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Fractionation of Nucleic Acids by Isoelectric Focusing†

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ABSTRACT: The basis of a fractionation of nucleic acids on gel electrofocusing has been investigated with two model systems: (1) dinucleoside phosphates and trinucleoside diphosphates and (2) isoaccepting species of tRNA. Many of the oligonucleotides and all of the tRNA species examined here (Phe, Arg, fMet, from *Escherichia coli*) formed stable banding patterns in the pH 3–5 range. Most oligonucleotides focused essentially as single bands whose apparent p*I*'s seemed consistent with an isoelectric fractionation arising from protonation of ring nitrogens in A and C. The banding patterns obtained with different tRNA species could not readily be attributed to differences in primary structure. Each of several highly purified isoaccepting species of tRNA gave rise to complex, but rather similar banding patterns. The amino acid accepting activity of components separated from tRNA fMet decreased progressively with decreasing p*I*. Although the possibility of artifact arising from binding to ampholyte could not be excluded, much of the evidence suggests that the multiple forms represent stable conformers, arising from a progressive, but reversible, denaturation of the tRNA during the electrofocusing procedure. Possible explanations for the reversible denaturation and for the apparently anomalous p*I* range of the multiple forms are discussed.

Recent demonstration of viable mRNA preparations capable of programming the synthesis of specific proteins lead to the hope that several eukaryotic mRNA species will soon be isolated and characterized. However, the purification to homogeneity of discrete species of messenger or other trace RNA molecules may prove difficult in view of the limited choice of high-resolution procedures for fractionating nucleic acids of similar size but different primary structure. One of the parameters that may be used to define and to demonstrate charge differences inherent in the primary structure of many biopolymers is the isoelectric point (p*I*) of the molecule. Recently, the procedure of isoelectric focusing has been adapted for fractionating macromolecules according to differences in their p*I* (Kolin, 1955; Svennson, 1961; Vesterberg and Svennson, 1966; Haglund, 1971). In this procedure, amphoteric molecules migrate under an electric field in stable pH gradients where they equilibrate or "focus" at their p*I*. Isoelectric focusing generally offers superior resolution to

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most electrophoretic or chromatographic procedures, and has proven valuable for separating closely related proteins and other amphoteric biopolymers. Since nucleic acids are amphoteric molecules, we decided to explore their behavior in this system. For this purpose, we developed the pH gradients in polyacrylamide gels to realize much of the potential resolution offered by electrofocusing (Awdeh *et al.*, 1968; Hayes and Wellner, 1969; Drysdale *et al.*, 1971).

In preliminary experiments, we examined a variety of oligonucleotides and some low molecular weight polysomal RNA species. Somewhat to our surprise, we found that many of these preparations formed unique and highly reproducible banding patterns in acid pH ranges. This paper explores the basis for the behavior of nucleic acid on gel electrofocusing by reference to patterns obtained from two model systems: (1) dinucleoside phosphates and trinucleoside diphosphates and (2) isoaccepting species of tRNA.

Experimental Section

Materials

Oligonucleotides. Samples of Cytidylyl-(3′–5′)-cytidine (CpC) (Sigma Chemical Co., St. Louis, Mo., lot 57B-2060) and guanylyl-(3′–5′)-cytidine (GpC) (Sigma lot 71C-0520) were kindly donated by Dr. A. Rich (Massachusetts Institute of Technology). Guanylylcytidylylcystosine (GpCpC) (control no. 16-1-467), cytidylylcytidylylcytosine (CpCpC) (control no. 16-3-447), and guanylyluridylyluridine (GpUpU) (control no. 16-10479) were supplied as the sodium salts by Miles Laboratories, Inc., Elkhart, Ind.

tRNA. The following purified tRNA preparations from *E. coli* K-12MO7 were generously supplied by Dr. A. D. Kelmers, Biological Macromolecular Separations Technology Program, Oak Ridge National Laboratories, Oak Ridge, Tenn.: $tRNA^{fMet}$ (methionine acceptance 1413 pmoles/A_{260}; activity, 97%), lot 10-85; $tRNA^{Arg}$ (arginine acceptance 1266 pmoles/A_{260}; activity 70%), lot 15-141; $tRNA_2^{Phe}$ (phenylalanine acceptance 659 pmoles/A_{260}; activity, 43%), lot-2.

All were isoaccepting species, having been separated by Freon reversed-phase chromatography, as described by Novelli (1969). All were free from esterified amino acids. Samples were dissolved in TKM buffer (0.1 M Tris-HCl (pH 8.0)–10 mM $MgCl_2$–0.1 M KCl–2 mM 2-mercaptoethanol) at a concentration of 2 mg/ml and stored at −20°.

Methods

Gel Electrofocusing. Ampholines ((carrier ampholytes) LKB 8151, pH 3–5 (batches 13, 35, and 38) and LKB 8153, pH 5–7 (batch 18)) were obtained from LKB Produkter Ltd., Sweden. Gel electrofocusing was performed according to the protocol of Righetti and Drysdale (1971) in an apparatus from Metaloglass, Inc., Boston, Mass. This system generally gives better resolution than that ordinarily obtainable by isoelectric focusing in the conventional sucrose density gradients because the gel matrix both reduces diffusion and supports substances that may tend to precipitate at their p*I*. Electrofocusing was performed in cylinders (10 × 0.3 cm) of 5% polyacrylamide gel containing 5% (w/v) glycerol. The gels were maintained at 4° by circulating coolant. Unless otherwise stated, the ampholyte concentration in the gel was 2% (w/v). After a brief period of electrolysis to discharge persulfate and to initiate formation of the pH gradient, the sample (10–50 μg of tRNA or 0.05–0.40 A_{260} unit of oligonucleotides in 10% glycerol and 2% (w/v) ampholyte) was applied to the top of the gel under a buffering layer of 1% gel ampholyte. An electrolysis period of at least 5 hr was used to ensure equilibrium focusing. Frequently, electrolysis was continued for periods up to 72 hr to investigate possible redistribution of components on prolonged focusing.

Oligonucleotides were detected by scanning the gel immediately after electrofocusing at 260 nm in a quartz cuvet in a Gilford recording spectrophotometer fitted with a linear scan device. Peaks of absorbancy due to separated nucleotides were distinguished from those of the ampholytes by scanning replicate gels containing no RNA. tRNA was also detected by scanning at 260 nm or by staining with 0.1% Toluidine Blue in 2% acetic acid. Stained gels were cleared by washing in 1% acetic acid.

pH gradients were determined by measuring the pH of eluates from 0.5-cm sections of gel shaken for at least 1 hr with 0.5 ml of water. The pH was measured at room temperature (22°) with a Corning pH meter Model 10 fitted with a combination microelectrode. When possible, the p*I*'s of individual components were determined by eluting the ampholytes from the appropriate gel segment containing the RNA spike. The error in this estimation of the p*I* is probably of the order of 0.1 pH unit. The effect of urea on the apparent pI of components eluted from urea gels (Ui, 1971) may be neglected at the dilutions used in the present studies. Characterization of components on the basis of their p*I* rather than their position in the gel reduced possible errors in identification caused by small displacements in the pH gradient from the banding of high concentrations of nucleotides or from the use of different batches of ampholytes with the same nominal pH range.

Analysis of Separated tRNA. Estimates of specific amino acid accepting activity of separated tRNA bands were obtained from fractions recovered from gel eluates. The identification and recovery of the tRNA was greatly facilitated by the fact that most fractions containing more than 10 μg of tRNA were clearly visible as opaque regions in the gel. Smaller amounts of tRNA were detected by scanning the gels at 260 nm immediately after electrofocusing. The tRNA was eluted from gel segments and subsequently separated from ampholytes by dialysis against TKM buffer in treated cellophane casing (Hughes and Klotz, 1960) (Visking Division of Union Carbide Corp., Chicago, Ill.) for 40 hr at 4°.

Aminoacylation activities of the isolated fractions were assayed by the method of Kelmers *et al.* (1965). Specific activity (pmoles of amino acid/A_{260}) of fractions of $tRNA^{fMet}$ were assayed at saturating levels of a highly purified sample of fMet synthetase, kindly provided by Drs. P. Schofield and J. F. Scott, Huntington Laboratory, Department of Medicine, Massachusetts General Hospital. The incubation mixture (0.25 ml) contained 25 μM Tris-HCl (pH 8.0), 2.5 μM $MgCl_2$, 25 μM KCl, 1 μM ATP, 1 nM L-[^{3}H]methionine, 1 μM 2-mercaptoethanol, 2–4 μg of tRNA, and approximately 0.1 mg of synthetase. After a 30-min incubation at 37°, the reaction was stopped by adding in the cold 0.25 ml of 20% trichloroacetic acid, followed by 0.1 ml of a solution of bovine serum albumin (1 mg/ml). The precipitate was collected on a Millipore filter, washed, dried, carried to 10 ml with Aquaflor (New England Nuclear, Boston, Mass.), and counted in a Beckman LS-250 liquid scintillation spectrometer.

Results

Preliminary experiments indicated that both the oligonucleotides and the tRNA could be concentrated into narrow zones at specific regions in the acidic portion of the gels.

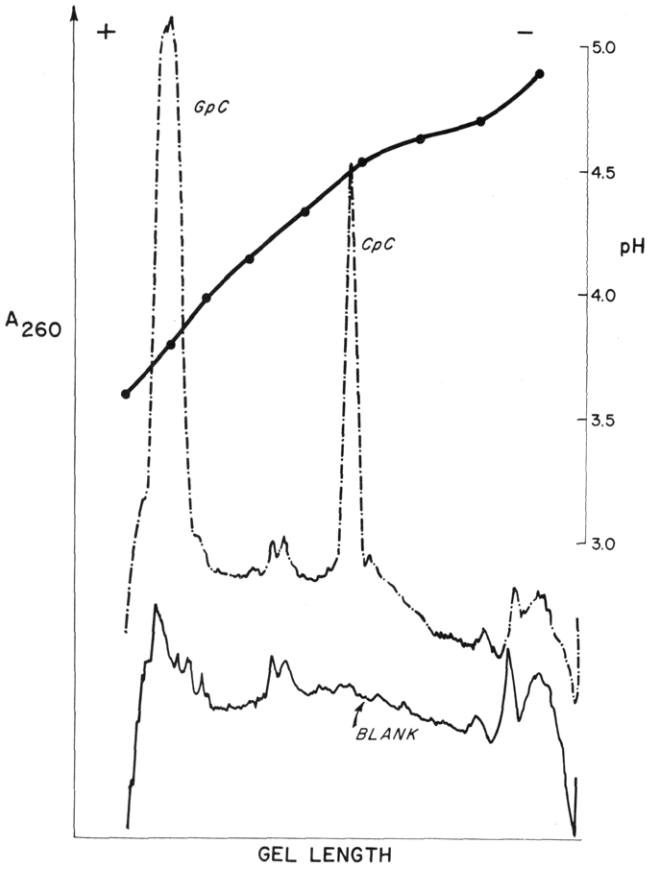

FIGURE 1: Separation of GpC and CpC. Samples, 0.15 OD of each were mixed and cofocused in 5% polyacrylamide gels containing 2% ampholyte, pH 3–5. After an electrolysis period of 6 hr, the gels and a control containing only ampholytes were scanned at 260 nm. The pH gradient in the gel is indicated by the solid heavy line.

In neutral or basic pH ranges, the RNA migrated the length of the gel and was compressed into poorly defined bands immediately above the anode. Since all of the RNA was found below pH 5, the most acidic ampholyte preparation buffering in the pH range 3–5 was used in subsequent experiments to effect a better separation.

TABLE I: Isoelectric Points of Some Oligoribonucleotides.[a]

	Obsd p*I*
Adenylyl-(3′–5′)-uridine (ApU)	3.6
Adenylyl-(3′–5′)-adenosine (ApA)	3.8
Cytidylyl-(3′–5′)-cytidine (CpC)	4.3
Guanylyl-(3′–5′)-cytidine (GpC)	3.9
Cytidylyl-(3′–5′)-cytidylyl-(3′–5′)-cytidine (CpCpC)	3.9
Guanylyl-(3′–5′)-cytidyl-(3′–5′)-cytidine	
($GpCpC_I$)	3.7
($GpCpC_{II}$)	4.1

[a] 0.15–0.40 A_{260} unit of the above oligoribonucleotides were subjected to electrofocusing in the pH 3–5 range. Estimates of p*I* were obtained from segments of gel containing the ultraviolet spike. p*I* values for GpU and GpUpU were not obtainable because they were below pH 3.

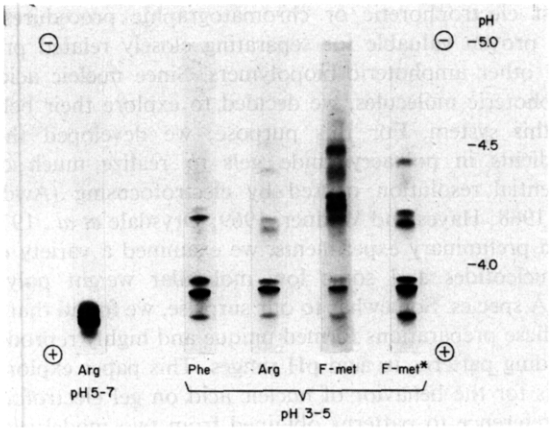

FIGURE 2: Fractionation of isoaccepting species of Phe, Arg, and fMet-tRNA by gel electrofocusing. Samples (25 μg) were subjected to gel electrofocusing for 6 hr in 5% polyacrylamide gels containing 2% ampholyte pH 3–5 or 5–7 as described in Methods. In the case of fMet*, the sample was incorporated directly into the gel before polymerization. In all others, the samples were applied to the top of the gel in a buffering layer of 2% ampholyte.

Dinculeoside Phosphates. ApA, ApU, GpU, GpC, and CpC were focused separately in the pH 3–5 range. Estimates of the p*I* of the major components in each preparation (obtained from eluates of sections of gel containing peaks of absorbancy over those of the ampholytes) are given in Table I. It should be noted that minor components may have been masked by the ampholytes and so may not have been detected here, nor would components with a p*I* less than pH 3, the lower buffering limits of the ampholytes.

With the exception of GpU, which presumably eluted at the anode, all other dinucleosides focused essentially as single peaks between pH 3 and 5. When cofocused in different combinations, ApU, ApA, GpC, and CpC banded at the same p*I*'s as when run individually. All four were separable in one gel. The separation of GpC and CpC is shown in Figure 1. Only two major components with p*I*'s corresponding to those of GpC and CpC, were evident above the background "noise" of the ampholytes. There was no indication of other components arising from possible interactions in this or any other combination of the above dinucleosides.

Trinucleoside Diphosphate. GpUpU, CpCpC, and GpCpC were subjected to electrofocusing, separately or together, in the pH 3–5 range. GpUpU migrated the length of the gel and either banded at the end of the gel or eluted into the anolyte. CpCpC focused as a single band at pH 3.9, 0.4 unit below CpC. A puzzling result was obtained with GpCpC, which separated into two major components, banding at pH 3.7 and 4.1, respectively. These components were apparently interconvertible. At low input levels, *e.g.*, 0.05 A_{260} unit, the more basic component was the greater. When focused in urea gels, the distribution at any one input level was markedly altered in favor of the more acidic component. Further experiments are in hand to characterize these components by other means.

tRNA. ISOACCEPTING SPECIES FROM *E. coli* K-12MO7. Patterns obtained by subjecting highly purified preparations of $tRNA^{fMet}$, $tRNA^{Arg}$, and $tRNA_2^{Phe}$ to different conditions of gel electrofocusing are shown in Figure 2. As with the oligonucleotides, the distribution of the tRNA was a function of the particular pH gradient developed in the gels. All of the tRNA species examined here banded below pH 5. In gels with a higher pH range, *e.g.*, pH 5–7, the RNA migrated

TABLE II: Amino Acid Acceptor Activities of $tRNA^{fMet}$ Isolates.[a]

Gel Fractions $tRNA^{fMet}$ Control (Unfractionated)	Amino Acid Acceptor Act. (pmoles/A_{260})	
	% Without Annealing 100%	% After Annealing 100%
Peak I (p*I* 4.5)	90	90
Peak II (p*I* 4.2)	75	85
Peak III (p*I* 4.0)	55	85
Peak IV (p*I* 3.8)	40	80
Peak V (p*I* 3.5)	20	75

[a] 1 ODU of $tRNA^{fMet}$ was fractionated in the pH range 3–5 as described in Methods. Peaks I–V (see Figure 5) were eluted from the gel and dialyzed against TKM buffer at 4° for 40 hr. The solution was divided into two aliquots. One portion was used as such; the other was first annealed at 65° for 10 min in the presence of 10 mM Mg^{2+}. Both preparations were tested for amino acid acceptor activity. The accepting activity of unfractionated $tRNA^{fMet}$ (control) was taken as 100%. Other experimental details are as in the legend to Figure 4.

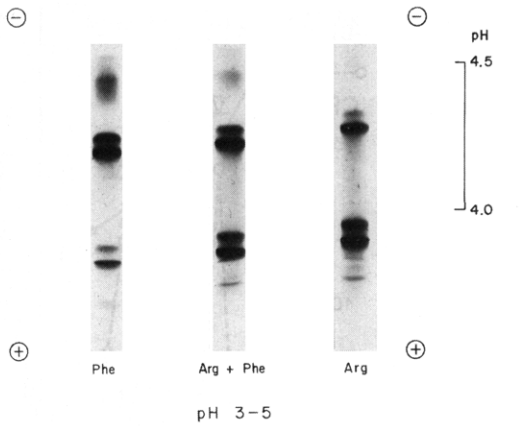

FIGURE 3: Banding patterns of $tRNA_2^{Phe}$ and $tRNA^{Arg}$, run separately and together. Details as in the legend to Figure 3.

the length of the gel to band immediately above the anode.

Perhaps the most striking feature of the banding patterns obtained in the pH 3–5 gels was the apparent heterogeneity shown by all of these highly purified tRNA preparations. For example, $tRNA^{fMet}$, consisting of at least 97% $tRNA^{fMet}$ by chromatography and amino acid accepting activity, separated after electrofocusing into about eight components in the pH range 3.5–4.5. The other tRNA species also gave more complex banding patterns than would be expected from their known purity. At least five bands were obtained from both $tRNA_2^{Phe}$ (43% pure) and $tRNA^{Arg}$ (70% pure). Most of the components formed well-defined, sharp bands, with the exception of those near pH 4.5, which were typically rather diffuse.

The main features of the patterns were quite reproducible as were the pH regions at which the various components banded. The patterns were stable for electrolysis periods of up to 72 hr. However, the relative distribution of the components from any one preparation was somewhat variable between experiments. Part of this variability could be attributed to experimental procedure. Usually, samples were applied from the cathode to the top of the gels after the pH gradient was partially formed. In this case, the RNA would migrate as the anionic form and would be unlikely to be exposed to a pH much below its p*I* before it reached an isoelectric state. The alternative method of focusing samples incorporated into the gel solution before polymerization introduces the possibility of artefact from interaction with other gel constituents such as persulfate. Of greater import in the present studies, is that the tRNA will converge on its apparent p*I* from both its anionic and its cationic forms. With most proteins which are unaffected by persulfate, the banding patterns given by the two procedures are usually indistinguishable. However, as shown in Figure 2, the mode of sample application had important consequences for the banding pattern of tRNA. When introduced at the cathode by the conventional procedure, $tRNA^{fMet}$ gave rise to at least eight components. Most of the RNA banded in the upper half of the gel above pH 4.0.

By contrast, when the tRNA was distributed throughout the gel before electrofocusing, only six major components were obtained. Although all six appeared to have counterparts in the control gel, their distribution was displaced toward the more acidic forms.

Neither the number of components nor their relative amounts were appreciably altered over a fivefold range of tRNA input (10–50 μg). However, high inputs of tRNA caused a substantial shift in the pH gradient and a concomitant shift in the positions of the RNA components in the gel. Components present in gels with high RNA inputs were, therefore, more reliably characterized by their measured p*I* than by their relative position in the gel.

Another striking feature of these patterns was the overall similarity in the number and distribution of components obtained from different tRNA species. Indeed, the banding patterns of all species examined here appeared to have several elements whose positions in the gels were identical or at least very similar. This point is demonstrated in Figure 3, which depicts the patterns obtained by cofocusing $tRNA^{Arg}$ and $tRNA_2^{Phe}$. When run separately, $tRNA_2^{Phe}$ resolved into six major components and $tRNA^{Arg}$ into five major components. The banding patterns were qualitatively similar, but quantitatively quite different. Most of the $tRNA_2^{Phe}$ banded above pH 4.0, whereas most of the $tRNA^{Arg}$ banded below pH 4.0. The pattern obtained from the mixture showed about eight components. Although elements unique to each species could be discerned on close inspection, the positions of several components, notably the two major fractions of $tRNA^{Arg}$ near pH 4.2 appeared to coincide so closely with the corresponding bands from $tRNA_2^{Phe}$ that they were not readily distinguishable.

Specific Activity of Isolates. In efforts to account for the polydispersity in the different tRNA species, sufficient amounts of the major fractions of one species ($tRNA^{fMet}$) were isolated to allow estimates of their amino acid accepting activities. Results from this experiment are given in Figure 4 and Table II. All fractions were found to be biologically active. However, most of the recovered $tRNA^{fMet}$ was considerably less active than the input material. The specific activity of the isolates varied according to their apparent "isoelectric points." The most basic fractions had the highest activity, the diffuse bands near pH 4.5, having 90% of the

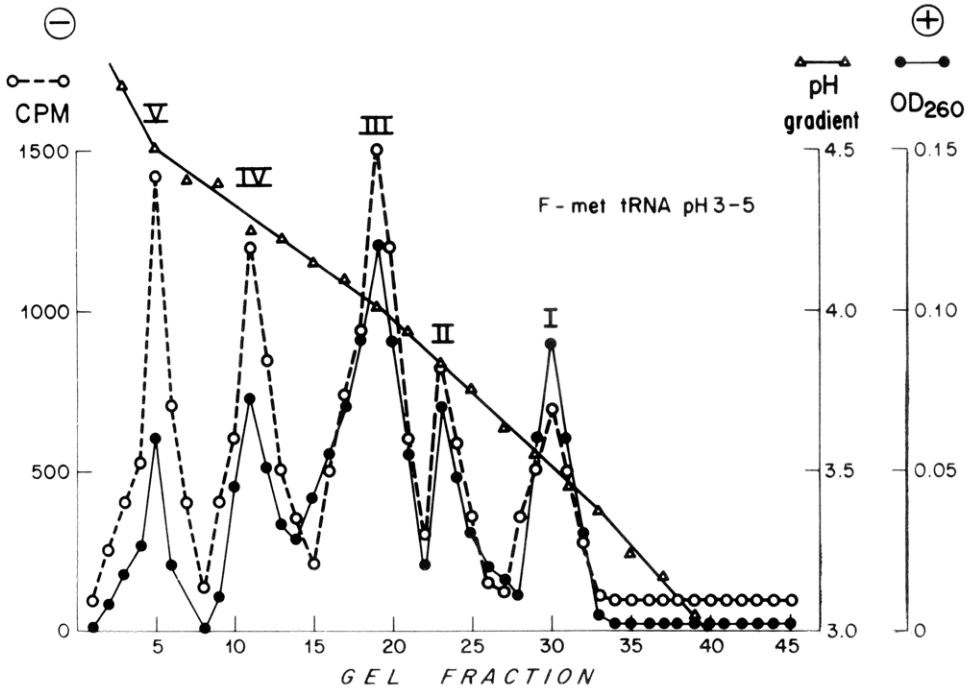

FIGURE 4: Amino acid accepting activity of isolates. $tRNA^{fMet}$, 1 ODU, was fractionated in a 5% polyacrylamide gel containing 3% ampholine, pH 3–5. At the end of the run the gel was sliced into 45 segments, each 2 mm thick. Each slice was placed in a small dialysis bag, carried to 0.3 ml with TKM buffer, and dialyzed for 40 hr at 4° against three changes of TKM buffer. The amount of RNA recovered was determined by the A_{260}. Samples of 1–4 μg of tRNA were assayed for amino acid accepting activity with L-[^{3}H]methionine as described in Methods. The total recovery from the gel, in terms of OD_{260} was 75% of the amount applied. The total recovery in terms of amino acid acceptor activity was 50% of the activity of the unfractionated material. The acceptor activities of each peak, expressed as per cent of the activity of unfractionated $tRNA^{fMet}$, are given in Table II. These results were routinely confirmed. (●) OD_{260}; (○) cpm; (△) pH gradient.

amino acid accepting activity of the original preparation. With decreasing p*I*, there was a progressive decrease in the activities; those fractions banding at pH 3.5, exhibited as little as 20% of the original activity. In this experiment, the total recovery in terms of A_{260} was 75% of the input. The total recovery in terms of amino acid accepting activity was 50%. The major peak, p*I* 4.0, accounted for approximately 30% of the tRNA and 40% of the amino acid accepting activity.

Since these figures were based on the measured activity of the unfractionated material, it was concluded that the multiple forms of differing activity were not contaminants, but, rather, artefacts generated by the electrofocusing procedure. Several experiments were performed to attempt to account for this phenomenon. The fact that the RNA banded only at specific pH regions in the gel spoke strongly against a nonspecific precipitation or aggregation. When extracted into TKM at 4°, isolates from $tRNA^{Arg}$ migrated in the 4S region on electrophoresis in a 6.5% polyacrylamide gel. Two observations indicated that the fractions were interconvertible. First, the amino acid accepting activity of the fractions of lesser activity could be largely restored on heating to 65° in TKM buffer. The results in Table II demonstrate the substantial recovery of activity of isolates of $tRNA^{fMet}$ after annealing. Secondly, when extracted into TKM buffer and refocused, each of the isolated components redistributed to generate many of the components found in the original fractionation. This same redistribution also occurred when the separated fractions were incorporated into the top of a second gel without prior extraction into TKM (Figure 5).

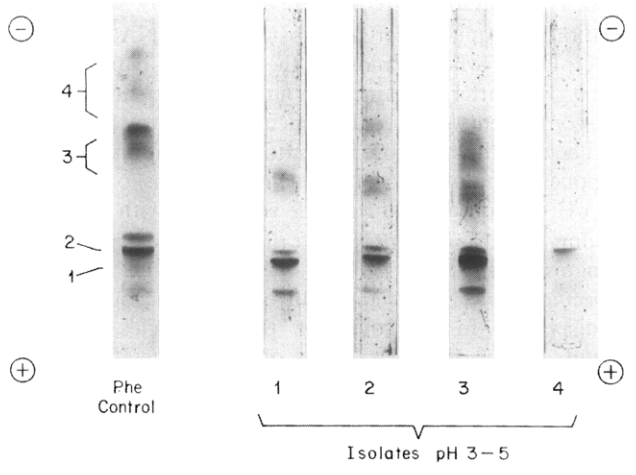

FIGURE 5: Refocusing isolates of $tRNA_2^{Phe}$. $tRNA_2^{Phe}$ (50 μg) was fractionated in the pH range 3–5. The gel was sectioned to isolate the regions indicated. Fractions 1–4 were incorporated directly into the top of new gels, then subjected to electrofocusing once more in the pH range 3–5, 2% ampholyte. The electrolysis period was 18 hr.

Because of the metal-chelating properties of the LKB Ampholines (Haglund, 1971), it seemed possible that the multiple forms of tRNA might arise from the loss, during electrofocusing, of discrete amounts of a dissociable cation such as Mg^{2+}. Such an event might be expected to produce a series of molecules of differing conformation (and p*I*) and differing biological activities (Penniston and Doty, 1963; Fresco *et al.*, 1966; Sueoka *et al.*, 1966; Reeves *et al.*, 1970). This consideration led us to examine the banding pattern of the tRNA as a function of its Mg^{2+} content. For these studies, $tRNA^{fMet}$ was heated to 65° in the presence of EDTA, then dialyzed at 4° for 18 hr against EDTA to replace dissociable

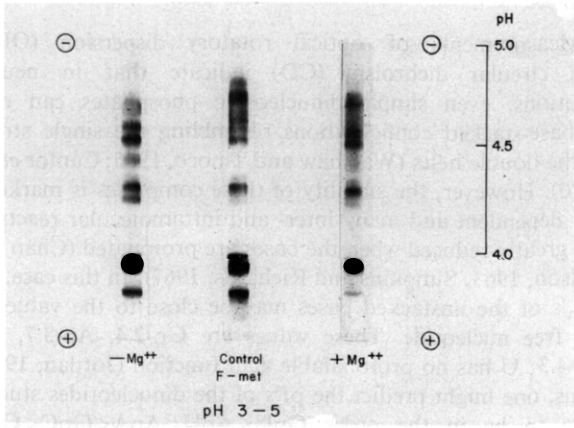

FIGURE 6: Isoelectric pattern of $tRNA^{fMet}$ as a function of its Mg^{2+} content. A solution of $tRNA^{fMet}$ was heated to 65° in 50 mM EDTA at pH 7.2, 20 mM phosphate buffer. One portion was subsequently dialyzed against this solution for 24 hr to remove dissociable Mg^{2+}, then for a further 24 hr against 20 mM sodium phosphate (pH 7.2) at 4° (−Mg^{2+}). Another portion was dialyzed against TKM buffer for 48 hr (control). The remaining portion was dialyzed against the phosphate buffer (pH 7.2), containing 20 mM Mg^{2+} for 24 hr, heated to 65° for 10 min and slowly cooled (+Mg^{2+}).

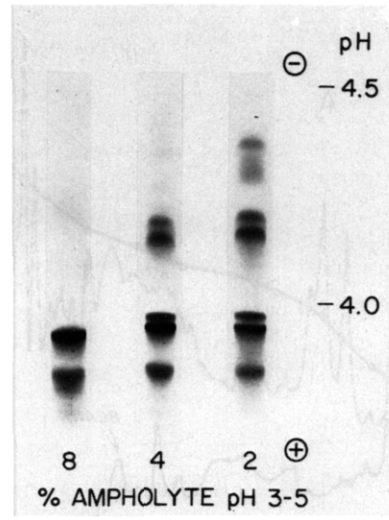

FIGURE 7: Banding pattern of $tRNA_2^{Phe}$ as a function of gel ampholyte concentration. $tRNA_2^{Phe}$ (25 μg) was subjected to electrofocusing in 5% polyacrylamide gels containing 2, 4, or 8% ampholyte, pH 3–5. All samples were applied in a solution of the same ampholyte concentration as that in the gel. In this experiment, electrofocusing was conducted for a period of 18 hr to ensure equilibrium focusing.

cations with Na^+ (Lindahl *et al.*, 1966). Another portion was dialyzed at 4° for the same period against a solution containing 10^{-2} M Mg^{2+}, then heated at 65° for 10 min to insure maximum saturation of the tRNA with Mg^{2+}. Both preparations were subjected to gel electrofocusing in the pH range 3–5. The results (Figure 6) show that neither treatment appreciably altered the resulting pattern.

The known susceptibility of polynucleotide complexes to change in ionic strength, led us to assess the importance of this factor on the banding patterns. Because equilibrium electrofocusing cannot be achieved in the presence of salts, we could only regulate the ionic strength in the gel through the ampholyte level. The banding pattern of $tRNA_2^{Phe}$ over a fourfold range of gel ampholyte concentration is shown in Figure 7. The results demonstrate a profound effect of ampholyte concentration on the number and relative distribution of the bands in the gel. At an ampholyte level of 2%, at least six components were detectable. About half of the RNA banded above pH 4.0. With increasing ampholyte levels, the number of components decreased as the pattern shifted to the more acidic forms. This shift in banding pattern was accompanied by a redistribution of the RNA so that the progressive loss of RNA above pH 4.0 was balanced by a corresponding increase in the amount of RNA below pH 4.0. At ampholyte levels of 8%, all of the RNA was apparently converted into the two most acidic components banding below pH 4.0. This result suggests that part of the variability in banding patterns noted previously may derive from exposure of the RNA not only to differing pH transitions but also to differing ionic strengths in the gel. Much of this variability can be controlled by standardizing the experimental procedure.

The dependence of the banding pattern on the ionic strength in the gel suggested that other factors affecting helix–coil transitions might be important and led us to investigate the effect of urea, a known denaturant of tRNA, on the banding pattern. For these experiments, $tRNA_2^{Phe}$ was exposed to 6 M urea for 30 min, then fractionated in gels containing 2% ampholyte and 6 M urea. The resulting pattern was similar to that obtained at high ampholyte concentration. In contrast to the control, which showed about six major components with p*I* ranging from 3.5 to 4.5, only two major components, p*I* 3.5 and 3.7, were observed in the urea gels (Figure 8). The relative amounts of these components were again consistent with interconversion of the RNA bands rather than a loss of RNA from the gel. It should be noted that the tRNA studied here contained only 43% $tRNA_2^{Phe}$ by accepting activity. Consequently, it is difficult to determine from these experiments whether the two components in the urea gel represent two classes of the same molecules in different stages of denaturation or, perhaps, two different species of tRNA at the same stage of denaturation.

Discussion

This paper describes a rather unexpected fractionation of some nucleic acids on gel electrofocusing—unexpected, because nucleic acids might be considered too acidic to have isoelectric points above pH 3, the lowest practical limits for maintaining their chemical stability and for electrofocusing with presently available ampholytes. Our results with oligonucleotides and with different species of tRNA, indicate, nevertheless, that many polynucleotides give unique and highly reproducible banding patterns in the pH 3–5 range. However, the basis of the banding patterns of some of these substances is rather puzzling and not all may represent a genuine isoelectric fractionation.

The behavior of dinucleoside phosphates and trinucleoside diphosphates seemed entirely consistent with an isoelectric fractionation arising from protonation of the bases. Only one major peak was obtained from each of the dinucleoside phosphates studied here. All were apparently separable from one another when run in the same gel with no apparent intermolecular complex formation.

Presumably, a mixture of molecules exists in thermodynamic equilibrium at the isoelectric point (Simpkins and Richards, 1967). For example, in the case of CpC, the forms H+CpC, CpCH+, H+CpCH+, and CpC, may be expected. Although most nucleotides studied here focused essentially as single peaks, a suggestion of heterogeneity was occasionally evident within some peaks. This heterogeneity was difficult to quantitate because of rapid diffusion within the gel after electro-

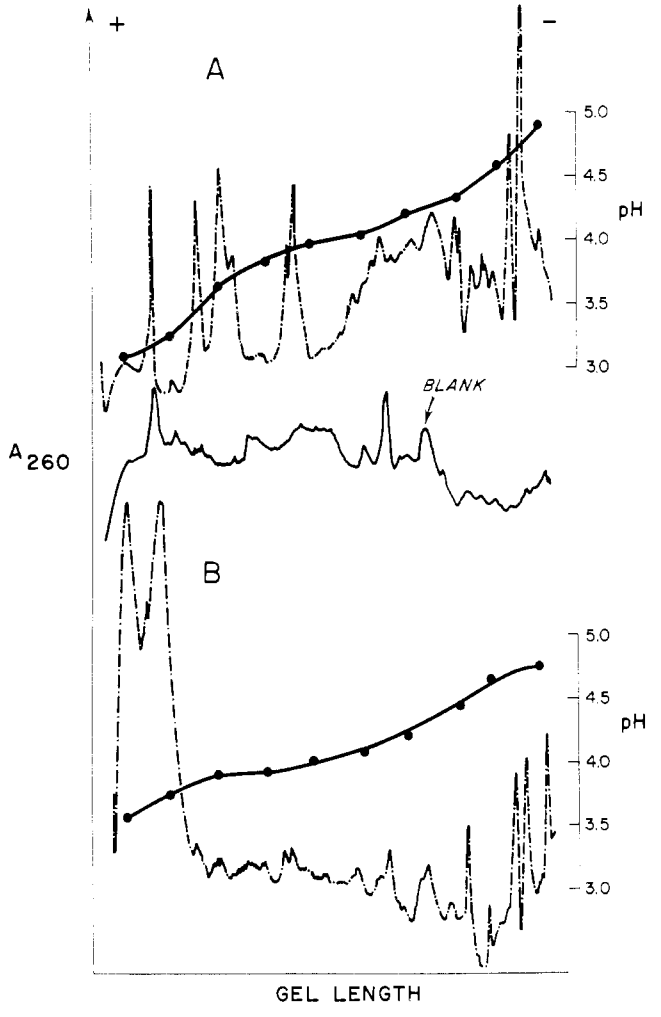

FIGURE 8: Effect of urea on banding pattern of $tRNA_2^{Phe}$. $tRNA_2^{Ph}$ (25 μg) was fractionated in gels containing 2% ampholyte, with or without 6 M urea. After 6-hr electrolysis, the gels were scanned at 260 nm. (A) Control, no urea. (B) Urea gel.

focusing. If this heterogeneity does exist, it might be studied by scanning the gels *in situ* during electrofocusing (Catsimpoolas and Wang, 1971).

In considering the *pI*'s of oligonucleotides, it should be noted that the apparent pK_a's of the bases depend on a variety of factors, including the position of the phosphates (3′ or 5′), base sequence, base stacking, hydrogen bonding between base pairs, chain length, and helical content. Such inter- and intramolecular events are also dependent on the concentration of reactants, the temperature and ionic strength of the solution and the hydrophobicity of the environment. In polynucleotides, some of these factors can alter the apparent pK_a of bases by at least 1.5 pH units (Michelson, 1963; Hartman and Rich, 1965). In general, the apparent pK_a's of bases in helical regions decrease with increasing inter- and intramolecular interactions and with increasing temperature and ionic strength. However, when protonation is involved in the development of an ordered structure, *e.g.*, poly(rA), the converse applies. Thus, the pK_a of the base in the ordered structure increases with chain length and the structure is destabilized by high ionic strength. Further details on this subject can be found in reviews by Felsenfeld and Miles (1967), Michelson *et al.* (1967), and Voet and Rich (1970).

Measurements of optical rotatory dispersion (ORD) and circular dichroism (CD) indicate that in neutral solutions, even simple dinucleoside phosphates can exist in base-stacked configurations resembling the single strand of the double helix (Warshaw and Tinoco, 1965; Cantor *et al.*, 1970). However, the stability of these complexes is markedly pH dependent and many inter- and intramolecular reactions are greatly reduced when the bases are protonated (Chan and Nelson, 1963; Simpkins and Richards, 1967). In this case, the pK_a's of the unstacked bases may be close to the values in the free nucleotide. These values are Gp-2.4, Ap-3.7, and Cp-4.3; U has no protonatable acid function (Jordan, 1960). Thus, one might predict the p*I*'s of the dinucleotides studied here to be in the order GpU<ApU<ApA<GpC<CpC, which, interestingly, was the observed result. In most instances, the measured p*I* was close to that expected from the pK_a of the bases if the dinucleotides were behaving as random coils. For example, CpC was isoelectric near pH 4.3, the pK_a of Cp. GpC banded near pH 3.9, 0.4 pH unit lower than the pK_a of C (G presumably not contributing to the positive charge at this pH). The p*I* of ApA (3.8) was also close to that expected from the pK_a of A (3.7), assuming that the isoelectric form represents half-protonation of both A's. The apparent agreements with this rationalization may, however, be entirely fortuitous since the validity of extrapolating from our experiments to others with more standardized conditions is questionable.

tRNA. The basis of the fractionation of different tRNA species is rather puzzling and is certainly not immediately explicable in terms of differences in primary structure. Each of several different isoaccepting species of tRNA gave rather similar banding patterns with many more fractions than could reasonably be expected from their known purity. Measurements of the amino acid accepting activity of isolates of $tRNA^{iMet}$ indicated that the heterogeneity represented discrete stages in a graded, but reversible, denaturation or inactivation of the tRNA during the electrofocusing procedure. Because gel electrofocusing is an equilibrium method, it seems likely that each band represents an essentially homogeneous population of molecules frozen into the same conformation. Thus the differences in their amino acid accepting activity may reflect differences in their rate of renaturation rather than differences in the proportion of chargeable molecules in any one fraction.

Three major problems in interpretation now arise. Why does tRNA reach apparent isoelectric states between pH 3 and 5? How do the multiple forms arise? Why do they differ in biological activity? In seeking an explanation, the following observations should be considered: (1) tRNA banded only in the pH 3–5 range, irrespective of the pH range developed in the gel; (2) the number and relative distribution of components depended in part on the mode of sample application; (3) the more acidic components predominated when the tRNA was focused in gels containing urea or high ampholyte levels; (4) the biological activites of the isolated fractions decreased progressively with their apparent p*I*'s; (5) the fractions were reactivated by annealing in TKM buffer at 65° but not on prolonged dialysis against this buffer in the cold; (6) isolated fractions redistributed when refocused with or without prior extraction from the gel segments.

The explanation of the apparently anomalous p*I* range is perhaps the key to our understanding of the other phenomena. Since tRNA contains more acidic groups than basic groups, one might expect it to reach an isoelectric state only when the excess acidic phosphates are protonated or are

otherwise neutralized. It is therefore difficult to understand why the tRNA banded near pH 4 in our experiments. One possibility is that the excess phosphates are balanced by tightly bound organic or inorganic cations. However, the possible presence of organic cations such as spermine may be discounted in our samples (A. D. Kelmers, personal communication) and the experiments with EDTA suggest that bound metals are unlikely to account for the banding pattern. Moreover, if the multiple forms represent the removal of discrete amounts of a dissociable cation during electrofocusing, only the same or more acidic components would be expected from any one fraction on refocusing (unless, of course, this factor were replaced from the gel constituents—a possibility we cannot exclude). A more disturbing possibility is that the banding pattern arises from an interaction with ampholytes in the pH gradient. Although the exact chemical composition of the LKB Ampholines has not yet been reported, it is likely that they contain polyamine functions that might bind tRNA as do other polyamines (Hirschman *et al.*, 1967; Lat and Sober, 1967). If so, it is necessary to account for the pH dependence of the banding patterns. Perhaps the most obvious explanation is that the anomalous p*I* range and multiple forms of the tRNA are due to direct binding to specific ampholytes in the pH 3–5 range. This explanation seems consistent with many of our findings, *e.g.*, the dependence of ampholyte level, the effect of urea, and perhaps the progressive, but reversible, inactivation of the tRNA. However, if the interaction involves polyamine functions, one might expect the tRNA to bind more readily to ampholytes in higher pH regions that presumably contain proportionately more polyamine functions. Alternatively, binding may not be specific for any class of ampholytes but may be a consequence of the protonation of the bases. In this case, the banding pattern might represent an isoelectric fractionation of a tRNA–ampholyte complex. Such a model would also accommodate many of our findings.

One final possibility is that the banding pattern represents a genuine isoelectric fractionation of tRNA at the low ionic strengths and temperatures prevailing under our particular conditions of electrofocusing. The multiple forms might then arise from conformational transitions in the tRNA as the bases are protonated. Such conformational transitions would presumably be sensitive to changes in both the ionic strength and pH in the gel during electrofocusing—hence, the observed dependence on the ionic strength (ampholyte levels) and the importance of the mode of sample application. Given this explanation, the banding pattern in urea would be consistent with the activities of the isolated fractions. Our results indicated that the most acidic fractions had the lowest specific activity and were therefore likely to be most denatured (Figure 4). This finding agrees with observations that the forms denatured in urea were the most acidic components (Figure 8). Unfortunately, although this explanation also accommodates most of our experimental results, it is inconsistent with the widely held view that native tRNA is more acidic than acid-denatured tRNA. This dilemma might be resolved by analysis of the isolates by ORD or CD. Experiments along these lines are in hand.

Since all tRNA molecules have common structural features, it is tempting to speculate that the qualitative similarities in the banding pattern of different species represent conformational transitions common to all tRNA molecules. In this case, the quantitative differences in relative amounts may reflect different susceptibilities of the common structures to denaturation. For example, $tRNA^{fMet}$ had proportionately more of the fractions near pH 4.5 and fewer of the less active fractions below pH 4 than either $tRNA^{Arg}$ or $tRNA_2^{Phe}$. Consequently, it might be concluded that $tRNA^{fMet}$ had a more ordered structure and was less susceptible to denaturation than the other tRNA species. This interpretation is consonant with the conclusions of Egan *et al.* (1970), who inferred a more compact structure for $tRNA^{fMet}$ than for $tRNA_2^{Phe}$ from gel filtration studies.

Stable conformers in tRNA have been demonstrated by a variety of other procedures (see review by Cramer, 1971). For example, conformational transitions have been observed among several species of yeast tRNA from their melting profiles (Sarin *et al.*, 1966). Interconvertible forms, dependent on Mg^{2+}, have been found (Sueoka *et al.*, 1966; Lindahl *et al.*, 1966). Five transitional states in the melting of tRNA from yeast, markedly dependent on ionic strength have been described by Römer *et al.* (1970). It would be of interest to know what relationship, if any, exists between the multiple forms observed on electrofocusing and the conformers observed by these and other methods.

In conclusion, our results indicate that many polynucleotides will be amenable to isoelectric fractionations with present techniques of electrofocusing. Because nucleic acids tend to be rather insoluble in acidic solutions, it seems advisable to conduct the separations in some support medium such as the gels used in the present studies. All nucleic acids that we have examined so far have been isoelectric below pH 5. Because the effective separating pH range is much narrower than that for proteins, isoelectric focusing may not be as useful for resolving complex mixtures of polynucleotides as it has been for proteins. The basis for fractionating polynucleotides by isoelectric focusing is also likely to prove more complex than that for proteins. Our results with tRNA indicate that secondary and tertiary structures are more important determinants of the banding pattern than is primary structure. However, many polynucleotides appear to be readily denatured by the prevailing conditions of pH and ionic strength at which they are isoelectric. As a result, multiple forms of differing conformation and p*I* may be produced from a homogeneous molecule.

When applied to protein separations, gel electrofocusing has often indicated unexpected heterogeneity in apparently homogeneous proteins. Although methodological artefacts have occurred, most instances of heterogeneity have been attributable to structural differences in the protein before electrofocusing (Righetti and Drysdale, 1972). Some differences may however be rather subtle and have as yet, no apparent biological significance. In the case of nucleic acids, the possibility of artefact cannot be dismissed. Even if ampholyte binding can be discounted, the formation of multiple forms by conformational changes during electrofocusing is likely to pose problems in interpretation—especially since few nucleic acids can be as readily characterized as tRNA. Thus, although isoelectric focusing may yet prove a useful adjunct to existing procedures for nucleic acid separations, the technique may find more favorable application in studies of structural transitions or intermolecular associations in well characterized molecules.

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