CHROMATOGRAPHIC STUDIES
ON AMINOACYL-ACCEPTOR RNA USING PAPER SHEETS OF DIETHYLAMINOETHYL-CELLULOSE

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INTRODUCTION

Partition paper chromatography was used by BENDICH AND ROSENKRANZ[1] to fractionate DNA according to molecular size. Since substituted celluloses are used extensively for column fractionation of nucleic acids, the paper sheet forms of anion-exchange cellulose offer further possibilities for separations of this kind. In unpublished experiments, N. B. FURLONG AND F. J. BOLLUM of this laboratory established that oligodeoxyribonucleotides up to 100 nucleotide units would migrate on DEAE-cellulose paper. Since amino acid-acceptor RNA is of this approximate size, its behavior on various anion-exchange cellulose papers was investigated.

Many investigators have fractionated acceptor RNA from larger molecular weight RNA on ion-exchange cellulose columns; several have observed the separation of one specific type of amino acid-acceptor RNA from the main portion or a change in the ratio of 2 or 3 amino acid-specific acceptor activities[2–4]. Other fractionation technics, such as countercurrent distribution[5,6], chromatography on methylated albumin[7] or hydroxylapatit[8], and column electrophoresis[9] also have been successful in separating amino acid-specific RNA's.

The present work demonstrates that paper chromatography also fractionates acceptor RNA according to amino acid-specific types and describes a number of conditions that affect the chromatographic behavior of RNA. A preliminary note on this subject has been presented[10].

MATERIALS AND METHODS

Preparation of the paper

Two sheets, $8^1/_2 \times 22$ in. of DEAE-cellulose paper (Whatman, DE-20) were clamped on a glass frame for descending chromatography (Kurtz-Miramon frames, Kensington Scientific Co., Berkeley, California) and irrigated successively with 200 ml 1 *M* ammonium bicarbonate, 200 ml water, 200 ml 0.1 *N* HCl and water until no acid could be detected by taste (less than 0.005 *N*). The ammonium bicarbonate seemed to be more satisfactory than NaCl in removing the yellow-colored impurities. The papers were dried, stored at room temperature, and used within three weeks.

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Condition for chromatography of [14C]-amino acyl-RNA

Prior to application of the sample, the paper was clamped on a glass frame for descending chromatography and placed in 0.05 M NaCl–0.02 M acetate buffer, pH 5.6, until it was wet several cm past the origin. About 15 min prior to application of sample, the paper was taken out of the solution to reduce the moisture content at the origin area. The sample was applied and the paper placed in the first developing solution, 0.2 M NaCl–0.02 M acetate buffer, pH 5.6, which causes mononucleotides to move with an R_F of 0.4–0.6 and most amino acids to move even faster. This served to move out the radioactive amino acid and adenine nucleotides leaving the RNA at the origin. After 2 h the developing solution was changed to 0.58 M NaCl–0.02 M acetate buffer, pH 5.6, to cause the RNA to migrate; NaCl concentrations less than 0.4 M failed to move acceptor RNA. To provide a reference for the rate of the migration of the RNA, a nucleotide was spotted at the origin when development with the higher salt solution was started. A pH of 5.6 was chosen to minimize the spontaneous hydrolysis of the RNA-amino acid ester bond. As will be pointed out later, some chromatograms were developed at room temperature and some at 4°. For fractionation of acceptor RNA's, the lower temperature was used routinely. Radioactive areas were located and measured quantitatively by a 4π, windowless, gas-flow strip scanner coupled with an integrating unit (Vanguard Instrument Co., LaGrange, Illinois).

Preparation of [14C]-amino acyl-acceptor RNA

The amino acyl-acceptor RNA was prepared from *Escherichia coli* by the method of OFENGAND *et al.*[3], except that DEAE-cellulose column was used in place of ECTEOLA-cellulose. RNA concentration was calculated from the extinction at 260 mμ, assuming that 1 mg RNA/ml in 0.01 N NaOH has an extinction coefficient of 30 in a 1 cm light path[3,11]. The enzyme mixture was prepared from the 100,000 × g supernatant of *E. coli* extracts by first adding streptomycin, 1 % final concentration, discarding the precipitate, and fractionating the supernatant with ammonium sulfate. The enzyme fraction precipitated between 0.5 and 0.65 % saturation with ammonium sulfate[12]. The enzyme and RNA were dialyzed and stored at −20°. The reaction mixture (0.5 ml) consisted of 0.1 M Tris chloride buffer (pH 7.3 at 25°) or cacodylate buffer pH 7.0, 0.01 M Mg acetate, 0.01 M ATP, approximately 0.2 μC [14C]-amino acid, 0.25 mg acceptor RNA, and the enzyme preparation. After 15 min at 37°, the reaction mixture was acidified to pH 5 with acetic acid and stored at −20°. For chromatography, aliquots of 0.01–0.35 ml were added as a single spot the wet DEAE-cellulose paper. Since the RNA has a higher affinity for the DEAE than ATP or other components of the reaction mixture, it forms a compact spot not more than 2 cm in diameter with the largest aliquot.

Uniformly labeled [14C]-amino acids were obtained commercially and were of specific activities greater than 50 mC/mmole.

RESULTS

Resolution of RNA into amino acid-specific types

RNA esterified with different radioactive amino acids was chromatographed on DEAE-cellulose paper, and the separation achieved for four amino acyl-RNA's is
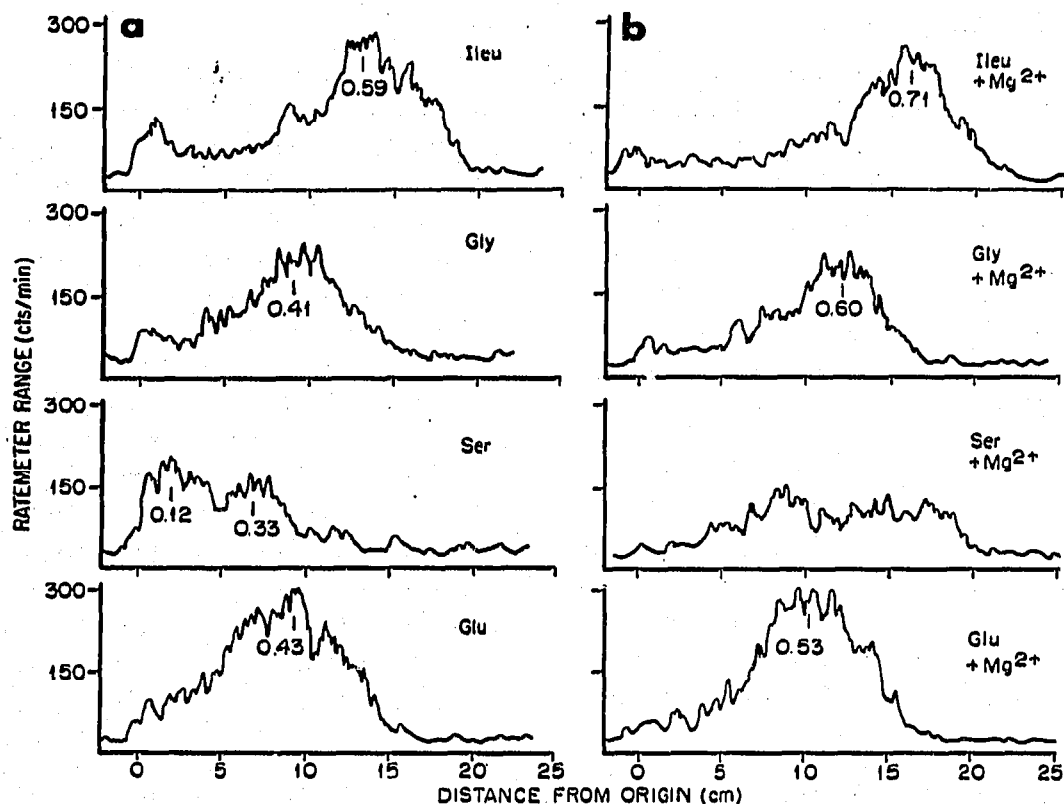
Fig. 1. Chromatography of RNA specific for four different amino acids. Uniformly labeled [^{14}C]-amino acids were esterified to the acceptor-RNA, and 0.05–0.15 ml aliquots of the reaction mixture were applied to DEAE-cellulose paper. (a) The paper was irrigated at 4° with 0.2 M NaCl–0.02 M acetate buffer, pH 5.6, for 2 h and then with 0.58 M NaCl–0.02 M acetate buffer, pH 5.6, for 2.5 h. (b) The paper was irrigated for the same time and with the same solution as for Fig. 1a with the addition of 0.01 M MgSO$_4$. The positions of the free radioactive amino acids were all at distances greater than 25 cm from the origin. The figure shown under each peak is the mobility relative to GMP.

shown in Fig. 1a. Isoleucyl-RNA moved about 13 cm in 2.5 h in 0.58 M NaCl, whereas the presence of 0.01 M Mg^{2+} in this developer increased the rate of migration (Fig. 1b). In addition Mg^{2+} also reduced the amount of material that was commonly found at the origin of the chromatogram (cf. later sections).

Seryl-RNA separated into two peaks of radioactivity (Fig. 1a) when chromatographed in the absence of magnesium. It is unlikely that this separation is a reflection of the contamination of the radioactive serine with radioactive glycine since the mobility of glycyl-RNA is greater than either peak of seryl-RNA. Furthermore, in the presence of magnesium during chromatography, this seryl-RNA was partially degraded, whereas glycyl-RNA was not (Fig. 1b). Previously, the separation of certain specific amino acyl-RNA's into two fractions has been shown using methylated albumin columns[7] and counter current distribution[13].

Further comparison of the amino acid specific RNA's is shown in Table I. A remarkably similar behavior of the charged RNA's which are specific for a given class of amino acids was noted in that aspartyl- and glutamyl-RNA had similar mobilities, and isoleucyl-, valyl-, and alanyl-RNA were all in the fastest group. It has been reported that on methylated albumin glutamyl- and aspartyl-RNA have very similar elution characteristics[7]. Thus, it would appear that the two RNA's

TABLE I

RELATIVE MOBILITIES OF AMINO ACYL-RNA's

Following application of samples, the DEAE-cellulose paper was irrigated first with 0.2 M NaCl–0.02 M acetate buffer, pH 5.6, for 2 h and then the higher NaCl concentration-acetate buffer. R_{GMP} refers to the mobility relative to 5'-GMP applied to the origin at the time the higher concentration of NaCl was initiated.

Exp. No.	[^{14}C]-amino acyl-RNA	Developing conditions	R_{GMP}
1	ileu	0.58 M NaCl	0.55
	lys		0.66
	leu	4°	0.46
	arg		0.43
	his		0.44
	phe		0.32
2	ileu	0.58 M NaCl	0.59
	val		0.64
	ala	4°	0.66
	asp		0.38
	glu		0.32
3a	ileu	0.60 M NaCl	0.59
	gly		0.41
	ser	4°	0.33, 0.12
	glu		0.43
3b	ileu	0.65 M NaCl	0.48
	gly		0.35
	ser	25°	<0.1
	glu		0.32

specific for the two acidic amino acids are themselves rather similar structurally.

In control experiments, the radioactive amino acid was chromatographed under conditions identical to those used for RNA chromatography. The aliphatic amino acids migrated rapidly in 0.2 M NaCl, and no radioactivity was detected in the area normally occupied by the RNA; the same was true for lysine, glutamic acid, aspartic acid, serine, threonine. On the other hand, phenylalanine did not move out entirely and a slight trail of radioactivity was found in the RNA areas as well as a small amount (<0.1 %) at the origin; histidine behaved similarly. Since some commercially obtained phenylalanine samples did not behave in this manner, contaminants were probably responsible. Despite this interference, the position of the phenylalanyl- and histidyl-RNA was detectable. With tryptophan, so much radioactivity remained in the RNA region and at the origin that a reliable estimate of the position of the tryptophanyl-RNA could not be made, using the present technic.

A variety of factors affect the migration of amino acyl-RNA on DEAE-cellulose paper. In reporting these experiments, two chromatographic properties of the RNA were studied: (1) the rate of migration under various conditions and (2) the fraction of the total that was found at the origin following the migration of the major portion of the RNA. The latter is due, presumably, to an irreversible binding of the RNA to the paper and may be of significance in explaining the behavior of RNA on substituted celluloses.

Salt concentration

The effect of salt concentration on the rate of leucyl-RNA migration is shown in Fig. 2; the rate is dependent on salt concentration up to 0.7 M NaCl where the maximum rate was achieved, as evidenced by the position of the ultraviolet absorbing material as well as the radioactive zone. The change in salt concentration also affected
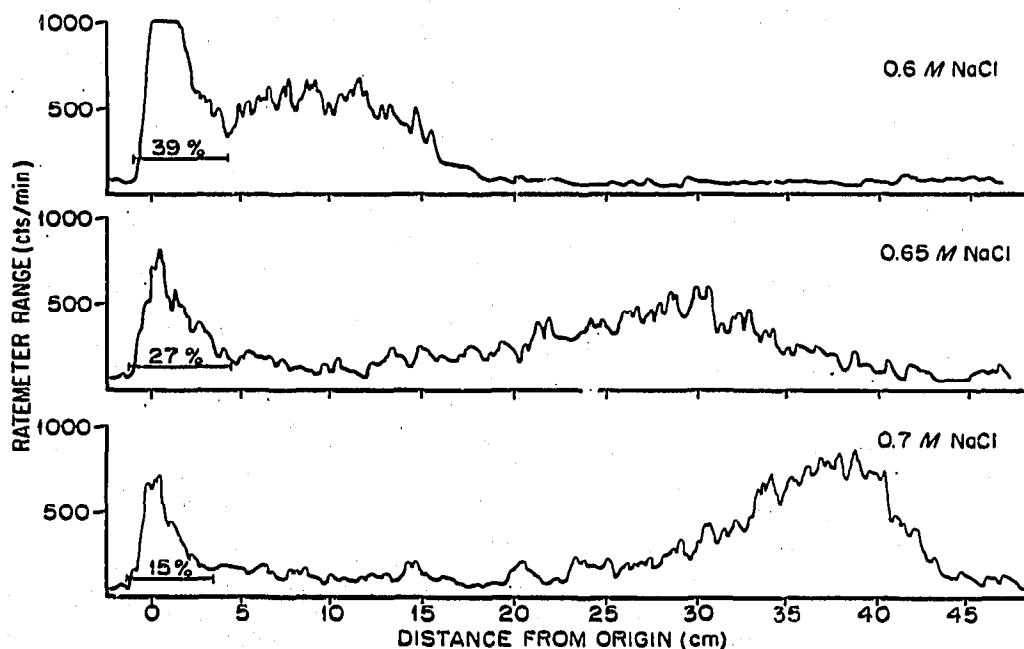

Fig. 2. Effect of salt concentration on [14C]-leucyl RNA chromatography. Samples (0.1 ml) of reaction mixture containing [14C]-leucyl RNA prepared as described in Methods were applied to DEAE-cellulose paper. Irrigation at 25° was with 0.2 M NaCl–0.02 M acetate buffer, pH 5.6, for, $2^{1}/_{2}$ h and then various concentrations of NaCl–0.02 M acetate, pH 5.6, for 4 h. The free amino acids had moved off the end of the paper under these conditions. All strips were surveyed at same setting of the count rate meter. Figure at origin indicates the proportion of total radioactivity measured that occurred at the origin area.

the proportion of material that remained at the origin, as much as 39 % of the leucyl-RNA remaining at the lowest salt concentrations. The 15 % at the origin after 0.7 M NaCl appeared to be a lower limit since NaCl concentrations up to 5 M did not reduce this value.

Examination of non-migrating RNA

If the material that migrated was different from that which remained at the origin, the rechromatography of each should result in single zones. Purified acceptor RNA, with no amino acid attached, was streaked on a DEAE-cellulose paper sheet, moved out in 0.65 M NaCl, and the forward and origin zones eluted separately with 1 M NaCl. Recovery from the origin zone was poor but sufficient to yield enough RNA to react with [14C]-leucine. Rechromatography of the material from each zone again gave two zones as detected by radioactivity and ultraviolet absorption, one at the origin and one that migrated in 0.65 M NaCl. Thus, it appears that the RNA found at the origin after chromatography was not necessarily different from that which migrated but resulted from irreversible adsorption of a portion of the RNA.

Temperature

The experiments immediately preceding were performed at room temperature (22–25°). At 4° there was a decreased rate of migration of leucyl-RNA, as would be expected, and there was a marked decrease in the amount of material left at the origin (Fig. 3). An approximately proportional reduction in migration rate occurred for GMP.
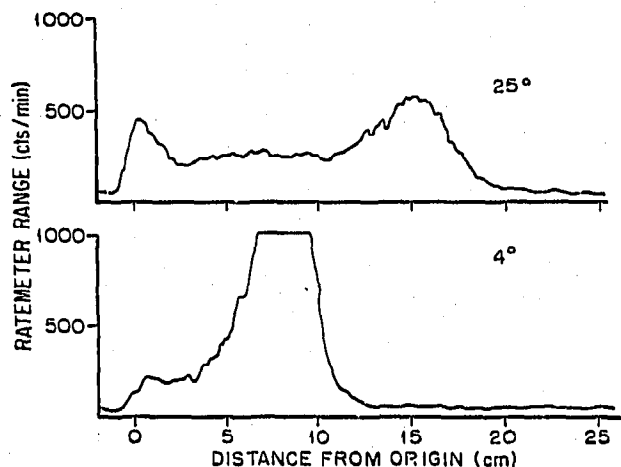

Fig. 3. Effect of temperature on [^{14}C]-leucyl RNA chromatography. Samples were the same as for Fig. 2. One paper remained at 4° before and after application of sample, the other paper was at 25°. Each was irrigated with 0.2 M NaCl–0.02 M acetate buffer, pH 5.6, for 2 h and then with 0.7 M NaCl–0.02 M acetate buffer, pH 5.6 for 1.7 h. At 4° the GMP, which was applied to the paper when 0.7 M NaCl was initiated, moved 0.58 of the distance attained at 25°. An ultraviolet-absorbing area was observed at the origin of the 25° paper but not of the 4° paper.

Time of contact with the paper

If diminished binding at the origin at 4° was due to a reduction in the rate of formation of the non-ionic bonds, it should be possible to observe an increase in the amount at the origin by extending the time of contact between the RNA and the DEAE-cellulose. Fig. 4 shows that the amount does increase significantly with time, the first few hours resulting in binding of about 10 % of the amino acyl-RNA near the origin. It should be noted that in all but the uppermost chromatogram the papers were irrigated with 0.2 M NaCl, pH 5.6, for 2 h; the papers then were allowed to hang in the humid atmosphere of the tank until irrigated with 0.7 M NaCl. In other experiments, 0.2 M NaCl, pH 5.6, was flowing through the paper constantly during the contact period, and similar results were obtained.

In addition to an increase in the binding at the origin, Fig. 4 also shows that the rate of migration of the RNA was reduced by extended time in contact with the paper. It is evident that certain characteristics of RNA undergo alteration in the presence of DEAE-cellulose resulting in more effective adsorption and slower mobility.

One explanation of these phenomena is that the secondary structure of the acceptor-RNA undergoes an alteration, such as the breaking of hydrogen bonds, during contact with DEAE-cellulose. The resulting structure could have a higher affinity for DEAE-cellulose because a greater flexibility in the molecule would result and allow a greater number of attachments between the primary phosphate and the DEAE. In addition to the electrostatic interaction, an increase in the extent of other
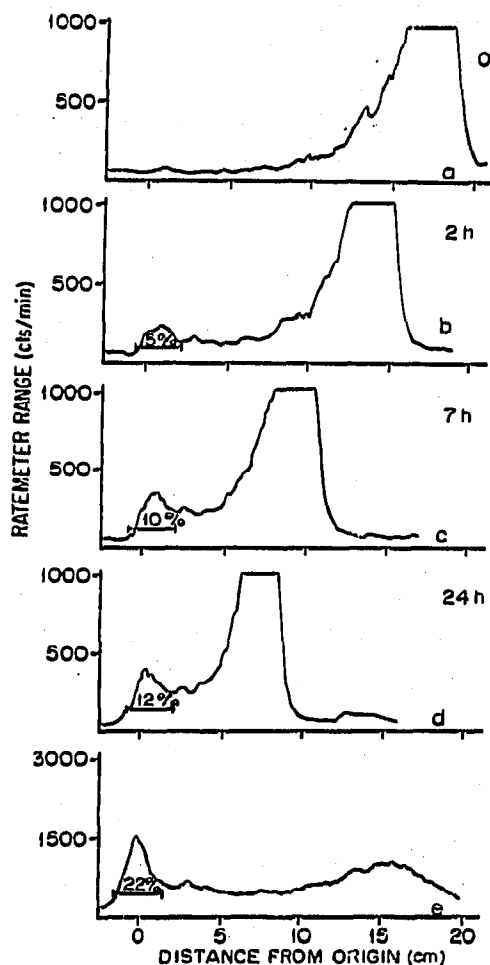
Fig. 4. Effect of time of contact of [^{14}C]-leucyl RNA with DEAE-cellulose. Samples of reaction mixture, 0.05 ml were prepared as in Fig. 2. (a) Paper was irrigated with 0.7 M NaCl–0.02 M acetate buffer, pH 5.6, for 1 h immediately after application of samples. (b) Paper was irrigated with 0.2 M NaCl–0.02 M acetate buffer pH 5.6 for 2 h and then with 0.7 M NaCl–0.02 M acetate buffer, pH 5.6 for 1 h. (c) After irrigation with 0.2 M NaCl–0.02 M acetate pH 5.6, the paper hung in the chromatography tank for 5 h and then was irrigated with the 0.7 M NaCl developer. (d) Paper hung 22 h before irrigation with the higher salt concentration. In 4a-d the chromatography was performed at 4°; in 4e, at 23° and irrigated as in 4b.

attractive forces between the RNA and the cellulose matrix of the paper could also occur.

Effect of urea

Since non-ionic bonds seemed to exist between the RNA and the cellulose and would be largely unaffected by salt solutions, 6 M urea was added to the usual developer. The effect of urea was to prevent any amino acyl-RNA from remaining at the origin and to cause 4 amino acyl-RNA's (ileu, gly, ser, glu) to migrate at the same rate as GMP.

Amount of RNA

The amount of RNA applied to the DEAE-paper can be varied at least 20-fold without appreciably changing the migratory behavior. Fig. 5 shows that the positions of the peaks were quite similar whether 0.02 or 0.4 ml of the reaction mixture was applied.
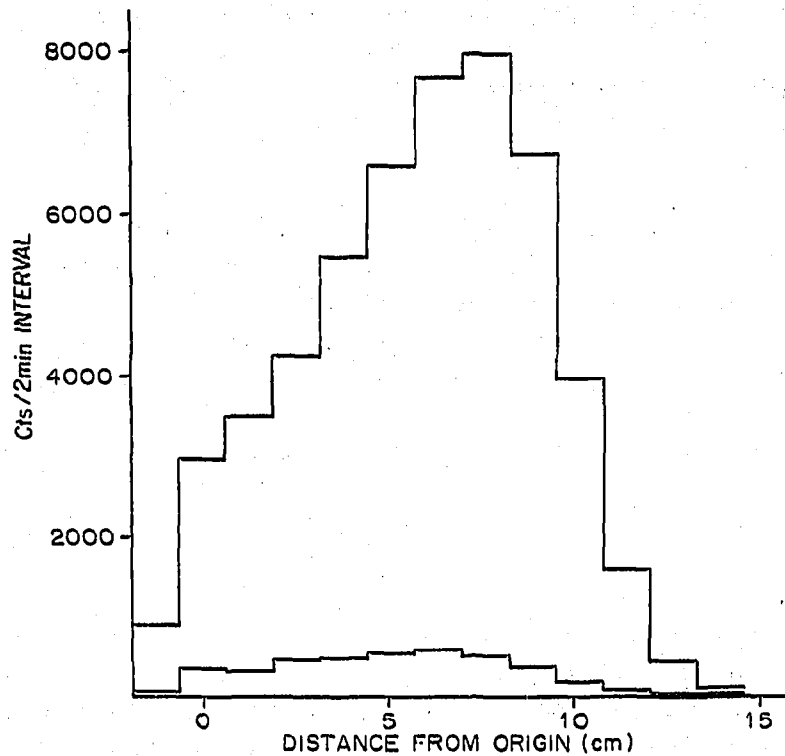
Fig. 5. Chromatography of different amounts of [¹⁴C]-leucyl RNA. Samples of a reaction mixture, 0.02 ml and 0.4 ml, were used. The [¹⁴C]-leucyl-RNA migrated in 0.58 M NaCl–0.02 M acetate buffer, pH 5.6, at 4° for $2^{1}/_{2}$ h. The data shown are the counts integrated over 2 min intervals, scanning at 25 cm/h through 1 cm apertures.
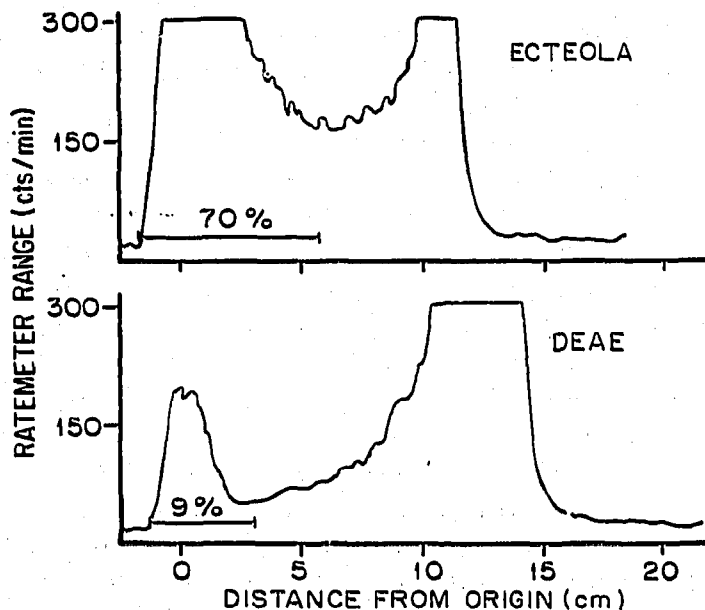
Fig. 6. Comparison of ECTEOLA-cellulose paper with DEAE-cellulose paper. Samples of reaction mixture, 0.05 ml containing [¹⁴C]-leucyl RNA, were applied to each paper which had been converted to chloride form as described in Methods. Both papers were irrigated at 24° with 0.2 M NaCl–0.02 M acetate buffer, pH 5.6, for 4 h and then 1 M NaCl–0.02 M acetate buffer, pH 5.6, for 1.25 h. The GMP which was applied at the time 1 M NaCl was started moved 0.7 as far on ECTEOLA as compared to DEAE paper.

The fraction of RNA irreversibly bound at the origin is independent of the amount of sample applied; e.g., samples of 0.02, 0.05 and 0.1 ml of isoleucyl-RNA were chromatographed and the amount remaining at the origin was 8.5, 4.8 and 5.9%, respectively.

ECTEOLA-cellulose paper

Because ECTEOLA-cellulose is also commonly used for chromatography of nucleic acids, it was compared with DEAE-cellulose, both materials being used as paper sheets. Fig. 6 demonstrates that, under the same conditions, leucyl-RNA behaves quite differently on ECTEOLA-cellulose. The material at the origin presumably was due, in part at least, to the anomalous behavior described for DEAE paper, the extent being predictably minimal on the latter because of the 1 M NaCl. No further exploration of the ECTEOLA paper was made. Experience on ECTEOLA-cellulose columns indicated that a strong binding of RNA occurred that was broken only by partially degrading the RNA[14].

DISCUSSION

Among the technics available for the fractionation of amino acyl acceptor-RNA (see Introduction), the DEAE-cellulose paper method is simple, inexpensive, and rapid. Furthermore, it can be adapted to a preparative scale; an 18-cm wide sheet has been used to chromatograph 3 mg of acceptor RNA and 100 % recovery of the acceptor activity for 4 amino acids was obtained by eluting the developed chromatogram with 1 M NaCl-6 M urea (JACOBSON AND NISHIMURA, unpublished data). Combination of DEAE-cellulose paper chromatography and other technics, such as methylated albumin chromatography, could prove productive in purifying certain acceptor RNA's since the relative mobilities of several of the amino acyl-RNA's are different on the two media. The effect of urea was to abolish the resolution of specific acceptor RNA's on DEAE-cellulose just as it was to cause oligodeoxyribonucleotides to migrate according to molecular size[15].

Some insight into the nature of the interaction between acceptor RNA and DEAE-cellulose is given by these experiments. There are apparently two phenomena to be explained: (1) the nature of the binding of various amounts of the acceptor RNA at the origin, and (2) the alteration in the RNA which results in the slower migration rate illustrated in Fig. 4. With regard to the RNA found at the origin, two types of forces are operative since the amount varied between 15 and 40 % with salt concentration but could not be reduced below this lower amount by further increasing the salt concentration. This indicates that non-ionic interactions occur between the RNA and the DEAE-cellulose resulting in attachments that cannot be dissociated by salt. Since these bonds form slowly but at a discernible rate, in terms of hours at 4° (Fig. 4), there are apparently many such bonds per RNA molecule. When only a few non-ionic bonds exist, they may not be sufficiently stable to prevent the RNA from migrating at higher salt concentration but they may be able to supplement the ionic interaction of the acceptor RNA and DEAE-cellulose at lower salt concentrations and cause more RNA to be retained at the origin (Fig. 2).

Such forces would not be apparent in the chromatography of mononucleotides and the smaller oligonucleotides since only a very few such bonds could form per molecule. On the other hand, for a polymer consisting of 90-100 nucleotides there would

be the possibility for multiple attachments resulting in a strong interaction not reversible by high molarities of salt.

Other laboratories have reported irreversible attachment of various RNA's to anion exchange cellulose usually evidenced by incomplete recovery of the sample. GOLDTHWAIT AND KERR[14] found ribosomal RNA was bound by ECTEOLA-cellulose so strongly that the alkaline conditions employed for elution resulted in material of diminished sedimentation coefficients.

The nature of the forces resulting in irreversible binding of acceptor RNA at the origin can be surmised, speculating from the effects of urea, magnesium ion, and temperature. It is unlikely that hydrogen bonds are involved since they would be expected to be less stable at 25° than at 4°, whereas greater binding was found at the higher temperature. Urea, although often considered to break hydrogen bonds, is effective in disrupting hydrophobic bonds of DNA resulting in a denaturation of the molecule[16]. Therefore, the fact that urea completely removes the acceptor RNA from the origin position of the DEAE-cellulose does not necessarily indicate hydrogen bonding but that hydrophobic bonds may exist between the RNA and the paper. The fact that magnesium ion reduces the amount of RNA at the origin is not inconsistent with an hydrophobic bond interpretation since it is known that magnesium ion causes a configurational change in acceptor RNA to a more helical form[17]. Such a configuration would probably reduce the hydrophobic bonds possible by making the purine and pyrimidine rings less available.

The second phenomenon, the reduction in the rate of acceptor RNA migration as a function of contact time with the DEAE-cellulose, may be a reflection of distortion of the RNA molecule. This distortion may arise from the electrostatic interaction between acceptor RNA and the DEAE-cellulose, or a combination of electrostatic and non-ionic forces, which might disorganize some of the secondary structure of the RNA and result in a more flexible molecule. In a more extended, flexible state the RNA phosphate groups may be able to engage in more interactions per molecule with the positively charged secondary amine groups of the paper, thus resulting in a slower mobility. The retarded mobility resulting from prolonged contact with the adsorbent apparently is not due to the same phenomenon responsible for the binding of the RNA at the origin. While a parallel exists in that both effects progress with time, the binding at the origin never accounts for all the RNA that might be expected if the retardation was proceeding progressively. Rather, the two phenomena each seem to reach a limit and thus do not appear to be due to common mechanism.

SUMMARY

Conditions for the chromatography of amino acyl-acceptor RNA on paper sheets of DEAE-cellulose have been explored further. Amino acid-specific RNA's can be separated using this type of chromatography. It was found that significant amounts of RNA are bound to the paper so that (1) salt solutions were ineffective eluents of RNA, and (2) urea combined with salt was effective. Results suggest that non-electrostatic forces play a significant role in the adsorption of RNA to substituted cellulose.

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