Influence of pH on the Stability of Some Aminoacyl Transfer Ribonucleic Acids and Their Elution Pattern in Chromatography on Columns of Methylated Albumin Adsorbed on Kieselguhr

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(Z. Naturforsch. **25 b**, 845—848 [1970]; eingegangen am 8. Mai 1970)

At pH 5.3 and 4.5 the half life of valyl-, threonyl-, leucyl- and seryl-tRNA from *E. coli* K 12 is significantly higher than at pH 6.8. While no changes were observed in the MAK elution patterns of valyl- and threonyl-tRNA, leucyl-tRNA was eluted in two peaks at pH 6.8 and 5.3 and in one broad peak at pH 4.5. Seryl-TRNA – two peaks at pH 6.8 – was separated in three peaks at pH 5.3 and 4.5. Rechromatography of these peaks at the other pH suggests the existence of at least four species of seryl-tRNA in *E. coli* K 12.

Columns of methylated albumin adsorbed on kieselguhr (MAK) are useful to separate small amounts of nucleic acids according to their molecular weight, their extent of hydrogen bonding and their base composition (G-C-content). This technique, first introduced by LERMAN [1] and further developed by MANDELL and HERSHEY [2], is not only used to separate native from denatured DNA and DNA from ribosomal and soluble RNA, but also to separate different kinds of aminoacyl tRNA from each other, as reported by SUEOKA and YAMANE [3]. During the following years the method of S u e o k a was employed by several other investigators. They all used linear sodium chloride gradients in phosphate buffered solutions, the pH of which was 6.7 or 6.8. In some cases the pH of the gradient was adjusted to 7.0 [4], 6.3 [5], 6.0 [6], 5.5 [7] and 5.2 [8], but no effect of the pH-changes on the elution pattern of aminoacylated tRNAs was reported.

In the following paper we show two advantages of acidic elution buffers on MAK chromatography:

1) In some cases the resolution of aminoacylated tRNAs may be significantly improved, as shown by the fractionation of seryl [^{3}H]-tRNA.
2) The greater stability of aminoacyl tRNA at acidic pH results in a much better recovery of aminoacylated tRNA from the columns.

Experimental

Materials

Bovine albumin, fraction V, was obtained from Fluka AG, Buchs, Switzerland. Kieselguhr was supplied by Serva, Heidelberg. When we used kieselguhr from other sources, we did not get as good a separation of the four species of seryl tRNA as with the product mentioned above. The tritium-labelled amino acids were purchased from the New England Nuclear Corp. or from the Radiochemical Centre, Amersham, dithiothreitol from Calbiochem.

Preparation of E. coli transfer RNA

The cells (*E. coli* K 12) were cultivated in a yeast-citrate-medium and harvested after 3 hours in the midle of the exponential phase. Crude tRNA was prepared by a modification of the procedure of BRUBAKER and MCCORQUODALE [9]: The cells (7.5 g) were mixed with water (4 ml) and phenol (11 ml), freshly redestilled and equilibrated with TMK buffer (0.01 M Tris/HCl, pH 7.4, 0.001 M $MgCl_2$, 0.001 M KCl), by grinding in a mortar for 10 min. at room temperature. The resulting paste was centrifuged in a Sorvall RC-2 centrifuge (30 min., 10 000 rpm). The aqueous layer was carefully removed, made up to 2% with potassium acetate by addition of 0.1 vol. of 20% potassium acetate and precipitated by addition of 2 vol. of cold ethanol (−20 °C). The suspension was stored for 2 hours at 4 °C. The sediment, obtained by centrifugation (10 min., 10 000 rpm) was washed two times with cold 75% ethanol, treated with 2 ml 1 M NaCl and centrifuged. The tRNA was precipitated from the supernatant by

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addition of 2 vol. ethanol. The mixture was stored in the refrigerator for half an hour at 4 °C. After centrifugation the NaCl-treatment was repeated two times. The tRNA was dissolved in 2 ml 0.5 M Tris/HCl, pH 8.8, incubated for one hour at 37 °C, supplemented with 0.2 ml potassium acetate (20%) and precipitated by 4 ml ethanol. After centrifugation the sediment was dissolved in 2 ml water and freeze dried to eliminate traces of ethanol. Then the tRNA was dissolved in water to give a concentration of 10 mg/ml. The frozen solution was stable for some months.

To prevent RNase contamination the glass-ware and centrifuge tubes were heated to 180 °C for 3 hours before use.

Preparation of aminoacyl transfer RNA synthetases

A crude mixture of aminoacyl tRNA synthetases was isolated from *E. coli* K 12 as follows: The cells (2 g) were suspended in TMK buffer (7 ml), supplemented with dithiothreitol (0.001 M) and glycerol (10%; the same buffer was used for the following steps), and homogenized by ultrasonic disruption. After centrifugation (120 min., 100 000 *g*) the supernatant was dialyzed overnight against buffer. The dialyzate was then applied to a column of DEAE-cellulose ($Cl^{\ominus}$, 1.5×10 cm). After washing with buffer the column was eluted with buffer containing 0.25 M NaCl. The fractions with the highest absorbance were combined, dialyzed against buffer and made up to 50% by addition of glycerol. The enzyme preparation stored at −30 °C, was stable for many months.

Preparation of ^{3}H-labelled aminoacyl transfer RNA

a) A 0.7 ml reaction mixture contained: 1 mg tRNA, 0.3 ml ATP-buffer (2 mM ATP, 10 mM Mg-acetate, 10 mM KCl, 100 mM Tris/HCl, pH 7.8), 0.02 ml L-amino acid-^{3}H (1 μCi, 10 mμMol), a mixture of 19 cold amino acids (50 mμMol each), about 140 μg synthetase protein (the optimal amount was tested for each enzyme preparation) and the required amount of TMK-buffer, to make up the solution to 0.7 ml. The reaction was carried out for 20 min. at 37 °C. To test for amino acid acceptor activity the mixture was precipitated by addition of 0.7 ml cold 10% TCA. The precipitate was collected on membrane filters (MF 50, Sartorius-Membranfilter GmbH, Göttingen), washed with 5% TCA and dried for 5 min. at 120 °C. Radioactivity was measured in a Packard Tri-Carb-liquid-scintillation spectrometer, using toluene scintillation liquid.

b) For chromatographic separations, 2 mg of tRNA were incubated in twice the volume of reaction mixture. After 20 min. at 37 °C 15 ml of elution buffer were added and the solution applied to the column.

c) To test for the stability of aminoacyl tRNA, 25 mg tRNA were charged with an amino acid as described above and purified by phenol extraction. The aqueous layer was divided in to three parts and the tRNA precipitated by ethanol and washed twice with 75% ethanol. The precipitates were dissolved (at 0 °C) in 5 ml 0.05 M K-phosphate pH 6.8, 5.3 and 4.5 respectively and incubated at 30 °C. Aliquots of 0.7 ml were precipitated at different times with an equal volume of TCA, washed and counted as described above.

Preparation of MAK-columns

MAK was prepared by stirring a mixture of 20 g kieselguhr (wet weight) in 100 ml 0.1 M NaCl with a solution of 50 mg methylated albumin in 5 ml water for 10 min. at room temperature. The mixture was poured into a Pharmacia column (15 mm diameter) giving a height of about 12 cm. The top of the columns was protected by a layer of uncoated kieselguhr (about 0.5 cm). The columns were washed with 60 − 100 ml starting buffer, charged with the reaction mixture, washed with 90 ml of the same buffer and eluted with a linear gradient of NaCl in 0.05 M phosphate buffer. The NaCl concentration of the gradients is given in the legends. The radioactivity of the fractions was determined by counting 0.5 ml samples in 10 ml dioxane scintillation liquid, to which 2 ml of a 1 : 3 mixture of methanol and water were added to prevent phase separation.

Results

To prepare crude tRNA we used the method of BRUBAKER and MCCORQUODALE [9], slightly modified by an additional treatment of the RNA with 1 M NaCl to eliminate contaminating ribosomal RNA. The aminoacylation was carried out in the

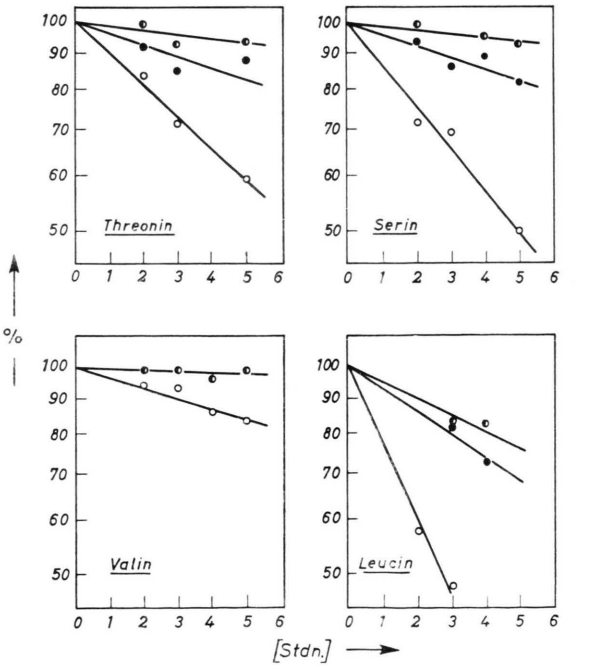

Fig. 1. pH-Dependent stability of aminoacyl tRNA. ^{3}H-Aminoacyl tRNA was dissolved in phosphate solution, pH 6.8 (−○−), 5.3 (−●−) and 4.5 (−◐−), and incubated at 30 °C. At the times indcated aliquots were taken, the tRNA was precipitated by TCA and the radioactivity of the precipitate measured.

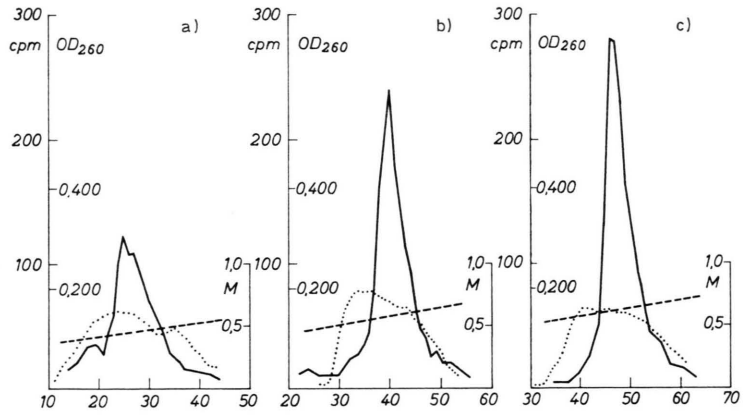

Fig. 2. MAK chromatography of threonyl [^{3}H]-tRNA at different pH. Gradient: (a) 0.3 to 0.85 M NaCl, 155 ml each, in 0.05 M K-phosphate pH 6.8. (b) 0.35 to 0.9 M NaCl, 155 ml each, in 0.05 M K-phosphate pH 5.3. (c) 0.35 to 0.9 M NaCl, 155 ml each, in 0.05 M K-phosphate pH 4.5. Column: MAK, 1.5 × 12 cm. Flow rate: 1 ml/min. Abscissa: Fractions to 3 ml; ordinate: radioactivity (—), gradient concentration (- - -), optical density (· · · ·).

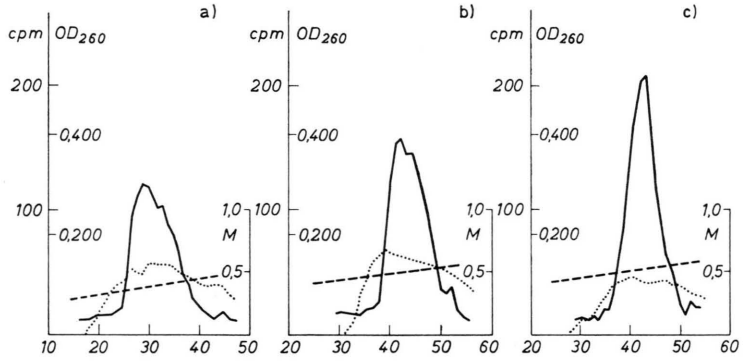

Fig. 3. MAK chromatography of valyl [^{3}H]-tRNA at different pH. For legend see Fig. 2.

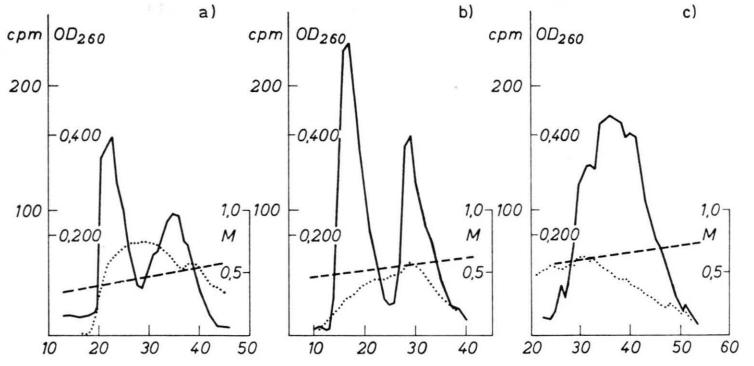

Fig. 4. MAK chromatography of leucyl [^{3}H]-tRNA at different pH. Gradient: (a) see Fig. 2. (b) 0.45 to 1.0 M NaCl, 1.55 ml each, in 0.05 M K-phosphate pH 5.3. (c) 0.45 to 1.0 M NaCl, 1.55 ml each, in 0.05 M K-phosphate pH 4.5. Column: see Fig. 2

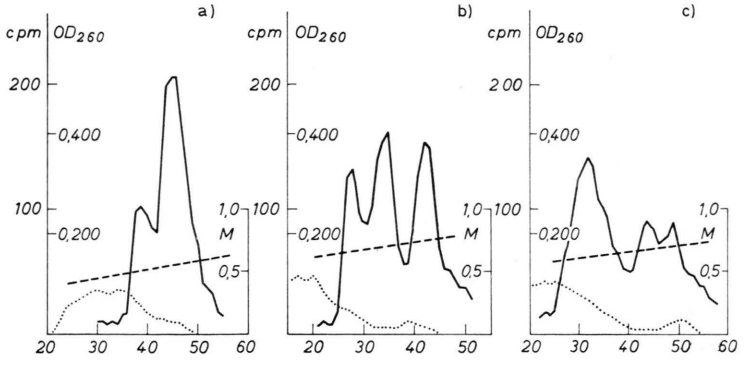

Fig. 5. MAK chromatography of seryl [^{3}H]-tRNA at different pH. Gradient: (a) Fig. 2. (b) 0.5 to 1.0 M NaCl, 140 ml each, in 0.05 M K-phosphate pH 5.3. (c) 0.5 to 1.0 M NaCl, 140 ml each, in 0.05 M K-phosphate pH 4.5. Column: see Fig. 2.

usual way, addition of CTP (10 mM) showed only a little effect on the binding of amino acids (about 5%) and was omitted in the following experiments.

Fig. 1 shows semi-logarithmic plots of the aminoacyl tRNA splitting reaction, which follows first order kinetics. Seryl-, threonyl- and leucyl-tRNA at pH 6.8 have a half life of 5 to 6 hours, valyl-tRNA is much more stable. At lower pH (5.3 and 4.5) the stability of the four aminoacyl tRNA species rises significantly.

In order to study the effect of pH on the elution pattern of aminoacyl tRNA from MAK-columns, seryl-, threonyl-, leucyl- and valyl-tRNA were eluted from the columns at pH 6.8, 5.3 and 4.5.

As shown in Fig. 2 and 3 threonyl- and valyl-tRNA give only one peak at pH 6.8, 5.3 and 4.5. The separation of the two peaks of leucyl-tRNA (Fig. 4) is similar at pH 6.8 and 5.3, while one peak only is observed at pH 4.5.

A most interesting observation is the pH-dependent change of the elution pattern of seryl-tRNA (Fig. 5): Instead of two peaks at pH 6.8 three main peaks are found at pH 5.3 and 4.5. Rechromatography of each of these peaks shows that four different types of seryl-tRNA (A, B, C, D) can be separated by MAK chromatography of *E. coli* K 12 tRNA. Table 1 shows the distribution of the four species

pH of elution buffer	number of peaks	species of seryl-tRNA
6.8	2	A +BCD
5.3	3	A +B +CD
4.5	3	AB+C +D

Table 1. Distribution of the four species of seryl-tRNA in the patterns of MAK chromatography at different pH.

in the chromatographic pattern: The first peak of pH 6.8 (A) gives the first peak at pH 5.3 and 4.5. The second peak at pH 6.8 (B C D) gives the second and the third peak at pH 5.3 (B and C D) and the first, the second and the third peak at pH 4.5 (B, C and D). The first peak at pH 4.5 (A B) can be divided by rechromatography at 5.3 into the first and the second peak (A and B), while the second and the third peak of pH 4.5 (C and D) give the third peak at pH 5.3 (Fig. 6).

In most of the pH 5.3 separations a small additional peak of variable extent was observed at the end of the third peak. This may be an artefact of the tRNA preparation or the aminoacylation.

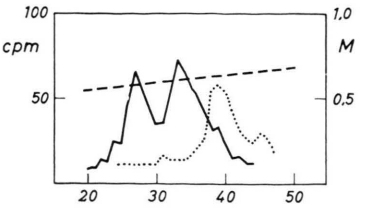

Fig. 6. Rechromatography of fractions from the pH 4.5-chromatography. The pooled fractions 30–35 (—) and 43–49 (- - -) respectively of the chromatography at pH 4.5 (Fig. 5) were rechromatographed after addition of 1.5 mg uncharged tRNA at pH 5.3. Column: MAK, 1.5 × 12 cm. Gradient: 140 ml 0.5 M NaCl and 140 ml 1.0 M NaCl in 0.05 M K-phosphate, pH 5.3.

Discussion

As can be seen from the above experiments, the recovery of radioactivity in the chromatography of aminoacyl [^{3}H]-tRNA at room temperature is much better at acidic than at neutral pH. This is in good agreement with earlier experiments of ISHIDA and MIURA [10].

Decreasing pH shifts the salt concentration, necessary to elute aminoacyl tRNA in MAK chromatography, to higher molarity (Figs. 2–5). In the case of the four amino acids tested, the separation at pH 5.3 is as good as or better than that at neutral pH, while the amount of tRNA-bound amino acids is increased by about 30 per cent.

Chromatography at pH 4.5 as compared to pH 5.3 results in a further increase in the yield of aminoacyl radioactivity, but only in the case of seryl-tRNA in a good separation. As for the other three amino acids used in these experiments, the chromatography at pH 4.5 showed one peak only. The results of the chromatography at pH 5.3 and the rechromatography at pH 4.5 and vice versa suggest the existence of four species of seryl-tRNA in *E. coli* K 12.

A similar result was obtained by ISHIKURA and NISHIMURA [11] with *E. coli* B. They used DEAE-sephadex chromatography followed by reverse phase chromatography. The tRNA requirement for our separation, however, is much lower. Therefore, MAK chromatography at different pH can be useful, if only small amounts of tRNA are available, as, for example, in research on the involvement of tRNA in regulatory mechanisms. On the other hand methyl-albumin silicic acid offers the possibility to apply the same method to larger amounts of tRNA.

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