peak shown in curve B, II of Figure 6, however, provides direct evidence that the valine-RNA fraction is not homogeneous. Further improvements in the present fractionation scheme will depend on (*a*) more complete removal of amino acids from the S-RNA fraction prior to attachment of a single labeled amino acid; (*b*) greater attention to details for preservation of the rather unstable bond of dye to RNA; and (*c*) further refinement of the rather arbitrary fractionation procedure itself. There is also evidence[19] for the presence in some preparations of S-RNA of RNA not involved in amino acid transfer reactions.

A variety of other aldehyde addition reagents and diazonium couplers are also available for trial. We have found that hexazonium pararosaniline[20] couples well with 2-hydroxy-3-naphthoic acid hydrazono-RNA, with production of a red, dye-bound RNA which gradually precipitates out of 2.2 *M* phosphate buffer pH 7.5 without the addition of n-propanol. We have, however, had little experience as yet with other features of this dye-RNA. The periodic acid-Schiff reaction[21] has been too nonspecific in its attachment to RNA to be promising as an aldehyde addition reaction.

The relative ease of achieving partial separations of S-RNA molecules by purely physical means [6–9, 22, 23] (also cf. Figure 6, control curve A, peak II) suggests that differences other than arrangement of nucleotide sequence may distinguish them. The possibility of specific differences in secondary or tertiary structure may thus also be considered. The "recognition" by a given S-RNA molecule of an activating enzyme bearing a particular amino acid is a case in point. To accomplish this purpose, some large surface fit of RNA to enzyme might be involved.

Summary.—A method of fractionation of S-RNA molecules has been described which is based on the ability of periodate to oxidize to aldehydes the unesterified 2′ and 3′ hydroxyl groups of the terminal ribosyl group of S-RNA. S-RNA previously stripped of amino acids is relabeled with a single amino acid, L-valine, which becomes esterified to the 3′ or 2′ hydroxyl group of the terminal ribose, thus blocking periodate oxidation of this amino acid-bearing RNA molecule. The remaining free 3′ and 2′ hydroxyl groups of the terminal ribosyl groups present in the remainder of the molecules in the S-RNA are oxidized by periodate. 2-Hydroxy-3-naphthoic acid hydrazide is now added, and forms a hydrazone with the aldehyde groups. Upon addition of tetrazotized *o*-dianisidine to this solution of hydrazono-RNA, a blue dye is formed by coupling of the diazonium compound with the hydroxy-naphthoic acid hydrazono-RNA. This dye-bound RNA is sufficiently different in solubility from the original RNA in concentrated phosphate buffer at pH 7.5 to be separable by stepwise addition of n-propanol. A partial purification of valine-RNA has been achieved, with thus far a 12-fold enhancement of specific activity. This procedure appears to have a general applicability to the separation of other aminoacyl-RNA molecules from the family of closely related RNA molecules generally believed to play a role in the coding of amino acids for the sequence-aligning step in protein synthesis.

The authors wish to express their indebtedness to Mrs. Barbara Bingham for fine technical assistance, and to Drs. Robert B. Loftfield and Mahlon B. Hoagland for helpful advice and criticism.

* This is publication No. 1,000 of the Cancer Commission of Harvard University.

This work was supported by grants from the American Cancer Society, Inc., the U. S. Atomic Energy Commission, and the U. S. Public Health Service.

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*A NEW REACTION OF CYCLIC ALIPHATIC ANHYDRIDES**

BY D. RITTENBERG† AND LAURA PONTICORVO

DEPARTMENT OF BIOCHEMISTRY, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY

Communicated April 11, 1960

During the course of another investigation, it was found that the addition of catalytic amounts of pyridine to maleic anhydride induces a chemical reaction of some violence which yields carbon dioxide and a black brittle residue. Further studies show that this reaction is a general one, occurring with most aliphatic cyclic anhydrides and with all tertiary amines tested except triethanolamine. Primary and secondary amines (e.g., aniline α-naphthylamine, diethylamine, morpholine) do not catalyze the reaction. The anhydrides and active amines tested are listed in Table 1. The reaction varies in rate with the various anhydrides; it is rapid