Application of a Spectrophotometric Method to the Determination of the Composition of Oligonucleotides Obtained from Cysteine Transfer Ribonucleic Acid

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1. The application of methods for determining the composition of oligonucleotides from u.v.-absorption spectra is described. 2. In the first method absorbances at selected wavelengths were read from the spectra of oligonucleotides in solution in 7M-urea which had been recorded at acid and alkaline pH values. 3. In the second method absorbances were sampled automatically at regular time-intervals during scans at acid and alkaline pH of each spectrum, converted into digital signals and recorded on paper tape for computer processing. The holmium spectrum in the region of the holmium peak at 333.7 nm was superimposed on each nucleotide spectrum. The position of this peak maximum was used as a standard reference point in the computer-based analysis. 4. By using either method the composition was calculated by a least-squares procedure by using a library of values for five standard nucleotides obtained in a similar manner. 5. The methods gave satisfactory compositions for mixtures of mononucleotides as well as for five dinucleoside monophosphates. 6. Methods of minimizing the effects on the nucleotide composition of spectral changes due to base stacking are discussed. 7. The compositions of some oligonucleotides obtained during an investigation of the nucleotide sequence of tRNA^{Cys} were determined and agreed with the sequences found by other methods.

Several publications describe methods for the determination of the composition of oligonucleotides by spectrophotometric methods.

Lee *et al.* (1965) presented a multicomponent spectrophotometric analysis, applying a least-squares procedure to absorbance values measured at 2 nm intervals between 230 and 328 nm. Although the content of 2'(3')-CMP and 2'(3')-GMP could be determined accurately at pH 2, the optimum results for all nucleotides were obtained at pH 12.

Toal *et al.* (1968) applied a linear programming formulation to determine the composition of di-, tri- and tetra-nucleotides eluted after a two-dimensional 'mapping' procedure. Absorbances were read at 2 nm intervals between 230 and 284 nm from spectra recorded at pH 1, 7 and 12. Constraints were applied in the calculation according to the 'mapping' information.

A simpler method (Richards, 1968) was based on measurements at selected wavelengths of the absorbance of the nucleotide solution at pH 7 and 12. The contributions of 2'(3')-AMP and 2'(3')-CMP to the resulting difference spectrum were negligible, for the spectrum was dependent solely on the modification of the spectra of 2'(3')-UMP and 2'(3')-GMP resulting from ionization of the enol groups at pH 12. From

this difference spectrum the quantities of 2'(3')-UMP and 2'(3')-GMP present could be calculated, and the content of 2'(3')-AMP and 2'(3')-CMP were then determined from the absorbance readings at pH 7. A critical factor in this method was the ability to reset reproducibly the wavelengths at which the absorbance measurements were made.

It appeared that best use could be made of these different pH optima by measuring the spectrum of a nucleotide mixture first at about pH 1 and then again at pH 12 and combining the two sets of values to obtain an extended vector. A least-squares calculation could then be employed, using as a library values for the standard mononucleotides obtained in a similar manner.

The present paper describes the development of this technique and its application to the identification of oligonucleotides obtained during an investigation into the nucleotide sequence of tRNA^{Cys} from baker's yeast (Holness & Atfield, 1976*a,b,c*). To avoid phasing problems between scans, part of the holmium spectrum in the region of the holmium peak at 333.7 nm was superimposed on the spectrum of each nucleotide at each pH value. The precise position of the peak maximum was used as a standard starting point for all spectra and avoided the necessity for repeated

checks on the location of the chart paper on the flat-bed recorder and on the wavelength calibration of the spectrophotometer.

Experimental

Materials

The preparations of the nucleotides 2'(3')-AMP, 2'(3')-CMP, 2'(3')-GMP and 2'(3')-UMP were each the mixture of the 2'- and 3'-isomers as supplied by Calbiochem, Los Angeles, CA, U.S.A. Pseudouridine was also obtained from Calbiochem. The dinucleoside monophosphates were supplied by Sigma Chemical Co., St. Louis, MO, U.S.A.

T2 RNAase* (EC 3.1.4.23) was obtained from Sankyo Co. Ltd., Tokyo, Japan.

Buffer solutions

The compositions of the buffers used were as follows. Buffer A (pH 1.1) contained 3M-KCl (25ml) and 3M-HCl (50ml), diluted to 100ml with water. Buffer B (pH 12.8) contained 3M-KCl (25ml) and 5M-NaOH (30ml), diluted to 100ml with water. Buffer C was prepared from 0.02M-formic acid in 7M-urea, adjusted to pH 3.8 with 2M-formic acid.

Spectrophotometric measurements and computer treatment of the results

Spectra were recorded by using a Unicam SP.800 spectrophotometer fitted with an SP.850 scale-expansion accessory.

In methods 1(a) and 1(b) (see below) absorbances were read from the preprinted charts on which the output from the spectrophotometer was recorded. To maintain accurate wavelength alignment during a run and from day to day it was essential that two requirements were met. The first was that the chart paper should be held in strict mechanical register with the position of the dispersing element. After prolonged use some movement of the chart with reference to the recorder carriage could occur. The second requirement was that the pen should be exactly perpendicular to the chart when the scan switch was in the 'scan' position. In fact replacement of pens after occasional cleaning, or substitution of new pens usually of length differing from the original, led to incorrect or loose alignment at this point. The wavelength calibration was checked daily by recording part of the holmium spectrum.

After recording the spectra at acid and alkaline pH on the calibrated chart, absorbances were read from the chart at 2nm intervals over the wavelength range 250–300nm (method 1a). These values were considered as a single linear array for calculation purposes.

The absorbances at acid pH formed the first 26 members of the array and those at alkaline pH occupied positions 27–52 in the array.

In method 1(b), to make more efficient use of the available information, absorbances at acid pH were read at 2nm intervals between 240 and 310nm and those at alkaline pH between 256 and 310nm, thus giving an array with 64 values. In both these methods the spectra of the standard mononucleotides were recorded on the preprinted charts and the absorbance values read from the charts to form the library values. Absorbance values were transferred manually to paper tape for processing via a teletype terminal connection by using the Maximop system developed at Queen Mary College on the twin ICL 1900 installation.

With methods 2(a) and 2(b) the output from the SP.850 accessory, which normally was led to a slave chart recorder, was led directly to a Stogate series III data-logging system (Stogate Technical Developments Ltd., Billingshurst, Sussex, U.K.) with output to an Addo Facit paper tape punch.

To avoid phasing problems between spectra it was essential that all spectra were started at the same point. The solutions of the oligonucleotides studied here were transparent to light above 320nm. It was thus possible to commence a scan of the spectrum of a nucleotide at about 337nm with a holmium glass filter in the sample light-path, superimposing part of the holmium spectrum with no mutual interference with the nucleotide spectrum. Absorbance values were recorded at 2s intervals commencing at about 337nm with the spectrophotometer set in the 'slow' scan mode. The first ten absorbance values covered the region of the holmium peak at 333.7nm. At about 330nm the filter was removed from the light-path by remote control and the spectrum of the oligonucleotide in solution in buffer A was recorded without interruption. About 140 absorbance values were recorded over the range 337–220nm.

Close to its maximum the holmium absorbance peak at 333.7nm was symmetrical, and by examining the first ten absorbance values the position of the peak maximum in the array could be defined with an accuracy better than 0.1nm. The position of this peak maximum in the array was taken as the starting point for a new array of 110 values obtained by interpolation.

The final 100 values of this array, which covered the wavelength range 315–235nm, were combined as described for method 1(a) with a similar set obtained during the recording of the spectrum of the oligonucleotide in solution in buffer B to give a final array of 200 absorbance values. Such composite spectra for each of the standard mononucleotides obtained in a similar manner formed the library.

Each composite spectrum was normalized to a total absorbance of unity and the nucleotide compo-

* Abbreviation: RNAase, ribonuclease.

sition of the sample calculated by a standard least-squares procedure. Where the initial computation indicated negative contributions from any of the standard mononucleotides, the composition could be recalculated by omitting such mononucleotides from the library.

As a measure of the accuracy of fit of the calculated composition to the observed spectrum, the total divergence (d) was calculated from the expression:

$$d = \sum_{i=1}^{i=n} (E_i - E_i')^2$$

where E_i is the normalized absorbance at wavelength i , E_i' is the absorbance at wavelength i derived from the computed composition, and n is the number of wavelengths at which absorbances were recorded.

In fact the total divergence will decrease in proportion to the number of absorbance measurements included in each spectrum. To compare the accuracy of fit between methods 1(a), 1(b) and 2 the relative divergence, defined as the total divergence per 100 absorbance measurements, is tabulated.

Constancy of scan time

The success of methods 2(a) and 2(b) depended on the assumption that the time taken for the spectrophotometer to scan a given wavelength range was constant and reproducible from day to day. The holmium spectrum between 460 and 270nm was scanned by using the 'slow'-scan setting of the spectrophotometer and absorbance values recorded via the

Table 1. Calculation of the composition of mononucleotide mixtures from spectrophotometric data

Each mononucleotide mixture was prepared from standard (1mM) aqueous solutions of AMP, CMP, GMP and UMP (2'- and 3'-isomers) and of pseudouridine. Each mixture was diluted with 9 vol. of buffer C (0.02M-formic acid in 7M-urea at pH3.8) and the spectrum recorded in the usual manner. The first set of absorbance values were read from the chart at 2nm intervals between 250 and 300nm. The pH of the solution was then adjusted to 12 by the addition of 15M-KOH (10μl/ml). The spectrum was recorded at this pH and a second set of absorbance values obtained. These two sets were combined, normalized to a total absorbance of unity and used to determine the composition of the mixture as described in the text (method 1a). The sum of the squares of the deviations of the observed (E_i) from the calculated absorbance (E_i') at each wavelength (i), the divergence, was calculated for each nucleotide mixture from the expression:

$$\sum_{i=1}^{i=n} (E_i - E_i')^2$$

The divergence per 100 absorbance units or relative divergence is tabulated in the final column. Where negative contributions from any nucleotide were indicated the composition was recalculated omitting those nucleotides from the library. Recalculated compositions are indicated by the asterisk.

Mixture		Component present (nmol)					10 ⁵ × Relative divergence
		Ap	Cp	Gp	Up	Ψ	
1	Composition	20	20	20	20	20	0.8
	Analysis	26	21	19	16	18	
2	Composition	33	33	33	—	—	1.1
	Analysis	41	36	32	—8	—2	
	Analysis*	37	34	29	—	—	
3	Composition	—	33	—	33	33	1.3
	Analysis	8	37	—2	23	34	
	Analysis*	7	36	—	23	33	
4	Composition	20	40	20	20	—	0.3
	Analysis	24	41	18	19	—2	
	Analysis*	26	41	18	16	—	
5	Composition	—	—	50	—	50	0.5
	Analysis	7	2	47	—7	51	
	Analysis*	3	0	49	—	49	
6	Composition	25	—	25	50	—	1.2
	Analysis	34	1	24	44	—2	
	Analysis*	34	0	25	41	—	
7	Composition	17	17	33	33	—	0.6
	Analysis	22	18	32	30	—2	
	Analysis*	23	18	32	27	—	
8	Composition	20	—	40	20	20	0.9
	Analysis	26	0	39	16	18	

data-logging system at 2s intervals. The spectrum was scanned four times at 15 min intervals, the first scan starting 20 min after switching on the equipment. The time required to traverse the wavelength range between the holmium peaks at 453.2 and 279.4 nm was measured at about 10-day intervals and gave the following mean values (four experiments), $\pm$ s.d.: 278.0 ± 0.2 , 278.0 ± 0.1 and 278.3 ± 0.1 s.

The reproducibility of the scan times was further checked by repeatedly scanning the spectrum of 2'(3')-AMP at pH 1.1 and calculating the time required to traverse the wavelength range from the reference start point to the maximum of the 2'(3')-AMP absorption. The mean of five measurements, $\pm$ s.d., was 173.6 ± 0.2 s. In this wavelength region the scan speed was 0.44 nm/s.

Preparation of nucleotide samples for spectrophotometric measurements

Methods 1(a) and 1(b) were designed to be used with oligonucleotides in solution in buffer C, as they were eluted during the chromatography of enzymic digests of tRNA^{Cys} on columns of DEAE-cellulose (Holness & Atfield, 1976b). Eluates not containing nucleotide material were used as reference solutions. After the spectra had been recorded at pH 3.8,

15M-KOH (10 μ l/ml) was added to both sample and reference solutions and the spectra were recorded at the alkaline pH.

Method 2(a) was designed to be used with desalted oligonucleotide samples without prior digestion with T2 RNAase.

Method 2(b) was similar to method 2(a) but was used for oligonucleotide samples that had been digested to mononucleotides with T2 RNAase, and was as follows. The desalted sample was dissolved in 50 μ l of a solution (50 units/ml) of T2 RNAase in 0.05M-sodium acetate adjusted to pH 5.2 with acetic acid and incubated at 37°C for 2 h. The digest was diluted with water (2.0 ml) and two 1.0 ml portions were pipetted into separate Pyrex tubes (10 cm $\times$ 1 cm). Reference solutions were similarly prepared from 50 μ l of the T2 RNAase solution but without added oligonucleotide. Buffer A (pH 1.1, 50 μ l) was added to the first set of sample and reference solutions and the spectrum recorded as described previously. Similarly 50 μ l of buffer B (pH 12.8) was added to the second set of solutions and the spectrum again recorded.

The unit of activity of T2 RNAase is defined as that quantity of enzyme that leads to an increase of 1.0 in E_{260} in the acid-soluble nucleotides when incubated for 15 min at 37°C in 1 ml of a solution containing commercial yeast RNA (3 mg/ml) (Uchida, 1966).

Table 2. *Calculation of the composition of mononucleotide mixtures from spectrophotometric data*

Mixtures of mononucleotides were prepared from the standard nucleotide solutions as described in Table 1. Each mixture was diluted with 9 vol. of water and divided into two equal batches. Buffer A, pH 1.1 (50 μ l/ml), was added to one, and buffer B, pH 12.8 (50 μ l/ml), to the other. Absorbance values of each portion of the solution were recorded on paper tape by using the data-logging system and the 'slow' scan mode of the spectrophotometer. At each pH 100 values were combined and the composition was calculated as described in the text (method 2a).

Mixture		Component present (nmol)					$10^5 \times$ Relative divergence
		Ap	Cp	Gp	Up	Ψ	
1	Composition	50	—	50	—	—	0.1
	Analysis	46	0	52	1	1	
2	Composition	25	50	25	—	—	0.2
	Analysis	29	51	24	-5	2	
3	Composition	—	25	25	50	—	0.1
	Analysis	0	26	24	50	1	
4	Composition	—	25	25	25	25	0.2
	Analysis	2	26	25	21	26	
5	Composition	20	20	20	40	—	0.2
	Analysis	20	21	19	40	1	
6	Composition	37	25	13	13	13	0.2
	Analysis	38	26	11	13	14	
7	Composition	25	25	25	25	—	0.2
	Analysis	27	26	21	27	-2	
8	Composition	19	19	19	19	25	0.1
	Analysis	17	18	19	22	26	
9	Composition	51	11	11	11	11	0.2
	Analysis	57	15	4	9	16	

Analysis of mononucleotide mixtures of known composition

Mononucleotide mixtures were prepared from standard (1mM) aqueous solutions of 2'(3')-AMP, 2'(3')-CMP, 2'(3')-GMP and 2'(3')-UMP and of pseudouridine. Each mixture was diluted with 9 vol. of buffer C (methods 1a and 1b) or with 9 vol. of water (method 2a). Values for the nucleotide extinction coefficients were taken from the published data of Holley *et al.* (1965) and Madison *et al.* (1967).

Solutions of dinucleoside monophosphates were also prepared for analysis by each method.

Analysis of oligonucleotides of unknown composition

The majority of the simpler oligonucleotides obtained during the enzymic degradation of tRNA^{Cys} were analysed by methods 1(a) and 1(b). Methods

2(a) and 2(b) were used for the more complex oligonucleotides.

Results and Discussion

Table 1 shows the results obtained from the analysis of eight mixtures of known composition prepared from standard solutions of the four mononucleotides and of pseudouridine. Similarities in the spectra of solutions of 2'(3')-AMP, 2'(3')-UMP and pseudouridine at acid pH led to difficulties in determining the compositions of mixtures containing all three. At alkaline pH the absorption maximum in the spectrum of pseudouridine was displaced to shorter wavelength and by including measurements at pH 12 the content of pseudouridine could be accurately determined. However, there still remained some cross-contamination between 2'(3')-AMP and 2'(3')-UMP. This is shown in some compositions presented

Table 3. Calculation of the composition of dinucleoside monophosphates from spectrophotometric data

Solutions of dinucleoside monophosphates were prepared and analysed by one of the following procedures: 1(a), as described in the legend to Table 1; 1(b), as described in Table 1, except that absorbance values at pH 3.8 were recorded at 2 nm intervals between 240 and 310 nm and those at pH 12 between 256 and 310 nm; 2(a), as described in the legend to Table 2; 2(b), as procedure 2(a), but the compound was first hydrolysed to its constituent parts by enzymic hydrolysis with T2 RNAase as described in the Experimental section. Duplicate analyses were usually performed when using methods 2(a) and 2(b).

Dinucleoside monophosphate	Method of analysis	Component present (mol fraction/100mol)					10 ⁵ × Relative divergence
		Ap	Cp	Gp	Up	Ψp	
GpU	1(a)	6	7	51	33	3	0.6
	1(b)	6	1	50	44	-2	0.3
	2(a)	6	4	50	41	0	2.2
	2(a)	12	5	47	44	-8	1.8
	2(b)	0	1	50	48	1	0.8
	2(b)	0	2	49	49	-1	0.2
GpC	1(a)	13	52	56	-34	13	1.3
	1(b)	2	53	52	-11	4	0.4
	2(a)	3	46	58	-14	7	1.2
	2(a)	-1	45	63	-12	5	1.2
	2(b)	1	50	59	-10	-1	0.6
	2(b)	-3	48	62	-10	3	0.4
CpG	2(a)	-4	44	57	2	1	0.6
	2(a)	0	45	55	-3	2	0.8
	2(b)	-4	45	59	-1	1	0.2
	2(b)	0	47	52	0	1	0.4
CpU	1(a)	6	54	2	32	6	0.6
	1(b)	2	50	3	44	1	0.2
	2(a)	-1	50	2	47	3	0.2
	2(a)	5	53	-2	41	3	0.4
	2(b)	3	52	0	45	0	0.2
ApG	1(a)	29	-1	47	29	-4	1.1
	1(b)	53	-6	63	-6	-3	0.8
	2(a)	41	-6	60	3	2	0.8
	2(a)	43	-4	56	4	0	2.0
	2(b)	55	3	47	-10	4	1.0
	2(b)	58	0	53	-11	0	0.8

Table 4. Influence of the concentration of dinucleoside monophosphate (ApU) on the apparent composition as determined by spectrophotometric method 2(a)

A series of aqueous solutions of ApU were prepared and the concentration of each measured in terms of the E_{260} . The composition of the dinucleoside monophosphate was determined by method 2(a) in duplicate except at the two lower concentrations of ApU. At the lowest concentration the effect of amplifying by a factor of 5 the signal from the spectrophotometer before applying to the data-logger was examined (line 2).

Concn. of ApU (E_{260} units)	Component present (mol fraction/100mol)					$10^5 \times$ Relative divergence
	Ap	Cp	Gp	Up	Ψp	
0.075	30	3	26	31	11	31.6
	25	4	28	32	10	29.8
0.167	49	5	8	35	4	0.6
0.349	49	5	-2	44	4	0.8
	48	5	0	45	3	0.8
0.925	53	6	-5	45	1	0.4
	49	4	0	46	0	0.6
1.62	54	6	-4	44	0	1.0
	52	5	0	43	0	1.0

in Table 1, where the sum of the 2'(3')-AMP and 2'(3')-UMP content approximates to the correct value, but individually there is some discrepancy. The problem was overcome by Guschlbauer *et al.* (1965) by taking advantage of the conversion of 2'(3')-UMP into a product that was almost transparent throughout the wavelength range 240–280 nm by irradiation with u.v. light (254 nm). These authors recorded the spectra before and after exposure to u.v. light and calculated the composition of the mixture by a least-squares method by using a library of spectra of the four standard mononucleotides obtained under similar conditions.

Although Richards (1968) proposed that large numbers of readings were not necessary, an increase in the number of absorbance measurements to 200 (Table 2) led to a greater precision in the calculation as indicated by the lower value for the relative divergence. The mole fractions of 2'(3')-AMP and 2'(3')-UMP were given more accurately and with less 'cross-contamination'.

A more realistic test of the method is shown in Table 3, where the compositions of five dinucleoside monophosphates were determined by the methods described. The composition of GpU was determined by method 2(a) on six different occasions and the mean composition, $\pm$ s.d., was as follows: Ap, 0.07 ± 0.03 ; Cp, 0.03 ± 0.03 ; Gp, 0.51 ± 0.03 ; Up, 0.43 ± 0.05 ; Ψp , -0.04 ± 0.03 . In most cases the

agreement between duplicate assays performed on different occasions was commensurate with the standard deviation found for GpU.

In method 1(a), where absorbance readings above 300 nm were not included, spurious contributions from pseudouridine were encountered, but these were decreased by extending readings to 310 nm (method 1b).

In these methods, increased blank values at wavelengths below 250 nm were encountered when the pH of column fractions was adjusted to 12.8, and these were occasionally difficult to eliminate. With method 2 urea was omitted and buffer systems more transparent at these low wavelengths were used. Under these conditions, however, account must be taken of the environment of the chromophore. The formation of secondary structure and the consequent base stacking leads to a hypochromic effect, which is demonstrable even in dinucleotides (Toal *et al.*, 1968). The effect is greatly dependent on the composition and is most marked in oligonucleotides rich in 2'(3')-AMP (Staehelin, 1961).

Since the spectral change in progressing from an equimolar mixture of mononucleotides to an oligonucleotide is dependent on the composition, it is clear that this hypochromicity will have a marked effect on any calculation based on spectra. In methods 1(a) and 1(b), where spectra of the oligonucleotides were measured in solution in 7 M-urea, base stacking was minimized.

In the absence of urea, when method 2(a) is used this hypochromic effect can lead to erroneous values for the calculated composition (Table 3). The relative divergence is significantly raised, indicating the poorer agreement between the calculated and observed absorbance curves. When the sugar-phosphate bonds were broken by hydrolysis with T2 RNAase there was better agreement between the theoretical and the calculated compositions and a concomitant fall in the divergence (method 2b).

The minimum quantity of oligonucleotide that can be used to obtain a satisfactory composition is determined by the signal-to-noise ratio in the spectrophotometer, and, as shown in Table 4, line 2, merely amplifying the output from the spectrophotometer does not result in any gain in accuracy if the nucleotide concentration is too low. The series of determinations with ApU show that when the nucleotide concentration is decreased below 0.30 E_{260} unit/ml the calculated composition became unsatisfactory. Above this, duplicate determinations agreed well.

Table 5 summarizes the results of the spectrophotometric determination of the composition of some oligonucleotides obtained during studies of the nucleotide sequence of tRNA^{Cys}. These were performed during all stages of the development of the method. The agreement with the expected composition is fair with methods 1(a) and 1(b) but again indi-

Table 5. *Spectrophotometric determination of the composition of oligonucleotides obtained during enzymic hydrolysis of tRNA^{Cys}*

For definition of procedures used see the legends to Tables 1 and 2 and the Experimental section. The superscript * indicates that the composition was recalculated by omitting from the library those components that apparently made a negative contribution to the composition. For the more complex oligonucleotides the calculated composition is shown as 'Calc.'.

Oligonucleotide	Method of analysis	Component present (mol fraction/100mol)					10 ⁵ × Relative divergence
		Ap	Cp	Gp	Up	Ψp	
pGp	1(a)	3	3	87	12	-1	3.0
A-Gp	1(a)	53	1	46	0	-1	0.3
A-Gp	1(b)	52	1	56	-7	0	0.3
	1(b)*	47	0	53	—	0	0.3
U-Gp	1(b)	4	5	43	47	0	0.2
G-G-Cp	1(b)	4	35	61	-1	1	3.7
U-U-Gp	1(b)	7	2	38	53	0	0.2
A-G-Cp	1(b)	36	33	39	-10	2	0.8
	1(b)*	30	32	38	—	0	1.0
A-U-Gp	1(b)	28	3	34	35	0	0.3
G-A-G-Up	1(a)	26	8	49	13	5	0.7
U-A-U-Gp	1(a)	17	1	28	55	-1	0.5
A-Ψ-U-Gp	1(a)	30	2	32	15	21	0.5
G-A-G-Cp	1(b)	33	25	57	-14	0	0.5
	1(b)*	25	23	52	—	0	0.8
C-U-C-Gp	1(a)	-1	48	24	23	4	1.0
A-G-A-Ψp	1(a)	54	-1	37	-14	24	0.7
	1(a)*	46	—	33	—	21	0.9
	2(a)	46	0	27	4	23	0.2
pG-C-U-C-G-Up	2(b)	4	31	21	41	2	1.0
	Calc.	0	33	33	33	0	
A-G-A-Ψ-U-G-Cp	2(a)	26	16	33	16	8	1.0
	2(b)	26	16	28	17	12	1.0
	Calc.	29	14	29	14	14	
G-A-G-U-G-C-G-A-G-C-U-C	2(b)	16	27	39	14	4	1.2
	Calc.	17	25	42	17	0	
C-U-G-A-G-U-G-C-G-A-G-C-U-C	2(a)	18	27	39	8	7	3.8
	2(b)	16	25	33	18	8	0.4
	Calc.	14	28	36	21	0	

cates the mathematical problem caused by the similarity between the spectra of 2'(3')-AMP and 2'(3')-UMP.

Although a greater precision has resulted from the automatic recording of the absorbance values and the more precise wavelength calibration (Table 3), the divergence in the composition of oligonucleotides obtained experimentally is greater than might be expected. This is probably due to a combination of the presence of non-nucleotide contaminants which absorb in the u.v. region and also to heterogeneity in the oligonucleotide itself. The similarity of the spectra of standard pseudouridine and of pseudouridylic acid isolated by Holness & Atfield (1976b) confirmed that no error was introduced by using the library values for pseudouridine in calculating the compositions of these oligonucleotides.

For the longer oligonucleotides (Table 5), which were obtained by partial hydrolysis of tRNA^{Cys}, the increased sensitivity to trace nucleases that might lead to unwanted degradation made further purification steps unattractive.

Compositions deduced by this method are clearly more sensitive to trace contaminants than are compositions deduced by more conventional sequence studies, but even for the long oligonucleotides the method provided valuable confirmatory evidence.

The method described in this paper is restricted to the determination of the composition of those oligonucleotides containing only the five mononucleotides for which library values are available. There are therefore limitations in its use for oligonucleotides derived from tRNA molecules rich in minor nucleotides, for which complete spectral data are not yet available.

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