CHROM. 3802

SEPARATION OF OLIGONUCLEOTIDES DERIVED FROM THE PHENYLALANINE TRANSFER RIBONUCLEIC ACID OF *ESCHERICHIA COLI* B BY THIN-LAYER TECHNIQUES*

HANS GÜNTER GASSEN**
Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn. 37830 (U.S.A.)
(Received September 20th, 1968)

SUMMARY

The practical use of thin-layer chromatography and electrophoresis in the separation and identification of oligonucleotides derived from nuclease hydrolyses of a single transfer ribonucleic acid ($tRNA^{Phe}$ from *Escherichia coli* B) has been explored, with particular attention to limitations of oligonucleotide size and to subsequent recovery and degradation analysis at the 1–10 nanomole level. In this range, where thin-layer methods are most efficient, satisfactory recoveries from the adsorbent are limited to penta- and smaller oligonucleotides, and the subsequent analyses require methods of corresponding sensitivity. The latter need is satisfied by the "nucleoside analyzer" of UZIEL, KOH AND COHN. The most efficient use of thin-layer methods appears to be separation, in one dimension, of mixed peaks derived from a prior column separation of the original enzymic digest of the tRNA in question.

INTRODUCTION

When thin-layer chromatography (TLC) and thin-layer electrophoresis (TLE) were introduced several years ago[1], their convenience and increased sensitivity (10–20 fold), compared with paper chromatography, seemed to make them promising tools for nucleic acid sequence analysis, where many oligonucleotides must be separated from each other. However, in spite of common use in other fields, applications to the nucleic acids have been very limited[2,3]. This may be because thin layers have a limited capacity; overloading the plates causes band spreading and poor separation. On the other hand, the small amounts that can be handled, *i.e.*, 5–20 nmoles of purine or pyrimidine, are difficult to analyze quantitatively with the means available. Separation on thin layers seems to be limited to oligonucleotides smaller than hexanucleotides[2,4]. Larger oligonucleotides are not resolved, and recovery drops as the chain length increases.

However, it will be of interest in the future to compare RNA's of identical

* This research was jointly sponsored by the Max Kade Foundation and by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.
** Present address: Max-Planck Institut für Experimentelle Medizin, 34 Göttingen (Germany).

biological function but from different organisms, from which only small amounts of material may be obtained; so micromethods for RNA sequence determination may be required. Since the nucleoside analyzer developed in this laboratory enables one to analyze as little as 1–10 nmoles of oligonucleotide[5], the combination of both tools is advantageous for the identification of oligonucleotides at the thin-layer level of resolution. As far as we know, only enzymic digests of crude (mixed) tRNA's have been used, and the methods of identification have not matched the thin-layer sensitivity[2]. In order to assess the possibilities of this method, we used a ribonuclease hydrolysate of $tRNA^{Phe}$ (*E. coli* B), the oligonucleotide composition of which had already been determined[6]. Furthermore, the nucleoside analyzer gives us reliable results in the range of 1–5 nmoles of tRNA, the optimal amount to separate on two-dimensional TLC. If one separates 3 ODU* (~ 5 nmoles) of tRNA on a thin-layer plate, for example, one recovers a maximum of 0.1 ODU (about 5 nmoles) of a given dinucleotide. Usually about 10–20% of the OD is not nucleotide material, which will obscure the result if the identification is made by spectrum.

Thin-layer electrophoresis is routinely used in this laboratory for the separation of oligonucleotide mixtures obtained from column separations. Whereas we have found the mapping of oligonucleotides by two-dimensional thin-layer methods to be of limited value, the column–thin-layer method has been very useful in the separation and identification of small amounts of oligonucleotides. The separation and identification of a trinucleotide mixture obtained from a ribonuclease hydrolysis of $tRNA^{Phe}$ of *E. coli* B by TLE is discussed in this paper.

MATERIALS

The tRNA used had a "purity" of 65% (the ratio of amino acid acceptance to adenosine end groups) and was given to us by A.D.Kelmers of the Oak Ridge National Laboratory. Pancreatic ribonuclease A (EC 2.7.7.16), alkaline phosphatase (EC 3.1.3.1), and snake venom diesterase (EC 3.1.4.1) were purchased from Worthington Biochemical Corp. T_1-ribonuclease (EC 2.7.7.26) was obtained from Sankyo Chem. Co., Tokyo. All enzymes were used without further purification.

Two brands of precoated thin-layer foils were used:

(1) Eastman Chromagram No. 6064 cellulose without fluorescent indicator, 20 × 20 cm, was obtained from Distillation Products Inc., Rochester, N.Y.

(2) MN Polygram 300 cellulose without binder and U.V. indicator, 20 × 20 cm, was obtained from Brinkman Instr., Westbury, N.Y.

DEAE-cellulose (DE 32 Whatman) was used for column chromatography and Sephadex A-25 coarse (Pharmacia) for the desalting of the oligonucleotides[7]. All other chemicals were of the finest grade available.

METHODS

(1) Thin-layer electrophoresis apparatus

Thin-layer electrophoresis was performed in a locally constructed MARKHAM–

* Nonstandard abbreviation: ODU (optical density unit), the quantity of material in 1 ml of a solution giving an optical density of 1.0 with a light path of 1 cm. All other abbreviations conform with IUPAC-IUB Tentative Rules (*e.g.*, *Biochim. Biophys. Acta*, 108 (1965) 1) and supplements thereto.

SMITH[8] apparatus adapted for precoated plastic foils (Fig. 1). The coated side of the thin-layer sheet is held against the paper wick by its own tension. To assure even better contact, a flexible sheet of Teflon can be used to increase the pressure. Varsol —a high-boiling gasoline—was used as a coolant. It was stirred with a Teflon-coated stirring bar to maintain an even temperature throughout the electrophoresis vessel. The paper wicks (Whatman No. 3 MM or Schleicher and Schüll 2043B) were changed after every run.

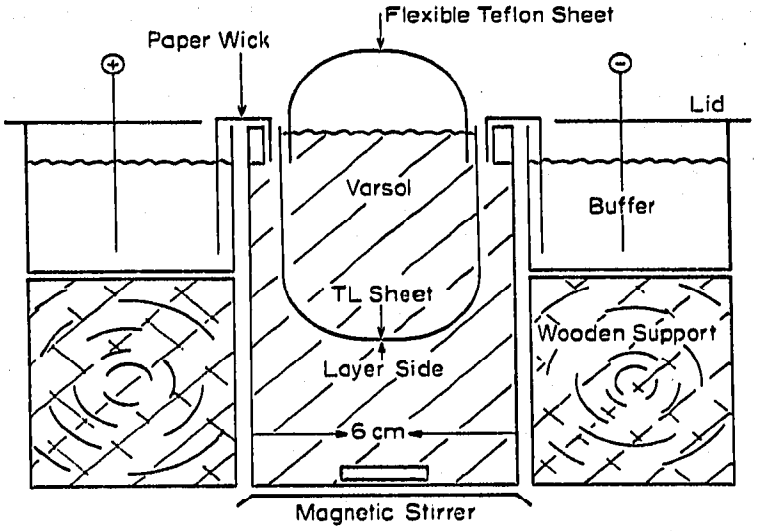


Fig. 1. Schematic drawing of electrophoretic apparatus. For explanation, see Methods.

(2) Application of the sample

The sample, about 5–10 μl per cm, was applied as a streak to the dry plate about 2 cm from one edge. The edge furthest from the sample was immersed in the buffer up to about 2 cm above the application line; when the solvent front almost reached the application line, the excess buffer was blotted with a filter paper. The other edge of the thin layer was then treated the same way. The two liquid fronts were allowed to flow together by capillary action, leaving the sample as a thin streak on the plate. It was then immersed in the buffer vessel in such a way that the wick and the layer overlapped about 1 cm at each end. Separation usually required about 35 min at 1300 V and 20–50 mA. After the separation, the plate was dried in a stream of warm air. The dry plate was immersed in a beaker of absolute alcohol and stirred for about 15 min; this procedure removed most of the $HCOONH_4$. Then it was dried again and the substances were located with the aid of an ultraviolet (U.V.) lamp.

(3) Elution of the samples from the plate

To obtain the sample in a small volume for final analysis, a special technique for the elution of the spots was developed (Fig. 2). The tip of a 0.4-ml plastic test tube (Beckman Cat. No. 314326) was cut off to leave a small hole in the bottom, and a small wad of cotton was pressed into the tube to act as a filter. The tube was then connected to a vacuum line, and the cellulose, which had been loosened with a spatula, was sucked into the tube. The tube containing the cellulose was placed on top of another 0.4-ml test tube, and 100 μl of water were placed in the top tube. The

whole assembly was shaken on a Vortex mixer to obtain a good suspension. The extraction was repeated with an additional 100 μl water, so that the bottom tube contained the eluted spot in 200 μl of water. It was concentrated to dryness under a stream of nitrogen[6].

(4) Analysis of the sample

All oligonucleotides and nucleotides were hydrolyzed to the nucleoside level and analyzed with the nucleoside analyzer of UZIEL, KOH AND COHN[5]. In a typical sample, 5 μl 1 *N* NaOH was added to 1–5 nmoles of oligonucleotide in 10 μl H_2O. The solution was incubated for 45 min at 80°, neutralized with 5 μl 1 *N* HCl, buffered with 5 μl 1 *N* NH_4Ac, pH 8.8, and incubated with 5 μl alkaline phosphatase (1 mg/ml) at 37° for 3 h. Alkali-sensitive nucleotides were hydrolyzed with snake venom phosphodiesterase (5 μl, 5 mg/ml) and phosphatase at 45° for 4 h at pH 8.8.

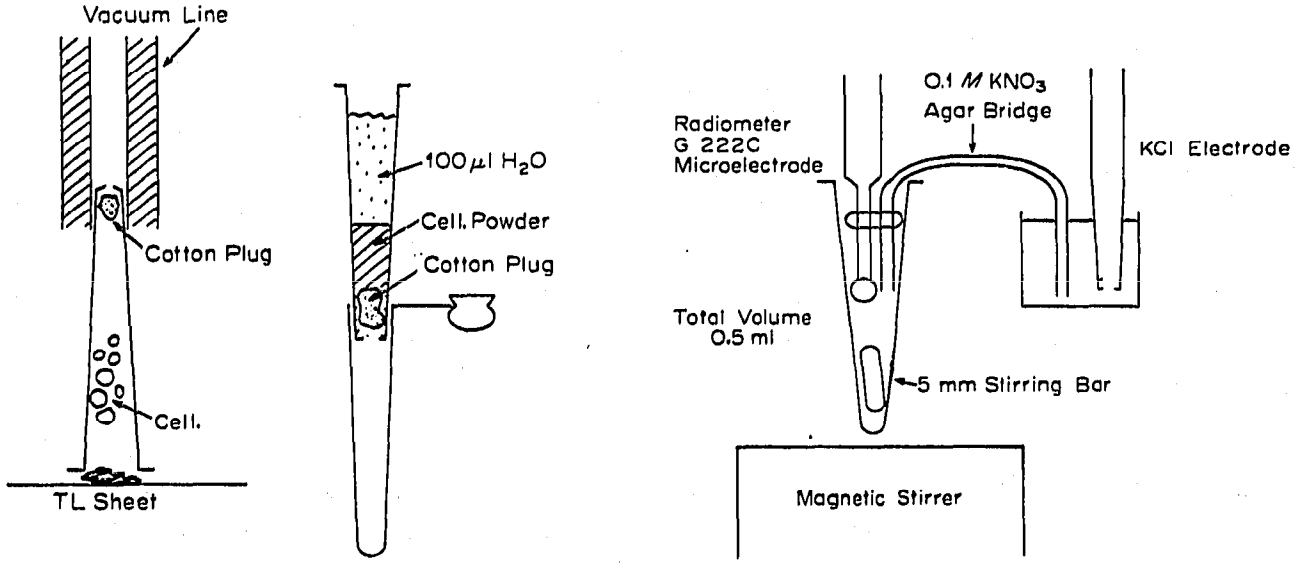


Fig. 2. Apparatus for elution of oligonucleotides from cellulose. The procedure is described in Methods.

Fig. 3. Apparatus for enzymic digestion of small amounts of tRNA's. With the technique shown, a salt-free digest is obtained.

*(5) Preparation of a ribonuclease digest**

In order to obtain samples free of salt, hydrolyses were performed in a micro-apparatus using a Radiometer pH-stat (Fig. 3). Four nmoles tRNA (2.6 nmoles $tRNA^{Phe}$) were dissolved in a 2-ml centrifuge tube in 0.5 ml H_2O. A small magnetic stirring bar was placed in the tip of the tube. The KCl electrode was replaced by a bridge consisting of a piece of plastic tubing, 0.1 cm diameter, which was filled with 3% agar in 0.1 *M* KNO_3. One end of the tubing was attached directly to a Radiometer microelectrode No. G 222C with the aid of a rubber band. The pH of the stirred solution was adjusted to pH 7.2. After the addition of 10 μl of ribonuclease (3 mg/ml), the pH was held constant for 6 h by the addition of 0.01 *M* NaOH. The solution was

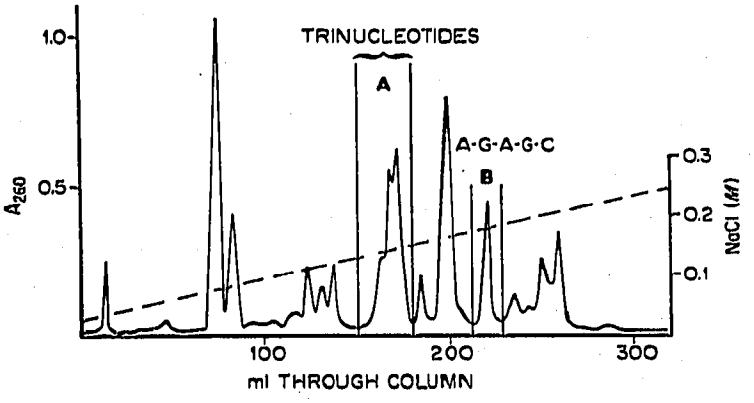
* The enzymatic hydrolysis of $tRNA^{Phe}$ on a larger scale and the separation of oligonucleotides by DEAE-cellulose column chromatography will be published in detail elsewhere[6].

then dried in a stream of air, dissolved in 20 μl H_2O and applied to the thin-layer plate.

Electrophoresis was carried out in 0.1 *M* $HCOONH_4$ adjusted to pH 2.5 (ref. 2). For chromatography, the solvent system of RUSHIZKY AND KNIGHT[9] was used. Otherwise the plates were treated as described in the section (2).

(6) Separation of oligonucleotide mixtures obtained from column separations

Twenty nanomoles tRNA (13 nmoles $tRNA^{Phe}$) were hydrolyzed with ribonuclease A and separated by DEAE column chromatography in 7 *M* urea (Fig. 4). Peak A, containing a mixture of trinucleotides, was desalted, concentrated to dryness, dissolved in 30 μl H_2O and applied as a 5-cm streak to a thin-layer plate. Conditions for the electrophoresis were as described in the preceding section. Three major and four minor bands were detected (Fig. 5). One-fifth of the material isolated from each of the major bands was used for the analysis of the nucleoside composition; another fifth was hydrolyzed by T_1 ribonuclease and separated by TLE, isolated and analyzed. Peak B (Fig. 4), containing the pentanucleotide A-G-A-G-Cp, was desalted and used for methodological studies.



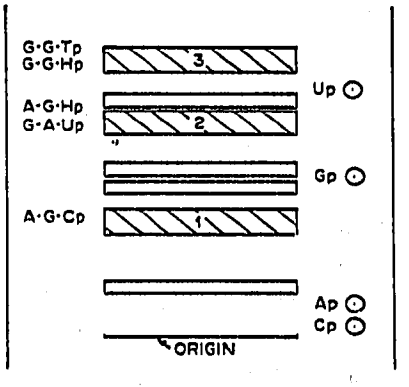


Fig. 4. DEAE-cellulose chromatography of a pancreatic ribonuclease digest of 20 nmoles $tRNA^{Phe}$ (*E. coli* B) (65%). Column: 100 × 0.5 cm. Gradient: 200 ml 7 *M* urea, 0.02 *M* Tris (pH 7.8); 200 ml 7 *M* urea, 0.02 *M* Tris, 0.3 *M* NaCl (pH 7.8). Flow rate: 0.2 ml/min. Fractions A and B were further analyzed (Fig. 5).

Fig. 5. Separation of trinucleotides (fraction A of Fig. 4) by thin-layer electrophoresis in 0.1 *M* $HCOONH_4$ (pH 2.5) at 1300 V and 30 mA. Separation time: 30 min. Sheet: 10 × 20 cm (Eastman Chromagram). Uridylic acid moved 7.8 cm. The composition of each band is given in Table III.

RESULTS

Recovery from plates

Since it was found that the recoveries from the layer dropped from 95% for mononucleotides to about 50% for a pentanucleotide, we tried to determine whether this was due to band spreading or to incomplete elution from the cellulose. As a test sample, the pentanucleotide A-G-A-G-Cp (part of this material came from peak B, Fig. 4, and part from previous hydrolyses) was used. Considering the first possibility, 16 nmoles of the pentanucleotide were applied as a 2-cm streak. After electrophoresis, the U.V.-detectable band (about 6 mm wide), a 4-mm section in front of it and a 6-mm section behind, were divided into 2-mm sections and eluted separately. All of the

remaining area, including the application line, was eluted together and analyzed (Table I). The U.V.-detectable band had a very sharp leading edge, and no oligonucleotide material could be discovered in front of it. However, there seemed to be a considerable tailing at the rear, which contained about 20% of the material.

TABLE I

SEPARATION OF THE PENTANUCLEOTIDE A-G-A-G-Cp BY THIN-LAYER ELECTROPHORESIS

The first 3 cm, including the application line, were eluted and analyzed, then a series of 2-mm strips was eluted and analyzed. The three entries between 4.0–4.2 and 3.6–3.8 indicate the U.V.-detectable band, which contains 50% of the material applied. In these experiments both layers, Eastman Chromagram and MN polygram were tested and gave nearly identical results.

Distance from origin (cm)	*A-G-A-G-C (nmoles)*	*Recovery (%)*	
4.4–4.6	0	—	
4.2–4.4	0	—	
4.0–4.2	2.8	18	
3.8–4.0	3.8	23	50%
3.6–3.8	1.4	9	
3.4–3.6	0.8	5	
3.2–3.4	0.6	4	13%
3.0–3.2	0.7	4	
0 –3.0	1.0	6	

For the second possibility, three samples (8 nmoles each) of the same pentanucleotide were applied to a thin-layer sheet. The first was eluted with 100-μl aliquots of H_2O, the second with 0.02 *M* NaCl, and the third with 0.5 *M* NH_4OH. Of the total material eluted, 90–95% was in the first 100 μl of solvent, and there was practically no difference among the three solvents chosen. However, the recovery in the case of this particular pentanucleotide was only 55–60%, and further washing of the cellulose did not improve the recovery. This experiment demonstrates the loss of material when oligonucleotides of higher chain length are applied to thin-layer plates.

Separation and identification of the trinucleotides

In order to determine the usefulness of TLE as a separation method for oligonucleotide mixtures, the trinucleotides from peak A (Fig. 4) were further separated by TLE. From other experiments (to be published elsewhere), it was found that this peak contained the trinucleotides A-G-Cp, A-G-Hp, G-A-Up, G-G-Tp and G-G-Hp. Table II shows the analysis of the major bands from the electrophoresis shown in Fig. 5. Bands 2 and 3 still contained two components each, which did not separate. Aliquots of the material from bands 1, 2 and 3 were hydrolyzed with T_1 ribonuclease, and again separated and analyzed. The data are shown in Table III. From the analysis and the R_E values, band 1 of Fig. 5 contains the trinucleotide A-G-Cp because T_1 hydrolysis yields A-Gp and free cytidylic acid. Band 2 (Fig. 5) yields A-Gp, A-Up and guanylic acid in equimolar proportions after T_1 hydrolysis; this is consistent with prior findings, and it can be concluded that this band contains A-G-Hp and G-A-Up, which do not separate under the conditions used. Finally, band 3 contains a mixture of G-G-T and G-G-H. In the separation of the trinucleotides, the recovery of the

TABLE II

NUCLEOSIDE COMPOSITION OF TRINUCLEOTIDES SEPARATED BY THIN-LAYER ELECTROPHORESIS

The trinucleotides were obtained by ribonuclease digestion of $tRNA^{Phe}$ and separated by DEAE column chromatography (Fig. 4) followed by thin-layer electrophoresis (Fig. 5). One-fifth of the material was hydrolyzed with snake venom phosphodiesterase and alkaline phosphatase. The numbers refer to one-fifth of the material.

Band No.	R_E[a]	*Nucleoside (nmoles)*					*Recovery (%)*
		Urd	*Guo*	*Ado*	*Cyd*	*Thd*	
1	44		1.9	1.9	1.9		74
2	87	2.0	3.9	3.9			76
3	112		8.0			1.0	71

[a] R_E = Distance moved given as percent of distance moved by uridylic acid.

TABLE III

IDENTIFICATION OF THE TRINUCLEOTIDES IN BANDS 1, 2 AND 3 (Fig. 5)

An aliquot of each band was hydrolyzed by T_1 ribonuclease and separated by electrophoresis as in Fig. 5. Layer: Eastman Chromagram. Each spot was then hydrolyzed to the nucleoside level and analyzed.

Band No. (Fig. 5)[a]	*No. of spots*	R_E[b]	*Nucleoside (nmoles)*					*Composition*	*Original trinucleotide*[c]	*Recovery (%)*
			Urd	*Guo*	*Ado*	*Cyd*	*Thd*			
1	2	0				1.4		Cp	A-G-Cp	52
		50		1.8	1.7			A-Gp		64
2	3	50		1.6	1.6			A-Gp	A-G-Hp	59
		65		1.2				Gp		44
		70	1.8		1.6			A-Up	G-A-Up	66
3	2	65		7.1				Gp	G-G-Tp	64
		100					1.6	Tp	G-G-Hp	60

[a] Gross analysis given in Table II.
[b] R_E = Distance moved given as percent of distance moved by Up (or by Tp).
[c] In Fig. 5, and therefore in A of Fig. 4.

components is about 70–75%. The recoveries from the T_1 hydrolysis were lower (45–60%), and the analysis showed a greater discrepancy in the nucleoside composition. However, with the amounts being separated and analyzed, *i.e.*, 1–2 nmoles, the somewhat low recoveries may be understood.

Mapping a ribonuclease digest of $tRNA^{Phe}$

Fig. 6 shows the two-dimensional separation of the ribonuclease digest of 4 nmoles tRNA (2.6 nmoles $tRNA^{Phe}$). Sixteen spots were detected, and each spot was analyzed. The data, including the R_E and R_F values, are shown in Table IV. The R_E values were quite consistent, whereas the R_F values were somewhat variable.

Table V shows the ribonuclease-produced oligonucleotides up to the pentanucleotides that have been proven to be constituents of $tRNA^{Phe}$ from *E. coli* B[6]. As can be seen (compare Tables IV and V), the method gives fairly good results up to the pentanucleotides. Although all longer oligonucleotides were not separated,

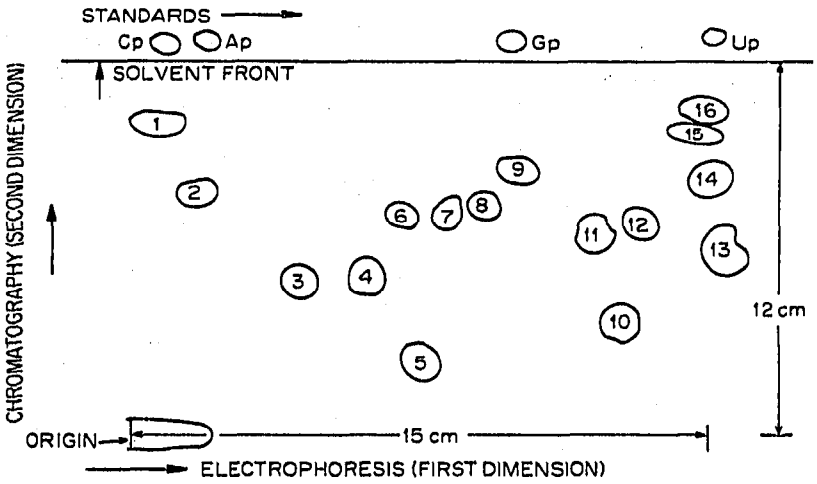


Fig. 6. Two-dimensional separation of a ribonuclease digestion of 4 nmoles tRNA (2.6 nmoles $tRNA^{Phe}$ from *E. coli* B). First dimension, TLE: 0.1 *M* $HCOONH_4$ (pH 2.5)at 1300 V and 50 mA; 50 min. Second dimension, TLC: *tert.*-butanol–0.5 *M* $HCOONH_4$ (pH 3.8)–isoamylalcohol (49:50:1); 8 h. Sheet: 20 × 16 cm, MN polygram. The nucleoside composition of the spots is given in Table IV.

TABLE IV

ANALYTICAL DATA FROM THE THIN-LAYER CHROMATOGRAPHY AND ELECTROPHORESIS ("MAPPING") OF 4 NANOMOLES OF tRNA (2.6 NANOMOLES $tRNA^{Phe}$)

The recoveries were calculated from the known composition of $tRNA^{Phe}$. Every spot from the thin-layer plate (Fig. 6) was eluted, and the total sample was analyzed.

Spot	R_F (× 100)	R_E[a] (× 100)	*Nucleoside (in nmoles)*					*Composition*	*Recovery* (%)
			Urd	*Guo*	*Ado*	*Cyd*	*Other*		
1	82	0				36.5		Cp	93
2	62	1			2.8	2.7		A-Cp	104
3	38	25				1.3	m^7Guo 1.0	m^7G-Cp	44
4	39	36		1.7	2.0	1.9		A-G-Cp	73
5	14	46		1.8	1.7	1.0		A-G-A-G-Cp	40
6	55	44		2.0		2.2		G-Cp	77
7	56	52		0.7	1.8			A-G?	
8	58	60	0.4			0.4		?	
9	68	65	0.6		0.8			A-Up	30
10	25	82	3.0	5.1	3.2			$(A\text{-}G_2)$Up	58
11	50	79	1.8	3.7	3.8			A-G-Hp G-A-Up	69
12	53	88		2.0		1.8		pG-Cp	73
13	44	102		7.2			2.0 Thd	G-G-Tp G-G-Hp	77
14	66	100	2.8	2.7				G-Up	106
15	78	98					Ψrd 1.2	Ψp	46
16	86	100	13.0					Up	50

[a] R_E = distance moved relative to Up (= 100).

TABLE V

OLIGONUCLEOTIDES UP TO THE PENTANUCLEOTIDES DERIVED FROM A RIBONUCLEASE DIGEST OF $tRNA^{Phe}$ (*E. coli* B)

Cp	A-Cp	A-G-Cp	G-A-G-Up
Up	G-Cp	A-G-Hp	A-G-G-Up
Ψp	G-Up	G-A-Up	G-G-G-Cp
	m^7G-Cp	G-G-Hp	A-G-A-G-Cp
	pG-Cp	G-G-Tp	

spots from other chromatograms, where electrophoretic movement of the hexa- and octanucleotide did occur, gave very poor analytical results that were of no help in the identification. Since the recoveries were calculated according to the $tRNA^{Phe}$ content of the tRNA used (65%), the high recoveries in the dinucleotide range can be understood. A-Up, for example, which is not found in ribonuclease digests of more pure $tRNA^{Phe}$, appeared in 30% yield and hence may be ascribed to the contaminating RNA's. On the other hand, G-G-G-Cp, which is derived from $tRNA^{Phe}$, could not be found in this map.

DISCUSSION

One of the serious problems attending the analytical use of thin-layer chromatography in nucleotide chemistry is the varying recovery from the plates. Whereas the recovery with mono- and dinucleotides reaches a satisfactory 90%, it drops with trinucleotides to about 70%, and the recovery for a pentanucleotide was usually 50%. The poor recovery is partly due to tailing of longer oligonucleotides. About 10–20% can be found in the tail, which sometimes spreads all the way to the application line. This tailing creates a second problem: it contaminates the slower components. However, with the pentanucleotide examined, the tailing accounted for only 20% of the loss. It is not clear what happened to the other 30% of the material. As pointed out by BIELESKI[10], the separation can be improved by raising the ionic strength of the buffer. This may improve the recoveries, but it considerably raises the current and temperatures that accompany it and so may cause degradation at the pH used.

The procedure of two-dimensional separation of oligonucleotides on paper was developed by RUSHIZKY AND KNIGHT[9]. The technique was adapted to thin-layer chromatography by BERGQUIST[2] because this method promised sharper separation, increased sensitivity and easier handling. In our hands, the method shows a number of limitations that raise questions as to its usefulness for RNA sequence determinations. Oligonucleotides longer than hexanucleotides cannot be separated under the conditions used. The poor recovery of these creates a serious problem, especially when isomeric compounds are found in the same spot. The small amounts that can be handled on thin-layer mapping require special analytical tools and usually allow only one subsequent analysis. Identification of oligonucleotides solely by their positions and intensities on the map may be dangerous, especially in the case of tRNA's, where rare nucleotides occur. However, it may be useful in the chemical modification of tRNA's, for example, where only the appearance or the disappearance of a certain oligonucleotide is to be shown. Otherwise, separation on DEAE-cellulose columns,

although requiring about three times as much material and a more time-consuming workup, gives much more reliable results.

On the other hand, it has been found that thin-layer procedures show a real advantage as a second separation method for column peaks containing small oligonucleotide mixtures. Usually these peaks are separated on a second column at a different pH or temperature. Because only a one-dimensional electrophoretic separation is used, one can apply as much as 10–20 ODU per 20-cm plate, and the pH of the buffer can easily be adjusted for optimal separation. Furthermore, the sample is obtained nearly salt free in a small volume, so that samples are ready for analysis within 2–3 h. If an enzymatic hydrolysate of a specific tRNA is analyzed, often the di-, tri- and tetranucleotides are not completely resolved in the first dimension; this is exactly the size range in which thin-layer electrophoresis can be applied successfully. For the separation of the tetranucleotides, it may be advisable to treat the sample with phosphomonoesterase first, to split off the terminal phosphate group.

The R_E values obtained for specific oligonucleotides may be an additional help in identifying the components. If the size of the oligonucleotides is known (*i.e.*, trinucleotide, tetranucleotide, etc.) the charge of the oligonucleotide at a given pH can be calculated quite exactly from the distance moved[11].

ACKNOWLEDGEMENTS

I am grateful to Dr. W. E. COHN, in whose laboratory this work was done, for his help in preparing this manuscript, and to Dr. M. UZIEL for much valuable advice and constructive criticism.

This research was supported by a personal fellowship from the Max Kade Foundation, New York.

REFERENCES

1 K. RANDERATH, in *Thin Layer Chromatography*, Academic Press, New York, 1963.
2 P. L. BERGQUIST, *J. Chromatog.*, 19 (1965) 615.
3 G. AUGUSTI-TOCCO, C. CARESTIA, P. GRIPPO, E. PARISI AND E. SCARANO, *Biochim. Biophys. Acta*, 155 (1968) 8.
4 A. YARON AND H. A. SOBER, *Anal. Biochem.*, 12 (1965) 173.
5 M. UZIEL, C. KOH AND W. E. COHN, *Anal. Biochem.*, in press.
6 M. UZIEL AND H. G. GASSEN, in preparation.
7 M. UZIEL AND W. E. COHN, *Biochim. Biophys. Acta*, 103 (1965) 539.
8 R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 52 (1952) 552.
9 G. W. RUSHIZKY AND C. A. KNIGHT, *Virology*, 11 (1960) 236.
10 R. L. BIELESKI, *Anal. Biochem.*, 12 (1965) 230.
11 D. SMITH, *Methods in Enzymology*, Vol. XII, Part A, Academic Press, New York, 1967.