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Occurrence and Distribution of Methylated Purines in the Ribonucleic Acids of Subcellular Fractions

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Until recently only two purine and two pyrimidine bases had been found in RNA. The first evidence that other components might be present was provided by the isolation of the nucleotide of 5-ribosyluracil (Cohn, 1957, 1958, 1960; Davis & Allen, 1957; Scannell, Crestfield & Allen, 1959). Cohn reported that in the course of the isolation of 5-ribosyluracil nucleotide he had observed other substances that appeared to be adenine derivatives with methyl groups on the C-2 or 6-amino positions of the purine ring. Adler, Weissman & Gutman (1958) detected a number of methylated purines in acid hydrolysates of yeast RNA. A careful study of RNA from a number of plant, animal, bacterial and viral sources has since led to the isolation of 2-methyladenine, 6-methylaminopurine, 6-dimethylaminopurine, 1-methylguanine, 6-hydroxy-2-methylaminopurine and 6-hydroxy-2-dimethylaminopurine (Littlefield & Dunn, 1958; Dunn & Smith, 1959). Smith & Dunn (1959) provided evidence for the occurrence of the methylated purines as true RNA components in 3',5'-phosphodiester linkage.

Dunn (1959) showed that there was a substantially higher proportion of methylated purines in the soluble RNA fraction of rat liver than in the microsomes. We have confirmed and extended this observation for several plant and animal tissues. The RNA in the soluble fraction of all tissues examined is much richer in these bases than is either microsomal or mitochondrial RNA. The portion of the soluble RNA that remains in solution at pH 5.2 is richer in methylated purines than is the fraction that is precipitated. We put forward the suggestion, based on calculations from our data, that all the methylated purines may in fact occur in soluble RNA, and that those found in particulate fractions are there because of the soluble RNA which was functionally associated with the particles at the time of their isolation. A preliminary account of some of this work has already appeared (Bergquist & Matthews, 1959).

MATERIALS AND METHODS

Chemicals. Samples of 6-amino-2-methylpurine, 6-methylaminopurine, 6-dimethylaminopurine, 6-methylaminopurine riboside, 2-amino-6-hydroxy-1-methylpurine, 6-hydroxy-2-methylaminopurine, 6-hydroxy-2-dimethylaminopurine, were generously donated by Dr J. D. Smith. Further samples of 6-methylaminopurine, 6-dimethylaminopurine, 2-amino-6-hydroxy-1-methylpurine and 6-

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hydroxy-2-methylaminopurine were obtained from Dr G. B. Hitchings.

Small amounts of contaminating material were removed from the authentic specimens by chromatography in solvents 1 and 3 (below) before measurement of ultraviolet-absorption spectra.

Paper chromatography. Solvents: 1, propan-2-ol-water (Markham & Smith, 1952); 2, butan-1-ol-water (Markham & Smith, 1949); 3, propan-2-ol-HCl (Wyatt, 1951); 4, butan-1-ol-formic acid (Markham & Smith, 1949); 5, saturated ammonium sulphate-sodium acetate-propan-2-ol (Markham & Smith, 1951). Whatman no. 3MM paper was used for solvents 1 and 5; Whatman no. 1 paper was used for all other solvents.

Plant and animal tissues. Plants of *Nicotiana tabacum* var. White Burley were grown under glass. When the plants were about 18 in. high the youngest fully-expanded leaf and the three leaves below were used.

Liver and spleen tissue were obtained from albino mice; liver was obtained from albino rabbits, and mammary adenocarcinoma and livers from C3H mice; S180 ascites tumour was grown in albino mice. The mammary adenocarcinoma was a spontaneous tumour which Dr J. F. Burton had obtained in the laboratory of the British Empire Cancer Campaign Society, Auckland, and which had been maintained by serial passage in C3H mice for 2 years. The C3H mice originated from Dr W. Heston, National Institutes of Health, Bethesda, Md., U.S.A. The S180 ascites tumour came from Dr C. Chester Stock, Sloan-Kettering Institute, New York.

Isolation of subcellular fractions. Mitochondria, microsomes and soluble fractions were prepared by conventional differential centrifuging of tissue homogenates in the following media. Medium A: medium A of Littlefield & Keller (1957); medium B: 0.25M-sucrose, 2 mM-EDTA, 35 mM-tris-chloride, pH 7.4; medium C: 0.25M-sucrose, 0.05M-tris-chloride, pH 7.6; medium D (for plant tissues): 0.5M-sucrose, 0.2M-tris-chloride. The following quantities of tissue were employed for each experiment: mouse liver, 40-120 g.; mouse spleen, 15-20 g.; rabbit liver, 60-80 g.; S180 ascites cells, 30-35 ml. of packed cells; tobacco leaf, 150-500 g; bean and pea embryos 150-300 g. For homogenization 10 vol. of medium was used for animal tissues and 5 vol. for plant tissues.

Mitochondria were isolated by centrifuging in the Spinco model L ultracentrifuge at 12000g for 15 min. The microsomal fraction comprised all the material in the mitochondrial supernatant fluid that sedimented after centrifuging at 105000g for 120 min. These particulate fractions were washed once or twice by sedimentation after resuspension in the homogenizing medium. The soluble fraction consisted of all the material that did not sediment under these conditions. On some occasions this soluble fraction was further fractionated into a pH 5.2 precipitate and a pH 5.2 supernatant fraction by the addition of ice-cold N-acetic acid (Hecht, Zamecnik, Stephenson & Scott, 1958), followed by centrifuging at 8000g for 5 min.

Subcellular fractions were prepared from S180 tumour, after lysis of the cells, by the method of Hecht *et al.* (1958).

Isolation of RNA nucleotides. The method is essentially that of Matthews (1958), which is based on suggestions of Markham (1955). The tissue sample or fraction is consecutively extracted with boiling 70% acid ethanol, boiling 95% ethanol, ether and acetone. The extracted residue is

then hydrolysed in alkali (10 times the pellet volume of 2N-KOH) without prior isolation of the RNA. When the RNA of subcellular fractions from the fairly large quantities (of the order of 100 g.) of tissue used in most experiments was being estimated, we introduced two extra stages, which assisted in the subsequent chromatographic resolution of the nucleotides. These are: (i) removal of some low-molecular-weight ultraviolet-absorbing substances that were not RNA components by dialysis of the ethanol-extracted residue before hydrolysis; (ii) removal of partially degraded protein material by heat and ethanol treatment after hydrolysis of the RNA to nucleotides.

The full procedure including these modifications was as follows. After isolation in the centrifuge, cell particles were resuspended in a measured volume of the same medium as that in which they had been washed. Ethanol (2 vol.), adjusted to 0.05N with respect to acetic acid and 1 mM with respect to Mg^{2+} ions, was added. The preparation was left overnight at 2°, centrifuged and the precipitate resuspended in 70% ethanol made 0.1N with respect to acetic acid, and boiled for 5 min. The supernatant was discarded after centrifuging and the pellet was further extracted for 5 min. with boiling 95% ethanol. This treatment effectively removes the free tissue nucleotides and some other low-molecular-weight ultraviolet-absorbing non-RNA materials.

The precipitate was extracted in 10 vol. of diethyl ether for 20 min. with occasional stirring. The supernatant was removed after centrifuging and replaced by 10 vol. of acetone-diethyl ether (1:1), and then with acetone. The final pellet was taken up in water to make a thin paste and all material in the tube was washed into a dialysis sac. It was dialysed for 12-14 hr. at 2° against 2-3 l. of distilled water or 0.1-1.0N-NaCl.

After dialysis, the contents of the sac were precipitated by the addition of 2 vol. of ethanol. After 6 hr. or more at 2°, the precipitate was collected by centrifuging and the excess of ethanol blown off in a warm air stream.

The dialysis step was introduced because it resulted in improved separations during subsequent chromatography of the nucleotides. In particular guanidic acid was resolved from the combined adenylic acid, uridylic acid and cytidylic acid, and the nucleotides were clearly separated from non-ribonucleotide material that remained near the origin. Appropriate tests on an extracted crude ribonucleoprotein fraction from pea-seedling mitochondria showed that this dialysis step led to no detectable loss of polynucleotide material.

After dialysis and precipitation a measured volume of 2N-KOH was added to the precipitate in a stoppered tube, which was then left at 18° or less for a minimum of 60 hr. On titration with $HClO_4$ at the end of the digestion period normality of the KOH was found to lie in the range 1.7-2.0N.

The alkaline hydrolysate was neutralized with $HClO_4$ and centrifuged. The precipitate of $KClO_4$ and protein was extracted twice with ice-cold water. Ethanol (2 vol.) was added to the combined supernatants and the pH adjusted to 4-5 with acetic acid. The solution was then brought to 55° in a water bath and maintained at this temperature for 5 min. It was then cooled to 2° in an ice bath and left in the cold for 60 min. Denatured protein and $KClO_4$ were removed by centrifuging and the supernatant was evaporated to dryness (a syrup often formed at this stage). The

nucleotides were taken up in a measured volume of water. The solution was again brought to 55° for 5 min., cooled for 60 min. at 2° and further protein removed by centrifuging. The solution of ribonucleotides was then chromatographed as preparative bands in solvent 1. In most experiments, the nucleotides from alkaline hydrolysates prepared by the above-described procedure were chromatographed consecutively in several solvents for the purpose of isolating compounds present in very small amounts. A number of tests of the heating and ethanol step described above showed that this treatment removed substances which interfered with chromatography of ribonucleotides in solvent 1 and facilitated elution of ribonucleotides with water from papers run in this solvent. Tests with mouse-liver mitochondria showed that negligible losses of RNA were incurred during the heating and ethanol step.

Estimation of total RNA. The ribonucleotides obtained from alkaline hydrolysis of the subcellular fraction RNA were given preliminary paper chromatography in solvent 1 and eluted in water (0.7–3.0 μ moles of nucleotides/12 in.-wide paper). A measured amount of the eluate was chromatographed again in solvent 1, the nucleotide area was eluted in 0.1 N-HCl and the extinction at 260 $m\mu$ measured. Another portion was evaporated to dryness and subjected to hydrolysis with N-HCl at 100° for 1 hr. Spots were run in solvent 3 and the base composition was calculated from the corrected molar extinction coefficients of Markham (1955). The total amount of RNA in the original sample of material was calculated from the molar extinction coefficients for the four nucleotides at 260 $m\mu$.

Comparison of methods for the isolation of ribonucleotides from RNA. Results with the above-described method were compared with those from salt extraction of RNA (Tyner, Heidelberger & LePage, 1953) and phenol extraction (Gierer & Schramm, 1956) with respect to base composition and yield of ribonucleotide material. Subcellular fractions from 60 to 80 g. of mouse liver were prepared. Portions of each fraction were used to test the recovery of ribonucleotides. Similar tests were made with tobacco leaf. The results of these comparisons may be summarized: (i) similar yields of mitochondrial and microsomal ribonucleotides are provided by all three methods; (ii) salt and phenol extraction of pH 5.2 precipitate and pH 5.2 supernatant RNA give substantially lower yields of ribonucleotides than our method (less than 10% with salt extraction); (iii) the base composition of the ribonucleotides obtained by the three methods varied. In other experiments with mouse-liver mitochondria, we found that the sum of the phenol-extracted RNA and the phenol-retained RNA (see Sibatani, Yamana, Kimura & Okagaki, 1959) gave a base composition similar to that of the nucleotides prepared by our method, although neither phenol-extracted RNA fraction alone resembled the values obtained by our method.

Other evidence suggests that our method does not overestimate RNA in the subcellular fractions. In particular, no contaminating compounds such as ribonucleoside 5'-phosphates were detected by electrophoresis in borate buffer. Also, 8-azaguanosine (used as an added test contaminant because it could be detected in low amounts on chromatograms by its fluorescence in u.v. light) was not adsorbed on to the crude nucleoprotein and did not co-chromatograph with the nucleotides from an alkaline hydrolysate, and finally recovery of nucleotide material with the appropriate absorption spectra after chromato-

graphy in solvent 5 was quantitative. The purine isomeric ribonucleotides occurred in the proportions expected from the alkaline hydrolysis of RNA.

Our method does not appear to underestimate RNA because the RNA was not isolated at any stage in the procedure, there was no evidence for degradation products of RNA in the extraction solutions and there were no losses of nucleotides in the steps designed to remove partially hydrolysed protein material.

Enzymic hydrolysis of nucleotides to nucleosides. Samples of nucleotides (0.01–0.1 μ mole) in 0.4 ml. of 0.2 M-acetate buffer, pH 5.6, were incubated for 6 hr. at 37° with 10–50 units of prostatic phosphomonoesterase (Burton & Petersen, 1960). Similarly, nucleotides in 0.05 M-tris-chloride buffer, pH 8.2, and 0.05 M-MgCl₂ were incubated at 37° with 5'-nucleotidase for the degradation of ribonucleotide 5'-phosphates to nucleosides.

Isolation and identification of methylated purines. Neutralized alkaline hydrolysates were run as preparative bands for 18–24 hr. in solvent 1 to separate the nucleotides from non-nucleotide material. The quantities of nucleotides used ranged from 5 to 80 mg. depending on the tissue and fraction being examined. A guanosine marker was included. An area of paper containing the combined adenosine, cytidine and uridine 2'-phosphates and 3'-phosphates, and down to, but not including, the guanosine marker, was eluted and chromatography by solvent 1 was repeated. The eluate was taken up in N-HCl and hydrolysed at 100° for 1 hr. Portions of the hydrolysate (1–5 μ moles of nucleotides/12 in.-wide paper) were run in solvent 3 (20 hr.) followed by solvent 2 (15 hr.) in the second dimension. The 2-amino-6-hydroxy-1-methylpurine and combined 6-hydroxy-2-methylaminopurine and 6-hydroxy-2-dimethylaminopurine spots were located by their ultraviolet fluorescence (Smith & Markham, 1950) and eluted. The 2-amino-6-hydroxy-1-methylpurine eluates from a number of chromatograms were combined in a measured volume of water and run in solvent 2 in the first dimension (15 hr.) followed by solvent 4 in the second dimension (30–72 hr.). The 2-amino-6-hydroxy-1-methylpurine was then estimated by ultraviolet spectrophotometry. This procedure, by which 2-amino-6-hydroxy-1-methylpurine was isolated, is called system I.

The 6-hydroxy-2-methylaminopurine and 6-hydroxy-2-dimethylaminopurine eluates were chromatographed in solvent 3 (20 hr.), followed by solvent 4 (30–50 hr.), to separate any contaminating adenine. The 6-hydroxy-2-methylaminopurine and 6-hydroxy-2-dimethylaminopurine area was then eluted and a measured volume was chromatographed as a short narrow band in solvent 3 in both the first and second dimensions. The temperature at this step was maintained at 20° or less. Incomplete separation occurred at higher temperatures.

With some preparations adenine was removed by electrophoresis on paper in 0.05 M-phosphate buffer, pH 1.7, for 90–120 min. at 20 v/cm. Under these conditions adenine moves ahead of the two methylated purines. The area of paper containing 6-hydroxy-2-methylaminopurine and 6-hydroxy-2-dimethylaminopurine was eluted, run in solvent 1, eluted again and then chromatographed in solvent 3 in two dimensions as described above. These two procedures for the separation of 6-hydroxy-2-methylaminopurine and 6-hydroxy-2-dimethylaminopurine are termed system II.

A third system involving preliminary separation of nucleotides was as follows. After solvent 1 chromatography, as in system I, instead of being acid-hydrolysed the nucleotides were subjected to electrophoresis at 20v/cm. in 0.05M-formate buffer, pH 2.6, for 3 hr. (1–5 μ moles of nucleotides/4 in.-wide paper strip). The area immediately in front of the adenylic acid band and up to the front of the uridylic acid band was eluted, chromatographed in solvent 1, eluted and hydrolysed in N-HCl. Portions of the hydrolysate were chromatographed in solvent 3 followed by solvent 2 in the second dimension. The 2-amino-6-hydroxy-1-methylpurine area was chromatographed in solvent 1 (16–24 hr.), followed by solvent 4 in the second dimension (30–50 hr.). The combined 6-hydroxy-2-methylaminopurine and 6-hydroxy-2-dimethylaminopurine eluates were rechromatographed in the solvent 3 and solvent 2 two-dimensional system, eluted and run as a narrow band as for system II in solvent 3 in two dimensions (system III).

For the isolation of the methylated adenines, an area of paper that would include such compounds, even when their position could not be detected by ultraviolet photography, was cut from each of the chromatograms of the original acid hydrolysate used for the isolation of the methylated guanines by system II. The eluates from a number of papers for the 6-amino-2-methylpurine, 6-methylaminopurine and 6-dimethylaminopurine areas were combined. Each compound usually gave a single spot in solvent 2 in both first and second dimensions (15 hr. in each dimension). The spots were eluted and estimated by ultraviolet spectrophotometry.

The identity of the methylated purines was established by comparison of their ultraviolet spectra in 0.1N-HCl and 0.1N-KOH, and of their R_F values in solvents 1–4, with those of authentic samples.

In most experiments insufficient material was available to do analyses on duplicate batches of the tissue. In the final stages of the analyses, however, estimations of uridylic acid, and of the base ratios of the major bases, were made in triplicate. Estimations on methylated purines in microsomes and the pH 5.2 precipitate fraction were usually made on duplicate spots from the final chromatograms. For the other cell fractions the amounts present allowed only single estimations to be made. An indication of the error to be expected in estimates of the methylated purines is given in Table 1 for S180 ascites tumour. Two independent estimates are given for three bases from the whole soluble RNA fraction. Much greater variation in concentrations has occasionally been noted. For example, the values for 6-hydroxy-2-methylaminopurine and 6-hydroxy-2-dimethylaminopurine in mouse-liver fractions are generally much lower in the data of Table 4 than in Table 2. The data in Table 4 were obtained 2 years after those in Table 2 and are probably more reliable. It is not known to what extent variation in the animals in these two experiments contributed to the differences noted.

Spectrophotometry. Spots located by ultraviolet photography were eluted in 0.1N-HCl and spectra were determined against appropriate paper blanks. Spectra were also measured on the same solution after addition of 2N-KOH to give a final concentration of 0.1N. For quantitative estimation, the following millimolar extinction coefficients in 0.1N-HCl were used: 6-amino-2-methylpurine, 12.9 at 265 μ (Baddiley, Lythgoe & Todd, 1944); 6-methylaminopurine, 15.1 at 267 μ (Dunn & Smith, 1958);

6-dimethylaminopurine, 15.6 at 277 μ (Elion, Burgi & Hitchings, 1952); 2-amino-6-hydroxymethylpurine, 11.4 at 250 μ (Smith & Dunn, 1959); 6-hydroxy-2-methylaminopurine, 12.3 at 250 μ (Elion, Lange & Hitchings, 1956); 6-hydroxy-2-dimethylaminopurine, 14.5 at 258 μ (Elion *et al.* 1956); uridylic acid, 9.89×10^3 at 260 μ (Markham, 1955).

RESULTS

The ability of nucleic acid preparations to retain tenaciously a variety of impurities made it important to show that the methylated purines were derived from polyribonucleotides. This was especially true for the crude ribonucleoprotein used here as starting material for methylated purine isolation. We have used several criteria to show that the methylated purine bases probably occur naturally as nucleotides in RNA. These included: (i) the isolation of the 2'- and 3'-phosphates of 6-hydroxy-2-dimethylaminopurine (Smith & Dunn, 1959; Bergquist & Matthews, 1959) from mammary tumour from C3H mice; (ii) identification of 2-amino-6-hydroxy-1-methylpurine and 6-hydroxy-2-methylaminopurine ribonucleosides after digestion of salt-isolated RNA by the diesterase and 5'-nucleotidase present in crude snake venom with tobacco leaves; (iii) proof that the nucleotides of 2-amino-6-hydroxy-1-methylpurine, 6-hydroxy-2-methylaminopurine and 6-hydroxy-2-dimethylaminopurine as isolated from alkaline digests were 3'-(or 2'-) ribonucleotides and not 5'-nucleotides by their inability to form borate complexes both before and after 5'-nucleotidase treatment with rabbit-liver and mammary-tumour soluble fractions; (iv) dephosphorylation with prostatic phosphomonoesterase of the ribonucleotides of 2-amino-6-hydroxy-1-methylpurine, 6-hydroxy-2-methylaminopurine and 6-hydroxy-2-dimethylaminopurine obtained from alkaline hydrolysates; and characterization of the nucleosides by spectra, electrophoretic mobilities and R_F values as well as by the spectra of their bases obtained by mild acid hydrolysis with tobacco leaves, and soluble fractions from rabbit liver and mammary tumour; (v) inability to dialyse the 6-hydroxy-2-methylaminopurine of unhydrolysed samples of either phenol-prepared RNA or the crude ribonucleoprotein as used in our method through cellophane with either water or M-NaCl as the dialysis solution with pH 5.2 supernatant fraction from albino-mouse liver; (vi) identification of the ribonucleosides of 6-hydroxy-2-methylaminopurine and 6-hydroxy-2-dimethylaminopurine and their bases, after isolation by methods similar to those used by Littlefield & Dunn (1958) with mouse mammary-tumour soluble fraction.

The approximate molar proportions of the methylated purine bases from a number of preparations of

RNA nucleotides from animal sources and tobacco-leaf soluble fraction are presented in Tables 1-4. The uracil content of the preparations used as a basis of comparison was determined by submitting a measured portion of the uridylic acid from the samples to the chromatographic steps used for

isolating and estimating the methylated purine bases. In six tests recoveries of uridylic acid ranged from 45 to 55 %, with a mean value of 51 %.

Table 1 shows the distribution of methylated purines in the subcellular fractions of liver and mammary tumour. The soluble fraction contains

Table 1. *Approximate molar proportions of methylated purines in subcellular fractions from mouse liver, an adenocarcinoma from C3H mice and S 180 tumour in the ascites form*

Subcellular fractions from C3H mouse tissues were prepared in medium C; subcellular fractions from S180 cells were prepared from an homogenate in medium A. To provide sufficient material for analysis, two preparations of subcellular fractions from S180 ascites tumour were made on different days and the mitochondrial and microsomal fractions obtained from each of the two preparations were pooled. The soluble fractions were processed separately and one of the preparations was fractionated into pH 5.2 precipitate and supernatant. 2-Amino-6-hydroxy-1-methylpurine was estimated with system I; 6-hydroxy-2-methylaminopurine and 6-hydroxy-2-dimethylaminopurine were estimated after isolation in system II. —, Not examined.

Tissue	Fraction	Moles/100 moles of uracil					
		6-Amino-2-methylpurine	6-Methylaminopurine	6-Dimethylaminopurine	2-Amino-6-hydroxy-1-methylpurine	6-Hydroxy-2-methylaminopurine	6-Hydroxy-2-dimethylaminopurine
C3H mouse liver	Mitochondria	—	—	—	< 0.10	0.57	< 0.10
	Microsomes	< 0.10	< 0.10	< 0.10	0.08	0.60	< 0.10
	Soluble	< 0.10	0.78	0.55	0.86	5.20	1.36
C3H mouse tumour	Mitochondria	—	—	—	0.24	0.78	< 0.10
	Microsomes	< 0.10	< 0.10	< 0.10	0.14	0.82	< 0.10
	Soluble	< 0.10	4.20	1.16	1.03	12.40	3.20
S180 ascites tumour	Mitochondria	< 0.20	< 0.17	< 0.16	< 0.09	0.54	< 0.10
	Microsomes	< 0.17	< 0.15	< 0.14	< 0.08	0.65	< 0.06
	pH 5.2 precipitate	< 0.90	2.24	< 0.90	1.20	1.12	1.27
	pH 5.2 supernatant	2.42	3.48	2.16	3.20	6.20	2.18
	Total soluble*	—	—	—	2.50	4.40	1.86
	Total soluble†	—	—	—	3.02	5.20	2.00

* Calculated from the total number of moles of methylated guanines isolated from the pH 5.2 fractions examined separately.

† Estimated on whole soluble fraction.

Table 2. *Approximate molar proportions of methylated guanines in subcellular fractions from rabbit liver and mouse spleen and liver*

Rabbit-liver mitochondria and microsomes were sedimented and then washed twice each in medium A. Mouse-liver and -spleen mitochondria and microsomes were prepared in and washed once with medium B. The washings from the microsomal fractions were combined with the first microsomal supernatant to give the soluble fraction. 2-Amino-6-hydroxy-1-methylpurine was isolated by system III from rabbit-liver subcellular fractions and by system I from mouse tissues. 6-Hydroxy-2-methylaminopurine and 6-hydroxy-2-dimethylaminopurine were isolated by system III from rabbit and mouse subcellular fractions.

Tissue	Fraction	Moles/100 moles of uracil		
		2-Amino-6-hydroxy-1-methylpurine	6-Hydroxy-2-methylaminopurine	6-Hydroxy-2-dimethylaminopurine
Rabbit liver	Mitochondria	0.20	0.12	0.15
	Microsomes	0.16	0.02	0.06
	Soluble	4.71	2.40	3.02
Mouse liver	Mitochondria	0.27	1.60	0.22
	Microsomes	< 0.05	0.08	0.19
	Soluble	2.23	7.90	1.40
Mouse spleen	Mitochondria	< 0.10	< 0.10	< 0.10
	Microsomes	< 0.10	< 0.10	< 0.10
	Soluble	0.62	3.20	0.85

the greater proportion of the methylated bases. The soluble fraction of tumour was richer than that of liver in these minor RNA constituents.

Table 1 also provides data on subcellular methylated purine distribution in S 180 ascites tumour. The soluble fraction of S 180 cells also possessed a higher proportion of methylated purines than did the mitochondrial and microsomal fractions. The pH 5.2 supernatant is relatively richer in these compounds than the pH 5.2 precipitate.

Analysis of rabbit-liver subcellular fractions also showed that, like the mouse tissues examined, the highest proportions of methylated purines were in the soluble fraction (Table 2). Unlike the mouse tissues, however, the predominant methylated base in rabbit-liver fractions is 2-amino-6-hydroxy-1-methylpurine.

There are higher proportions of all methylated guanine bases in the soluble fraction from albino-mouse liver than in that from mouse spleen (Table 2). Microsomal RNA from normal albino-

mouse liver has a very low proportion of methylated guanines. Because of lack of material, the detectable concentrations for methylated guanines in spleen microsomal RNA were only 0.1 mole/100 moles of uracil. Thus it is not known whether the values of methylated guanine for the two microsomal fractions are similar.

The method for preparing RNA nucleotides for chromatography may have important effects on the proportions of methylated purine bases that can be isolated as well as on the total recovery of nucleotides noted earlier. Table 3 gives the proportions of methylated purines in the soluble fraction of *N. tabacum*, estimated by the procedure given in this paper and on RNA extracted by hot 10% NaCl. The salt procedure also gave substantially lower estimates for most of the methylated purines in mouse-liver subcellular fractions (Table 4). Not enough RNA was obtained by salt extraction of the pH 5.2 fractions for adequate analyses to be performed.

Table 3. *Approximate molar proportions of methylated purines in ribonucleic acid prepared by different methods from the soluble fraction of Nicotiana tabacum leaf tissue*

RNA nucleotides were prepared by the method described in this paper or by 10% NaCl extraction (Tyner, Heidelberger & LePage, 1953). 'Extracted RNA' is the fraction extracted by hot 10% NaCl; 'pellet RNA' is that RNA remaining in the denatured protein pellet after NaCl extraction and examined by the method described in this paper. 2-Amino-6-hydroxy-1-methylpurine was isolated by system I; 6-hydroxy-2-methylaminopurine was isolated by system II.

Method	Moles/100 moles of uracil					
	6-Amino-2-methylpurine	6-Methylaminopurine	6-Dimethylaminopurine	2-Amino-6-hydroxy-1-methylpurine	6-Hydroxy-2-methylaminopurine	6-Hydroxy-2-dimethylaminopurine
This paper	0.38	0.51	0.36	1.06	1.89	< 0.08
10% NaCl-'extracted RNA'	0.20	0.40	0.30	0.84	1.62	< 0.10
10% NaCl-'pellet RNA'	< 0.10	< 0.10	< 0.10	0.20	0.13	< 0.10

Table 4. *Influence of method of preparation of ribonucleic acid on proportions of methylated guanines in subcellular fractions from mouse liver*

Approximate molar proportions of methylated guanine bases in RNA, prepared as described in this paper or by 10% NaCl extraction, from subcellular fractions. Fractions were prepared from an homogenate in medium B; particles were washed twice before preparation of the RNA. 2-Amino-6-hydroxy-1-methylpurine, 6-hydroxy-2-methylaminopurine and 6-hydroxy-2-dimethylaminopurine were isolated by system III.

Method	Fraction	Moles/100 moles of uracil		
		2-Amino-6-hydroxy-1-methylpurine	6-Hydroxy-2-methylaminopurine	6-Hydroxy-2-dimethylaminopurine
This paper	Mitochondria	0.24	0.18	0.02
	Microsomes	0.10	0.30	0.06
	pH 5.2 precipitate	0.29	3.01	0.25
	pH 5.2 supernatant	8.00	8.25	3.50
	Total for soluble	0.34	3.71	0.70
10% hot NaCl extraction	Mitochondria	0.26	0.07	< 0.03
	Microsomes	0.06	0.03	< 0.01
	pH 5.2 precipitate	*	*	*
	pH 5.2 supernatant	*	*	*

* Insufficient RNA was extracted to be used for analysis.

DISCUSSION

There are considerable technical difficulties in isolating compounds that may be present in RNA in amounts as low as one base in 400 000. Nevertheless, our data show substantial differences in the proportions of methylated purines present in the ribonucleotides from the RNA of various cell fractions. However, we have failed to discover a suitable solvent for the isolation of 2-amino-6-hydroxy-1-methylpurine and 6-hydroxy-2-methylaminopurine ribonucleotides. Thus it has not been possible to prove that all the methylated purines discussed here existed in RNA.

Although it may be assumed that the methylated purines do in fact occur in polynucleotides, two sources of error in the data must be considered: (i) that the compounds may undergo transformation during isolation; (ii) that the methylated purines are located exclusively in one fraction of the cell and are present as contaminants in the other fractions. These will be considered in turn.

Brookes & Lawley (1960) have shown that 1-methyladenylic acid undergoes transformation into the ribonucleotide of 6-methylaminopurine under mild alkaline conditions. The above-mentioned work was reported after the experiments described here had been completed. Thus some of our results for the proportions of 6-methylaminopurine in subcellular fraction RNA may not represent the true situation *in vivo*; e.g. some of the results for 6-methylaminopurine in Tables 1 and 2 may be in error. D. B. Dunn & J. D. Smith (unpublished work) found that 6-amino-1-methylpurine is present in pig-liver RNA, but this compound is not present in *Escherichia coli* soluble RNA (Dunn, Smith & Spahr, 1960). We were unable to test for this base through lack of sufficient material. The possibility of a transformation of 2-amino-6-hydroxy-1-methylpurine derivatives into 6-hydroxy-2-methylaminopurine derivatives under alkaline conditions was eliminated by Smith & Dunn (1959).

The results reported in Tables 1 and 2 show that the soluble fraction is much richer in methylated purines than the mitochondrial or microsomal fractions. The occurrence of methylated bases in decreased proportions in the mitochondrial and microsomal RNA poses the question whether they are true components of these RNA or contaminants due to the presence of soluble RNA. The data in Table 4 show that methylated guanines are still present in twice-washed rabbit-liver mitochondrial and microsomal fractions. These minor components were present even in five-times-washed mitochondria of mouse liver. Thus if soluble RNA were contaminating the mitochondrial and microsomal fractions, it must be associated with these particles

in such a way as not to be removed by the washing procedure commonly used in cell-fractionation studies.

The highest proportions of methylated guanines are found in the pH 5.2 supernatant RNA. This fraction contains, on a molar basis, three to five times as much methylated guanines as the pH 5.2 precipitate RNA, but represents only about 30% of the soluble RNA as isolated from mouse liver. Both the pH 5.2 precipitate RNA and pH 5.2 supernatant enzymes are required for the transfer of amino acids to microsomal protein (Hoagland, 1960). As yet there is no evidence suggesting a function for the RNA in the pH 5.2 supernatant fraction in either amino acid transfer or incorporation. The methylated purines in the supernatant RNA may have some functional role in these steps in protein synthesis if the pH 5.2 supernatant RNA is shown to be required in the reaction. Alternatively, the pH 5.2 supernatant RNA may merely act as a store of fragments of RNA from the degradation of, or for the synthesis of, pH 5.2 precipitate RNA.

In the discussion that follows we draw two conclusions from our data: that pH 5.2 precipitate RNA contains on the average about one methylated purine per polynucleotide chain, and that published estimates of the amounts of soluble RNA associated with microsomes can be approximately accounted for by assuming that the methylated purines found in microsomes are in pH 5.2 precipitate RNA containing one methylated purine per polynucleotide chain.

The majority of the published estimates of the molecular weight of soluble RNA (Weiss, Acs & Lipmann, 1958; Hoagland, Stephenson, Scott, Hecht & Zamecnik, 1958; Dunn, 1959; Hecht *et al.* 1958; Preiss, Berg, Ofengand, Bergmann & Dieckmann, 1959; Tissieres, 1959; Singer & Cantoni, 1960; Zamecnik & Stephenson, 1960; Otaka & Osawa, 1960) were obtained by ultracentrifuging procedures on whole soluble-fraction RNA. The values range approximately from 1.7×10^4 to 4.0×10^4 , with a mean of about 2.9×10^4 . These values probably represent estimates of the molecular weight of pH 5.2 precipitate RNA, since ultracentrifugal analysis will emphasize the sedimentation behaviour of the major fraction in whole soluble RNA.

We have calculated from the proportion of methylated guanines in five samples of pH 5.2 precipitate RNA given in this paper and another (Bergquist & Matthews, 1962) that, if the average molecular weight of a nucleotide is 320, and if each molecule of the RNA contained only one methylated guanine, then the molecular weight of pH 5.2 precipitate RNA would be $3.1\text{--}5.5 \times 10^4$, with a mean value of 4.1×10^4 (S.E.M. 0.39×10^4). This is in

reasonable agreement with the mean value of 2.9×10^4 obtained by ultracentrifugal analysis. In the above calculations we have ignored the methylated adenines. If total methylated purine content had been used the calculated molecular weights would have been lower by 30–50%. Thus to a first approximation it is reasonable to assume that on the average there is about one methylated purine residue per unit of pH 5.2 precipitate RNA.

There are two sets of data in the literature on microsomal RNA that can be used to test, in a preliminary way, the proposition that the methylated purines found in microsomes are due to the presence of soluble RNA.

First, Hoagland & Comly (1960) estimated that 29–35% of the soluble RNA in the cell is required to saturate microsomal RNA during protein synthesis *in vitro*. Assuming one methylated guanine per unit of pH 5.2 precipitate RNA, we have calculated from five samples that 45% (range 24–60%) of the pH 5.2 precipitate RNA in the cell would have been associated with the microsomes. If 70% of whole soluble RNA was in the pH 5.2 precipitate, this gives a mean value of 31% for whole soluble RNA.

Secondly, Hoagland & Comly (1960) found that, after incubation in a medium for amino acid incorporation, an amount of soluble RNA equivalent to 1–5% of microsomal RNA was isolated with the microsomes. We have calculated from four sets of data with the same assumptions as above that 14% (range 2–22%) of the RNA in the microsome fractions would have come from pH 5.2 precipitate RNA. Thus for both the proportion of total soluble RNA associated with microsomes and the proportion of RNA in microsome preparations that may be soluble RNA, our calculations give figures of the same order as those of Hoagland & Comly. Thus within the errors in the various estimations involved the available data are consistent with the idea that methylated purines occur only in soluble RNA, but this proposition is, of course, by no means demonstrated.

For example, there are no independent estimates available for amounts of soluble RNA that might be associated with mitochondria. However, investigations (McLean, Cohn, Brandt & Simpson, 1958; Kalf & Simpson, 1959; Reis, Coote & Work, 1959; Roodyn, Reis & Work, 1961) have shown that mitochondria are able to incorporate amino acids into protein *in vitro*. Greengard & Campbell (1959) have demonstrated that transfer (soluble) RNA stimulates the incorporation of amino acids into mitochondrial protein. Hoagland (1960) interprets this finding to mean that transfer RNA diffuses into mitochondria and participates in the synthetic reaction. Thus it is probable that a portion of the material isolated as mitochondrial RNA is actually

soluble RNA so firmly bound to the particles that it sediments with the mitochondrial fraction (see also Siekevitz, 1959). The methylated purines we have found even in the most carefully washed mitochondrial fractions might well be part of such soluble RNA.

SUMMARY

1. A procedure is described for the quantitative isolation and estimation of ribonucleic acid ribonucleotides from subcellular fractions. This procedure gave similar yields to salt or phenol extraction for microsomal and mitochondrial ribonucleic acid, and higher yields for soluble ribonucleic acid.

2. In all tissues examined (mouse liver and spleen, two mouse tumours, rabbit liver, tobacco leaf) the highest proportion of methylated purines was found in the soluble ribonucleic acid.

3. For all subcellular fractions, the highest concentrations of methylated purines were found in fractions from a mammary adenocarcinoma of C3H mice.

4. When soluble ribonucleic acid was fractionated by adjustment to pH 5.2, the ribonucleic acid in the supernatant fluid contained a higher proportion of methylated purines than that in the precipitate.

5. It is suggested that all the methylated purines are located in soluble ribonucleic acid and that the methylated purines found in microsomal and mitochondrial preparations are due to soluble ribonucleic acid that is not removed from these particles during their isolation.

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Effects of 8-Azaguanine on the Composition of Ribonucleic Acids from Subcellular Fractions

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Several synthetic analogues of purine and pyrimidine bases that are inhibitory for growth are known to be incorporated into the nucleic acids of organisms, replacing some or all of the normal bases. Viruses containing unnatural bases in their nucleic acids may be less infectious than normal virus. For example: 8-azaguanine in the RNA of tobacco mosaic virus (Matthews, 1954) and turnip yellow-mosaic virus (Matthews, 1955), 5-bromouracil in the DNA of T2 phage (Litman & Pardee, 1956) and 2-thiouracil in the RNA of tobacco

mosaic virus (Francki & Matthews, 1959; Francki, 1960). It is, however, by no means established that this incorporation into complete virus particles causes a reduction in the rate of virus multiplication. Even less is known about the relationship between incorporation of analogue and growth inhibition in bacterial, plant and animal cells, although there is indirect evidence that the analogue may inhibit the production of specific proteins through the production of ineffective RNA containing the analogue (e.g. Creaser, 1956; Chantrenne & Devreux, 1960*a, b*; Aronson, 1961*a, b*).

Previous analyses on the incorporation of analogues into RNA have been carried out on preparations from whole cells. In the work described below we have observed substantial differences in

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