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The Pseudouridine Contents of the Ribosomal Ribonucleic Acids of Three Vertebrate Species

NUMERICAL CORRESPONDENCE BETWEEN PSEUDOURIDINE RESIDUES AND 2′-*O*-METHYL GROUPS IS NOT ALWAYS CONSERVED

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The pseudouridine contents of the rRNA species of HeLa cells, mouse L-cells and *Xenopus laevis* cultured kidney cells were examined. Pseudouridine, like 2′-*O*-methylation, was found to occur relatively frequently in each of the high-molecular-weight rRNA species. However, the numerical data do not support the idea that there is a general one-to-one relationship between pseudouridine residues and 2′-*O*-methyl groups in vertebrate rRNA.

In this paper we examine the pseudouridine contents of 18S and 28S rRNA from three vertebrate species. The values are compared with those available for the numbers of 2′-*O*-methyl groups in the respective rRNA molecules. The background to this investigation is as follows. Pseudouridine is relatively abundant in the rRNA of all eukaryotes so far examined (Dunn, 1959; Glitz & Decker, 1963; Lane, 1965; Amaldi & Attardi, 1968; Klootwijk & Planta, 1974), but is much less abundant in *Escherichia coli* rRNA (Nichols & Lane, 1967; Dubin & Gunalp, 1967). Also, the numbers of 2′-*O*-methyl groups are much higher in eukaryotic than in prokaryotic rRNA (Khan & Maden, 1976, and references therein). This apparent relationship between pseudouridine content and numbers of 2′-*O*-methyl groups in rRNA was first pointed out by Lane (1965) and Nichols & Lane (1967). More recently a close numerical correspondence was reported between pseudouridine content and numbers of 2′-*O*-methyl groups in HeLa-cell rRNA (Hughes *et al.*, 1976). Moreover, in HeLa cells pseudouridine is formed largely or entirely at the level of precursor rRNA in the nucleolus (Jeanteur *et al.*, 1968; Maden & Forbes, 1972), the site at which 2′-*O*-methylation of rRNA also exclusively occurs (Maden & Salim, 1974).

Before considering whether there might be any causal relationship between pseudouridine formation and 2′-*O*-methylation during ribosome maturation it is important to know, for rRNA from a number of species, whether the numerical correspondence between the two types of modified nucleotide is in general exact or only approximate.

Accurate estimates are now available for the numbers of 2′-*O*-methyl groups in the rRNA molecules of several vertebrates (Khan *et al.*, 1978) and also of *Saccharomyces carlsbergensis* (Klootwijk & Planta, 1973). These estimates were obtained by preparing rRNA specifically labelled in its methyl groups from cells grown in the presence of [^{14}C]-methylmethionine, 'fingerprinting' enzymic digests of the rRNA and summing the molar recoveries of all of the methylated oligonucleotides. No comparable method yet exists for pseudouridine determination owing to the lack of any specific radioactive label for this modified nucleoside. Therefore pseudouridine determinations have relied upon separation of this compound from all other compounds in total hydrolysates of rRNA. Achieving the required degree of separation for accurate quantification requires some care. From a number of possible methods we have used here the procedure of Hughes *et al.* (1976), with improvements. We have thereby marginally refined our previous estimate of the pseudouridine content of HeLa-cell rRNA and have determined the pseudouridine contents of the rRNA of mouse L-cells and *Xenopus laevis* cultured kidney cells, for which accurate data on 2′-*O*-methylation are available (Khan *et al.*, 1978). The present findings do not support the notion of a general exact correspondence between pseudouridine content and 2′-*O*-methyl groups in rRNA. In particular, *X. laevis* 18S rRNA was found to contain some 50 pseudouridine residues, but only 33 2′-*O*-methyl groups.

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Methods

Labelling of cells and preparation of rRNA

HeLa cells were labelled with [^{32}P]P_i and rRNA was prepared as described previously (Maden & Salim, 1974). ^{32}P-labelled rRNA was prepared from mouse L929 cells by similar general methods, and from *X. laevis* cultured kidney cells as described by Khan & Maden (1976). For labelling with [2-^{14}C]-uridine an initial inoculum of about 10^7 cells in a Roux bottle was labelled for 48h with 25μCi of uridine (55mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.). During this period the cells quadrupled and most of the uridine was incorporated. The rRNA species were separated on sucrose gradients (Maden & Salim, 1974). 28S rRNA was subjected to brief thermal treatment and re-centrifugation to liberate 5.8S rRNA (Maden & Robertson, 1974), and 18S rRNA was also re-centrifuged on a sucrose gradient (Maden & Salim, 1974) for further purification.

Alkaline hydrolysis of rRNA and electrophoresis

Samples of 18S or 28S rRNA containing several hundred thousand c.p.m. were taken up in 100–200μl of freshly prepared 0.2M-NaOH in small silicone-treated tubes. Smaller amounts of radioactivity were used for determinations on 5.8S rRNA. The tubes were sealed with Parafilm and were incubated at 37°C for 18–20h. Each of the resulting hydrolysates was subdivided and applied in the form of 8–12 samples, 15cm from one end of a 46cm×57cm sheet of Whatman 52 paper. The samples were applied as 1.2cm streaks, separated by gaps of 2.4cm. Electrophoresis was carried out in 5% (v/v) acetic acid in pyridine buffer, pH3.5, for 30–40min at 4.5kV. The products were located by radioautography. Separations of hydrolysates of ^{32}P-labelled rRNA were shown previously (Hughes *et al.*, 1976). Plate 1(*a*) shows a separation of a hydrolysate of [^{14}C]uridine-labelled 18S rRNA from HeLa cells; radioactivity is confined to pyrimidine mononucleotides and pyrimidine-containing alkali-stable dinucleotides. According to the requirements of the experiment two to four samples were used for base-composition analysis by scintillation counting. The remaining samples were used for pseudouridylate (Ψp) determination as described below.

Hydrolysis with T_2 ribonuclease

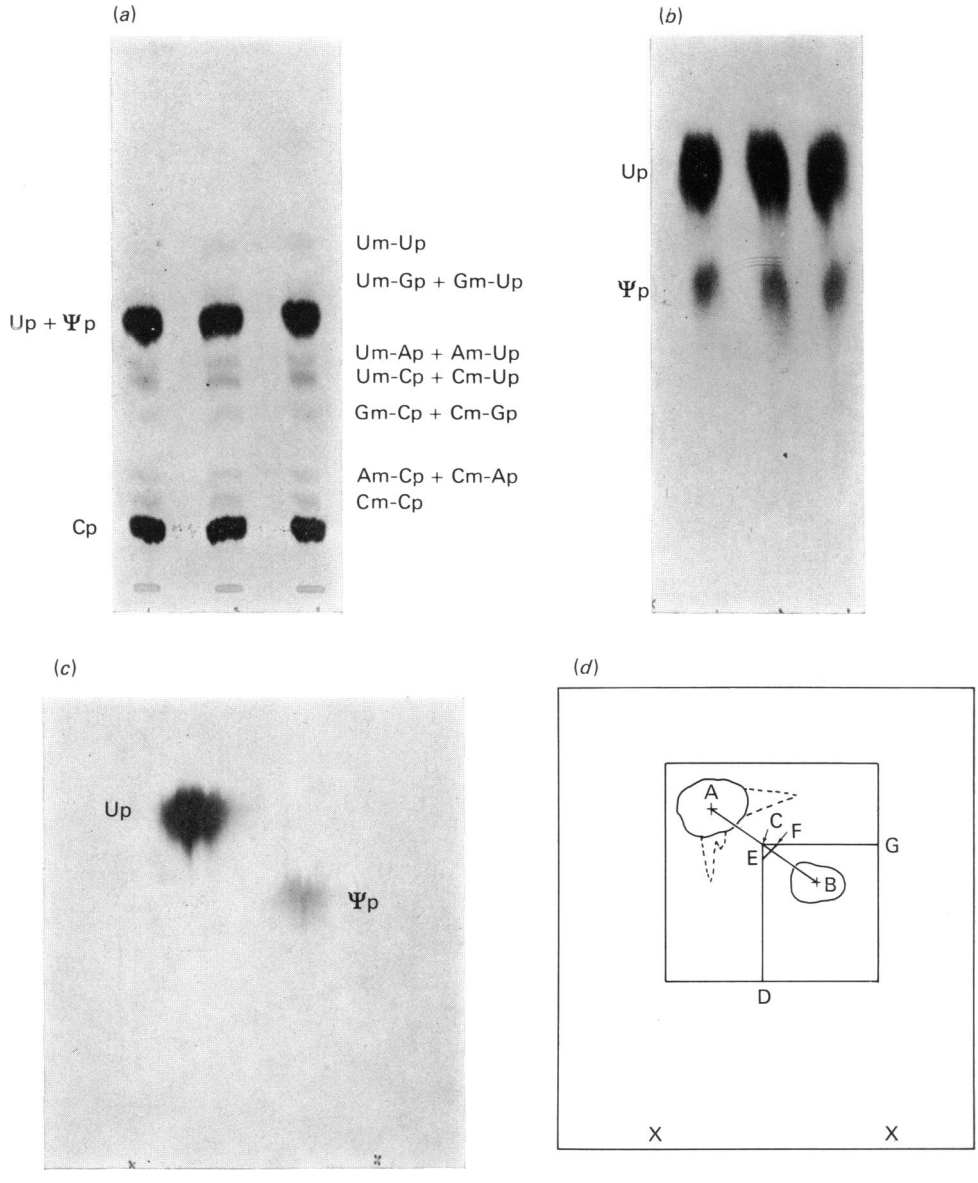
This was carried out with T_2 ribonuclease plus T_1 and pancreatic ribonucleases as described by Brownlee (1972, p. 210), by using a sufficient enzyme/substrate ratio to ensure complete hydrolysis. Electrophoresis was carried out as for alkaline hydrolysates. We previously expected that hydrolysis with T_2 ribonuclease would yield sharper separations of Ψp from Up than in alkaline hydrolysates in the final chromatographic step, because of the presence only of 3′-phosphates in the T_2-ribonuclease hydrolysates, as compared with the mixture of 2′- and 3′-phosphates in the alkaline hydrolysates (Hughes *et al.*, 1976). In fact there was no detectable difference in the degree of resolution obtained by the two methods, and both methods were used interchangeably in the present work.

Chromatography: first dimension

Ψp is not significantly separated from Up on electrophoresis at pH3.5, though it sometimes forms a slightly trailing edge to the main Up band. To separate the two compounds about eight Up plus Ψp spots from the electrophoretic runs were excised and stitched 15cm from one end of a 46cm×57cm sheet of Whatman 52 paper, in which correspondingly located holes had previously been cut. It was important to ensure from the radioautograph (Plate 1*a*) that any slightly trailing edge of the Ψp spot was included in the excised bands. The alkali-stable compounds Um-Gp, Gm-Up and Gm-Gp (Plate 1*a*) were excluded as far as possible, though this was not crucial as these compounds migrate slowly on chromatography (see below). Descending chromatography was carried out for 48h in propan-2-ol/HCl/water (44:11:9, by vol.) (Wyatt, 1951; Brownlee, 1972, p. 205), by which time the solvent had just reached the end of the paper. Plate 1(*b*) shows a chromatographic separation of the Up plus Ψp band of Plate 1(*a*). In such separations Up displayed an R_F of approximately 0.75 and Ψp an 'R_U' (mobility relative to Up) of 0.75 to 0.8. Thus the centre of the Ψp spot was 7–8cm behind that of the Up spot. However, because of variable trailing of the Up spot, re-chromatography was performed in a second dimension to effect complete separation.

Chromatography: second dimension

A few first-dimension runs showed unacceptable streaking and were rejected. Material from all good quality first-dimension runs was rechromatographed as follows. A 20cm strip was excised from 4cm ahead of the centre of the Up spot to 8cm behind the centre of the Ψp spot, and including all of the radioactivity in these two compounds. The strip was stitched over a hole 15cm from one end of another sheet of Whatman 52 paper, and parallel to the origin of the second chromatographic dimension. Chromatography was repeated for 48h in the same solvent at right-angles to the first dimension. The material was now resolved into a major Up spot and a minor Ψp spot (Plate 1*c*). Most of the material that had trailed in the first dimension (right to left) ran normally in the second dimension (upwards in Plate 1*c*), but there was also slight trailing from the main Up spot in the second dimension. Thus the Ψp spot was flanked on two sides by trailing arms of the Up



EXPLANATION OF PLATE 1

Consecutive steps in the separation of Ψp from Up

The experiment is from an alkaline hydrolysate of HeLa-cell 18S rRNA, labelled for 48h with [2-^{14}C]uridine. Up and Cp were labelled to equal specific radioactivity under these conditions. (*a*) Electrophoresis. The positions of the mononucleotides and pyrimidine-containing alkali-stable dinucleotides are indicated. Gm-Gp, mentioned in the text, is not labelled here, but migrates marginally ahead of Um-Ap and Am-Up (Maden & Salim, 1974). (*b*) Chromatography: first dimension. (*c*) Chromatography; second dimension. The steps are shown with the direction of migration upwards. The anode is at the top in (*a*). (In *c* the first dimension was from right to left.) (*d*) Method of quantification; details are described in the Methods section (XX, origin of the second dimension). A similar Figure for a hydrolysate of ^{32}P-labelled rRNA is shown in Hughes *et al.* (1976).

spots. However, it was found by re-running previously purified Up that, on average, only 0.15% of the Up migrated into the Ψp area as defined below.

The identity of the bulk of the more slowly migrating component as Ψp was confirmed in a large-scale run (Hughes, 1976) by elution of the material and spectral analysis at pH 7 and pH 12 (Wu & Allen, 1959). The possible presence of minor contaminants is considered below.

Quantification of chromatograms

In recent work only chromatograms that satisfied the following criteria were selected for quantification. (i) The centre of the Ψp spot was at least 7cm from the centre of the Up spot in each dimension, i.e. the line AB in Plate 1(*d*) was at least 10cm. (ii) There was no obvious evidence in the radioautograph of trailing of Up into the Ψp area, defined below. Most chromatograms met these criteria when both dimensions had been run until the solvent reached the end of the paper.

To obtain the maximum degree of reproducibility and the minimum contamination of Ψp by Up the following procedure was adhered to when counting chromatograms for radioactivity. A square of side 17–20cm was drawn to include all the radioactivity in Up plus Ψp (Plate 1*d*). To define the Ψp area the line AB was bisected at C and perpendiculars were drawn as in Plate 1(*d*) (A and B being the centres of the Up and Ψp spots). It was assumed that all the Ψp was contained below and to the right of the line DEFG. (It was found on re-running Ψp that this boundary is beyond the limit of any forward diffusion from the centre of the Ψp spot.) The entire 17–20cm square was then cut into 50–60 small squares for scintillation counting, using the line DEFG to separate 'Ψp' squares from 'Up' squares. When each chromatograph was counted for radioactivity the instrument background was carefully determined with several 'blank' vials containing scintillant only. The 'observed' fraction of Ψp was expressed as a percentage:

$$\frac{\text{radioactivity in } \Psi p}{\text{radioactivity in } (Up+\Psi p)} \times 100 - 0.15\%$$

where the radioactivities are corrected for background and 0.15% is the average spillover of Up into Ψp as discussed above.

Products that may co-migrate with Ψp

The 5′-terminal mononucleotide diphosphates, pCp and pAp, co-migrate with Up plus Ψp on electrophoresis (Brownlee, 1972, p. 215; B. E. H. Maden, unpublished work). pCp migrates in the present chromatographic system slightly ahead of Ψp (R_U>0.8) with streaking. Because of the method used for demarcating Ψp (Plate 1*d*) practically all pCp would be included in the Ψp area for scintillation counting. A correction for pCp was therefore made where necessary. pAp is not an end group of any of the rRNA species examined in this work (Khan & Maden, 1976).

Um-Gp, Gm-Up and Gm-Gp migrate fairly near to the main Up-plus-Ψp band in Plate 1(*a*). However, on subsequent chromatography the R_U values for Um-Gp and Gm-Up were approx. 0.4 and that for Gm-Gp was approx. 0.1.

Dihydrouridylic acid, if present, migrates ahead of Ψp in the present chromatographic system (R_U for dihydrouridylate = 0.9; Brownlee, 1972, p. 205). However, the dihydrouracil ring is alkali-labile (Brownlee, 1972, p. 46). Therefore dihydrouridylic acid could only be present in chromatographs of T_2-ribonuclease hydrolysates, and not in those of alkaline hydrolysates. No significant differences were found for the value of Ψp/(Up+Ψp) for T_2-ribonuclease hydrolysates as compared with alkaline hydrolysates. From the lack of material migrating between Ψp and Up in T_2-ribonuclease hydrolysates we believe that there is little if any dihydrouridylic acid in these rRNA species. There is, however, a single oligonucleotide in 18S rRNA containing an unidentified component that may possibly be dihydrouridylate (Hughes, 1976) (see 'Note Added in Proof').

Results

Table 1 shows the percentages of Up plus Ψp in hydrolysates of the rRNA from the three vertebrate species. Many determinations were carried out for HeLa-cell rRNA and *X. laevis* 18S rRNA, and essentially indistinguishable results were obtained by the alkaline and T_2-ribonuclease hydrolysis methods (Hughes, 1976; the present work). The results were generally in good agreement with published values, also shown. (The values for the other three mononucleotides were also in good agreement with published values; results not shown.)

The total numbers of Up and Ψp residues in the rRNA can be calculated from the percentage values if the molecular weights of the rRNA molecules are known. The molecular weight values given in Table 1 are based on hydrodynamic measurements (Petermann & Pavlovec, 1966), determination of relative electrophoretic mobility on polyacrylamide gels (Loening, 1968) and measurement of contour lengths on electron microscopy (Wellauer & Dawid, 1973, 1974). In the latter two methods the 16S rRNA of *Escherichia coli* was used as standard (Stanley & Bock, 1965; Fellner, 1974). The total numbers of mononucleotides per rRNA molecule were estimated from the molecular-weight values; a very minor correction was introduced (see the end of the legend to Table 1) to allow for the fact that small quantities of alkali-stable dinucleotides migrate separately from the mononucleotides (Maden & Salim, 1974) and were

Table 1. *Number of Up plus Ψp residues in vertebrate rRNA species*

Base compositions were determined as described in the Methods section. Values for the HeLa-cell 18S and 28S rRNA and the *X. laevis* 18S rRNA are each from approximately equal numbers of determinations by the alkali and T_2-ribonuclease methods. The other values are from the T_2-ribonuclease method only. All values for 28S rRNA are from 5.8S-RNA-free material. The published values, shown for comparison, were from alkaline hydrolysates and are from the following references: (1) Amaldi & Attardi (1968); (2) Willems *et al.* (1968); (3) Lane & Tamaoki (1967); (4) Birnstiel *et al.* (1968); (5) from the nucleotide sequence (Nazar *et al.*, 1975), allowing for an extra Up before the 3′-end of the HeLa sequence (Maden & Robertson, 1974); '(1,2)' signifies the mean between the values in these two references; (4) is the mean between two closely similar values obtained from two different labelling periods in the paper of Birnstiel *et al.* (1968). The molecular-weight values are from the following references: (6) Petermann & Pavlovec (1966); (7) Wellauer & Dawid (1973) [both reports gave 0.67×10^6 for mammalian 18S rRNA; (6) gave 1.64×10^6 and (7) gave 1.76×10^6 for mammalian 28S rRNA]; (8) Loening (1968); (9) Wellauer & Dawid (1974). 'Total mononucleotides' refers to the numbers of nucleotides released as or co-migrating with mononucleotides in alkaline hydrolysates per molecule of rRNA, assuming the molecular-weight values shown and allowing for small quantities of separately migrating alkali-stable dinucleotides. Thus HeLa-cell 18S rRNA contains approx. 2000 nucleotides and 38 equivalents of 2′-*O*-methyl groups. Therefore 76 alkali-stable dinucleotides are released. Of these about 26 co-migrate with Ap and Gp, and the remainder migrate separately from the mononucleotides (Maden & Salim, 1974). Therefore the total Up+Ψp content is calculated as 21.8% of 1950 nucleotides = 425 nucleotides. A similar very minor adjustment was made for alkali-stable components in the other rRNA molecules, including 5.8S RNA.

		Up+Ψp (% of total mononucleotides)	No. of determinations	Published values	Reference	$10^{-6}\times$ Mol.wt.	Reference	Total mononucleotides	Total Up+Ψp
18S	HeLa	21.8	14	(21.7)	(1,2)	0.67	(6,7)	1950	425
	L-cell	19.9	4	(19.3)	(3)	0.67	(8)	1950	390
	X. laevis	23.1	8	(22.9)	(4)	0.67	(8,9)	1950	450
28S	HeLa	15.8	13	(16.5)	(1,2)	1.70	(6,7)	5000	790
	L-cell	14.5	4	(16.0)	(3)	1.70	(8)	5000	760
	X. laevis	17.3	4	(17.5)	(4)	1.55	(8,9)	4500	780
5.8S	HeLa	22.1	5	(23.0)	(5)			156	36

therefore not included in calculating the 'percentage composition' in Table 1. The total contents of Up plus Ψp in the rRNA molecules were then calculated.

Table 2 shows the ratios Ψp/(Up+Ψp) expressed as percentages after chromatographic determination, as described in the Methods section. Values are shown for rRNA labelled with [^{32}P]P_i and with [2-^{14}C]uridine. The values, together with those in the last column of Table 1, permitted initial estimates to be obtained for the pseudouridine contents of the rRNA species ('observed' values in Table 2). Some minor points require mention before proceeding from these to the final 'suggested' values.

(1) The great majority of determinations of Ψp/(Up+Ψp) for a given RNA species and radioisotope were within ±0.3% of the mean values stated in Table 2. This represents a reproducibility of ±1.2 pseudouridine residues for 18S rRNA and ±2.4 pseudouridine residues for 28S rRNA.

(2) Some 'observed' values obtained from rRNA labelled with [^{14}C]uridine differ appreciably from those obtained from rRNA labelled with [^{32}P]P_i. The disparity for HeLa-cell 18S rRNA, for which many determinations were carried out, is outside the range of error indicated in the preceding paragraph. A possible explanation may be that during the 18h labelling period with [^{32}P]P_i the individual nucleoside triphosphate pools did not fully equilibrate with radioactive phosphate (Jeanteur *et al.*, 1968, appendix). As the number of pseudouridine residues is fairly small the nearest-neighbour relationships to other nucleotides may be non-random, and this would be reflected in a slightly non-random distribution of ^{32}P into Ψp with respect to Up in the RNA hydrolysates. (This possibility was not examined further because it was likely that any such effects would be small and not easy to quantify reliably by using rRNA labelled with [^{32}P]P_i for different periods.) There should be no comparable source of error with rRNA labelled with [^{14}C]uridine assuming, by analogy with tRNA (Altman & Smith, 1971), that pseudouridine is formed by rearrangement of uridine at the polynucleotide level. We have therefore weighted our final 'suggested' values for the pseudouridine contents of the rRNA molecules in favour of those obtained by using RNA labelled with [^{14}C]uridine. The differences between the values obtained with the two isotopes affect the final estimates for pseudouridine content by only a few residues (Table 2).

(3) Each of the high-molecular-weight rRNA species contains one or two pseudouridine residues

Table 2. *Estimated number of pseudouridine residues and 2'-O-methyl groups in vertebrate rRNA species*
The numbers of determinations of (Ψp/Up+Ψp) are shown in parentheses. For further details see the text.

		$\frac{\Psi p}{Up+\Psi p}$ (%)		Ψ residues per molecule					
				'Observed'		Corrections			Total 2'-O-methyl groups
		^{32}P	^{14}C	^{32}P	^{14}C	^{32}P	^{14}C	Suggested	
18S	HeLa	8.53 (8)	9.45 (7)	36	40	+1	+1	40±2	38
	L-cell	8.79 (3)	9.34 (5)	34	36	+1	+1	36±3	38
	X. laevis	10.84 (7)	10.72 (4)	49	48	+1	+1	49±3	33±1
28S	HeLa	7.26 (8)	6.93 (7)	57	55	0	+1	56±3	63±1
	L-cell	7.80 (3)	6.78 (4)	59	52	0	+1	54±4	63±1
	X. laevis	8.01 (5)	7.74 (4)	63	60	+2	+2	62±4	63±2
5.8S	HeLa	7.73 (5)	6.58 (6)	2.8	2.4	−0.8	−0.4	2	1.2

that are not released as free Ψp in the hydrolysates and are therefore not included in the 'observed' estimates of Ψp. The 18S rRNA molecules contain one residue of a hypermodified pseudouridine derivative (Saponara & Enger, 1974; Maden *et al.*, 1975), which is currently designated 3-(3-amino-3-carboxypropyl)-1-methylpseudouridine, or amΨ [see Brand *et al.* (1978) for discussion of nomenclature]. The 28S rRNA molecules each possess two pseudouridine residues in alkali-stable linkage: Ψm-Gp and Um-Gm-Ψp (Maden & Salim, 1974; Khan & Maden, 1976).

(4) The 28S rRNA from HeLa and L-cells each yield approx. 1 mol of pCp/mol of rRNA (Khan & Maden, 1976) and HeLa-cell 5.8S rRNA yields approx. 0.4 mol of pCp. [The other rRNA species in Table 2 possess pUp at the 5'-ends (Slack & Loening, 1974; Khan & Maden, 1976).]

The column headed 'Corrections' in Table 2 summarizes the net effects of points (3) and (4). The net corrections are slight or zero for the high-molecular-weight rRNA species. For example, for HeLa-cell 28S rRNA labelled with $[^{32}P]P_i$ the net correction is zero, as the two pseudouridine residues that do not co-migrate with Ψp, and must therefore be added to the observed value, are cancelled by the two phosphate groups of pCp, which co-migrates with Ψp and must therefore be subtracted. The effect of correction for pCp is appreciable for 5.8S RNA, however.

The last two columns of Table 2 show the suggested numbers of pseudouridine residues in the rRNA species based on the various factors discussed above, together with the numbers of 2'-*O*-methyl groups in the rRNA species calculated from the data in Tables 1 and 2 of Khan *et al.* (1978).

Discussion

The method for pseudouridine determination described in this paper was chosen from a number of possible ones because it is convenient for analyses of whole rRNA or (using one-dimensional chromatography) of individual oligonucleotides during sequence work, and also because the steps are performed sequentially without intermediate elution or other manipulation in which sample losses might occur. Dunn (1959) also used paper chromatographic (or chromatographic plus electrophoretic) methods in an analysis of the pseudouridine content of total 'microsomal RNA' from rat liver. His value of 7.5 mol of pseudouridine/100 mol of uridine was not unlike ours if calculated for combined 28S plus 18S rRNA. Amaldi & Attardi (1968) previously estimated the pseudoridine content of HeLa cell rRNA, using column-chromatographic separation of the products of alkaline hydrolysis. Ψp was only partly resolved from Up after a single fractionation, and it was necessary to re-run the Ψp shoulder to obtain further separation. For 28S rRNA they found that Ψp amounted to 1.1% of the total nucleotides. This corresponds to 55 Ψp residues per molecule, in close agreement with our value. For 18S rRNA they found that Ψp amounted to 1.5% of the total nucleotides, or 30 Ψp residues per molecule. This value is appreciably below our value. Because of our precautions to ensure complete separation of Ψp from Up we believe that our numerical estimates are accurate to within the limits stated in Table 2. A possible source of error in the column-chromatographic method might be incomplete recovery of partly purified Ψp for rechromatography after the first column-chromatographic separation.

According to the data in the last two columns of Table 2 HeLa-cell 18S rRNA contains approximately equal numbers of pseudouridine residues and 2'-*O*-methyl groups. HeLa-cell 28S rRNA contains slightly fewer pseudouridine residues than 2'-*O*-methyl groups. The data for L-cell rRNA are very similar to those for HeLa-cell rRNA. In *Xenopus* 28S rRNA the number of pseudouridine residues and 2'-*O*-methyl groups are very similar to each other.

In *Xenopus* 18S rRNA the number of pseudouridine residues is considerably in excess of the number of 2′-*O*-methyl groups. Since the latter number was determined by analysis of 'fingerprints' of T_1-ribonuclease hydrolysates of methyl-labelled rRNA (Khan *et al.*, 1978) it is unlikely to be in error by more than one methyl group. Even if the pseudouridine content has for some reason been overestimated by 10% (five pseudouridine residues), there are still approx. ten more pseudouridine residues than 2′-*O*-methyl groups in *Xenopus* 18S rRNA.

We conclude from these findings that pseudouridine residues and 2′-*O*-methyl groups do not necessarily occur in equal numbers in rRNA. The fact remains that the eukaryotic rRNA molecules for which data are available are relatively rich in both types of modified nucleotide. The general question remains unanswered as to what features in eukaryotic precursor rRNA are enzymically recognized for these two types of modifications.

Note Added in Proof (Received 19 April 1978)

On the basis of a recent report (Thomas *et al.*, 1978) we now consider it likely that the un-identified component mentioned at the end of the Methods section is N^4-acetylcytidine.

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