

The Occurrence and Distribution of Thymine and Three Methylated-Adenine Bases in Ribonucleic Acids from Several Sources

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It has been known for several years that deoxy-ribonucleic acid may normally contain 5-methylcytosine (Wyatt, 1951), 5-hydroxymethylcytosine (Wyatt & Cohen, 1953), and 6-methylaminopurine (Dunn & Smith, 1955, 1958*a*) in addition to adenine, guanine, cytosine and thymine. The first evidence of such additional bases naturally present in ribonucleic acid is the recent isolation of an unidentified nucleotide from the ribonucleic acid of yeast (Cohn, 1957; Davis & Allen, 1957). During studies on the incorporation of unnatural bases, Dr J. D. Smith and one of us (D.B.D.) each noted that digests of ribonucleic acid from *Escherichia coli* contained a compound with spectral and chromatographic characteristics which suggested that it was the riboside of thymine (J. D. Smith, unpublished work; Dunn, 1957). The present work has not only confirmed this finding but has indicated that three methylated adenine bases, 2-methyladenine, 6-methylaminopurine and 6-dimethylaminopurine, as well as thymine, are naturally present in small amounts in ribonucleic acid from a variety of sources. None of these compounds has been described previously as a component of ribonucleic acid, but all four are known to occur naturally as nucleosides or nucleotides, since thymine and 6-methylaminopurine occur in deoxyribonucleic acid (Kossel & Neumann, 1893; Dunn & Smith, 1958*a*), 2-methyladenine in the vitamin B₁₂-like Factor A extracted from *E. coli* and other sources (Dion, Calkins & Piffner, 1954; Brown, Cain, Gant, Parker & Smith, 1955) and 6-dimethylaminopurine in puromycin formed by the actinomycete *Streptomyces alboniger* (Waller, Fryth, Hutchings & Williams, 1953). The presence of 6-methylaminopurine in ribonucleic acid from yeast has also

been reported by Adler, Weissmann & Gutman (1958).

This paper describes the isolation of the ribosides and ribotides of these compounds from several ribonucleic acids and their identification by chromatographic, electrophoretic and spectroscopic methods. Preliminary results of this work have been reported (Littlefield & Dunn, 1958*a, b*).

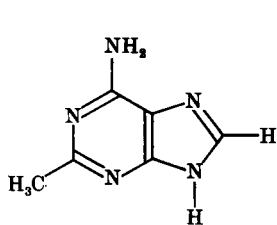
MATERIALS AND METHODS

Growth of bacteria. *E. coli* strains B/r and 15T⁻ and *Aerobacter aerogenes* were grown as previously described (Dunn & Smith, 1958*a*). For *E. coli* 15T⁻, medium A was supplemented with 100 mg. of thymine/l.

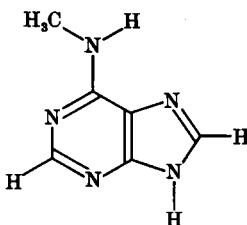
Staphylococcus aureus strain H was grown in aerated nutrient broth. The medium was inoculated with 1 part in 100 of an 18 hr. culture of the bacteria and it was then incubated at 37° for 6 hr.

Preparation of ribonucleic acid

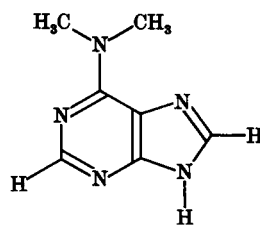
Bacteria. Strains of *E. coli* and *A. aerogenes* were extracted for 18 hr. with 60% (v/v) ethanol-water (pH 5) at 20° to remove soluble nucleosides and nucleotides. The cells were separated by centrifuging and washed twice with 60% (v/v) ethanol-water and once each with 90% (v/v) ethanol-water, ethanol and acetone. After drying in air, the nucleic acid was extracted with 0.1M-sodium citrate (pH 7.2) at 100° for 1-2 hr. (Smith & Matthews, 1957). The mixture was centrifuged at 10 000 rev./min. for 10 min. and the sediment re-extracted with citrate solution. The combined extracts contained 90-98% of the ribonucleic acid (RNA) from the bacteria and 60-80% of the deoxyribonucleic acid (DNA). In two preparations of nucleic acid from *E. coli* B/r, protein was removed from the solution by shaking with a chloroform-octanol mixture (Sevag, Lackman & Smolens, 1938). The RNA and DNA were precipitated from the citrate solution by adjusting to pH 4



2-Methyladenine



6-Methylaminopurine



6-Dimethylaminopurine

with acetic acid and adding ethanol to give a concn. of 67% (v/v). Where necessary, a few drops of *m*-MgSO₄ were added to aid precipitation. To ensure the removal of all free nucleotides, the precipitation from citrate solution was repeated 2 or 3 times, and the final precipitate washed twice with 60% (v/v) ethanol-water.

The soluble nucleotides were extracted from *S. aureus* with 5% (w/v) aqueous trichloroacetic acid at 20° for 10 min. After centrifuging, the trichloroacetic acid was removed from the residue by three extractions with ether. The citrate extraction was then carried out as above, but only about 50% of the RNA present in this material was liberated. It was possible to remove most of the remaining RNA from the residue by autoclaving it with a further portion of citrate buffer at 120° for 15 min. The material isolated by the two methods was united for analysis.

Yeast. Commercial yeast RNA (British Drug Houses Ltd.) which had been reprecipitated with ethanol (Markham & Smith, 1950*a*) was used for analysis.

Liver. A sample of RNA isolated from whole rabbit liver several years ago was available in the laboratory. Rat-liver microsomes prepared according to Zamecnik & Keller (1954) were washed with 60% (v/v) ethanol-water, ethanol and acetone before the RNA was hydrolysed with alkali. In one preparation the RNA was extracted in 0.1*M*-citrate (pH 7) at 100° for 15 min. and precipitated as described above.

Wheat germ. A sample of wheat-germ RNA was kindly supplied by Dr J. D. Smith. It had been isolated from commercially prepared wheat germ (Bemax, Vitamins Ltd.). This had been mixed with 0.14*M*-NaCl in a Waring Blendor for 15 min., the mixture centrifuged for 30 min. at 3000 rev./min. and the turbid supernatant separated from the precipitate which contained most of the DNA. Ethanol to give 67% (v/v) was then added to the supernatant and the precipitate separated by centrifuging. This material was adjusted to pH 7 and RNA extracted with *m*-NaCl at 100° for 10 min. After low-speed centrifuging for 15 min., the NaCl extraction was repeated on the residue. The combined extracts were centrifuged at 10 000 rev./min. for 15 min. before protein was removed according to Sevag *et al.* (1938) and the RNA precipitated with acid and ethanol. The RNA was washed successively in 60% (v/v) ethanol-water, ethanol and acetone and then allowed to dry in air.

Viruses. Tobacco mosaic virus was prepared by differential centrifuging (Stanley, 1937) and the RNA isolated according to Markham & Smith (1950*b*). A sample of RNA isolated from turnip yellow mosaic virus was obtained from Dr J. I. Harris. The virus, prepared according to Markham & K. M. Smith (1949), had been treated with 67% (v/v) acetic acid to separate the protein and nucleic acid (Fraenkel-Conrat, 1957).

Hydrolysis of ribonucleic acid

Hydrolysis to nucleoside 2'- and 3'-phosphates. Samples containing 20-70 mg. of RNA were incubated with 0.5-2 ml. of *m*-KOH at 30° for 18 hr. The mixture was neutralized with HClO₄, and acetic acid added to pH 4. Ethanol to give 67% (v/v) was added to precipitate any DNA and protein, and these together with insoluble KClO₄ were removed by centrifuging. The solution was evaporated to dryness in a stream of air at 50° (Markham, 1955). The dried material was mixed with a small volume of water and

cooled for 1 hr. at 5° to precipitate more KClO₄ and the aqueous solution of nucleotides separated from the residue by centrifuging. When necessary, this solution was evaporated at 50° to a convenient volume before being applied as lines 18 cm. long on 2-6 paper chromatograms.

Hydrolysis to nucleosides. A sample of nucleic acid from *E. coli* B/r containing about 50 mg. of RNA was mixed in 2 ml. of 0.1*M*-glycine buffer (pH 9) and incubated at 37° for 36 hr. with 2 mg. of rattlesnake venom. Chloroform was added to prevent bacterial contamination and aq. *n*-NH₃ added when necessary to maintain the mixture at pH 9. Acetic acid to pH 4 and ethanol to give 60% (v/v) were added to the hydrolysate to precipitate the remaining DNA and protein and these were then removed by centrifuging.

Conversion of nucleotides into nucleosides

The samples of nucleotides in about 0.5 ml. of 0.1*M*-sodium acetate buffer (pH 5) were incubated with 0.05 ml. of a solution of prostatic phosphomonoesterase (2 mg./ml.) (Markham & Smith, 1952*b*) for 3-6 hr. at 37°. After concentration by evaporation, the solution was used directly for chromatography.

Conversion of nucleosides into bases

Hydrochloric acid. *n*-HCl at 100° for 1 hr. was used to release the purines from their nucleosides (Vischer & Chargaff, 1948).

Perchloric acid. Thymine riboside was converted into thymine by heating with 72% (w/v) HClO₄ at 100° for 2 hr. (Marshak & Vogel, 1951). After dilution with an equal volume of water, the hydrolysate was applied to a chromatogram, which was subsequently run in solvent 1.

Periodate and alkali. Whitfield (1954) described the formation of cytosine from cytidine as a result of the successive effects of periodate and alkali. A modification of this method, previously used to convert 5-chlorouridine into the base (Dunn, 1957), was used successfully with thymine riboside. The nucleoside was incubated with excess of 0.1*M*-sodium periodate for 0.5 hr. at 20°. An equal volume of 0.1*M*-glucose was then added to remove the excess of periodate; after a further incubation of 0.5 hr. at room temperature, an equal volume of aq. *n*-NH₃ was added to raise the pH above 10. After incubation for 18 hr. at 37° the products were chromatographed in solvent 3; a substantial proportion of the nucleoside was found to be converted into the base.

Isolation of ribose from nucleosides

Purine nucleosides. Although the purine ribosides were hydrolysed with *n*-HCl or *n*-H₂SO₄, the excess of acid, or salt after neutralization, affected the *R_F* of ribose in solvent 5 which was used to separate the sugar. It was found that 10-20 μg. of these ribosides was hydrolysed satisfactorily by 0.01 ml. of 0.1*N*-HCl in 1 hr. at 100° in a sealed tube. When this small quantity of acid was allowed to dry on paper before chromatographing, it did not affect the behaviour of the ribose.

Pyrimidine nucleosides. The reaction of pyrimidine ribosides with hydrazine (Baron & Brown, 1955) was utilized to detect ribose in thymine riboside. The material (120 μg.) was treated with 0.01 ml. of 60% aq. hydrazine hydrate at 100° for 1 hr. Benzaldehyde (0.025 ml.) was then added

and the mixture warmed at 50° for 10 min. After the addition of 0.5 ml. of water and five extractions with ether, the aqueous layer was chromatographed in solvent 5.

Chromatography and electrophoresis

For separating the nucleotides, nucleosides and bases the solvent mixtures 1-4 were used. Solvent 5 was used for the chromatography of ribose.

(1) Propan-2-ol (680 ml.), 11.6*N*-HCl (176 ml.), water to 1 l. (Wyatt, 1951); (2) propan-2-ol (700 ml.), water (300 ml.) with NH₃ in vapour phase (Markham & Smith, 1952*a*); (3) butan-1-ol (770 ml.), water (130 ml.), 98% formic acid (100 ml.) (Markham & J. D. Smith, 1949); (4) butan-1-ol (860 ml.), water (140 ml.) with NH₃ in vapour phase (Markham & J. D. Smith, 1949); (5) ethyl acetate (300 ml.), water (300 ml.), acetic acid (100 ml.) (Jermyn & Isherwood, 1949).

Whatman 3MM filter paper was used for solvent 2 and for the isolation of the enzymically prepared nucleosides on solvent 4. For all other chromatograms Whatman no. 1 paper was used.

Paper electrophoresis was carried out according to Markham & Smith (1952*a*) with 0.05*M*-buffers.

Nucleotides were eluted in water for transfer to other chromatograms. Except for transfer to electrophoresis papers, more efficient elution of nucleosides and bases was obtained with dilute formic acid (0.01-0.1*N*).

Spectrophotometry

Compounds located according to Markham & J. D. Smith (1949) were eluted in 0.1*N*-HCl and spectra measured against appropriate paper blanks in a Unicam Model SP. 500 spectrophotometer. Spectra were also measured on the same solution after the addition of 2*N*-KOH to give a final concentration of 0.1*N*. For estimation the following molar extinction coefficients in 0.1*N*-HCl were used: uridine, 10.1×10^3 at 262 μ (Fox & Shugar, 1952); thymine riboside, 9.65×10^3 at 267 μ [extinction coefficient for thymidine (Fox & Shugar, 1952)]; 2-methyladenosine, 12.5×10^3 at 258 μ [extinction coefficient at maximum for 2-methyladenine β -xyloside (Baddiley, Lythgoe & Todd, 1944)]; 6-methylaminopurine riboside, 18.3×10^3 at 262 μ ; 6-dimethylaminopurine riboside, 18.5×10^3 at 268 μ (Kissman, Pidaaks & Baker, 1955). The extinction coefficient for 6-methylaminopurine riboside was determined from the change in absorption of a solution when it was heated for 1 hr. in *N*-HCl, by using the molar extinction coefficient for 6-methylaminopurine of 15.1×10^3 (Dunn & Smith, 1958*a*).

Chemicals

The nomenclature used in this paper for purines and pyrimidines is that of Bendich (1955). Gifts of 2-methyladenine, 6-methylaminopurine and 6-dimethylaminopurine were obtained from Dr D. M. Brown, Dr D. J. Brown and the American Cyanamid Co. respectively. Specimens of 2-methyladenine β -ribofuranoside picrate, thymine β -ribofuranoside, 6-methylaminopurine β -ribofuranoside and 6-dimethylaminopurine β -ribofuranoside were obtained from Dr G. B. Brown and his colleagues. The preparation of the first two compounds has been described (Davoll & Lowy, 1952; Fox, Yung, Davoll & Brown, 1956). The last two compounds were prepared by Dr A. Hampton by unpublished methods. The dimethylaminopurine ribo-

side was identical with that prepared by Kissman *et al.* (1955) (Dr G. B. Brown, personal communication). The 2-methyladenosine picrate was converted into the free nucleoside and separated from the picric acid by chromatography in solvent 3. A sample of 2-methyladenine γ -ribofuranoside isolated from vitamin B₁₂-like Factor A was obtained from Professor Dr K. Bernhauer.

Enzymic preparation of ribosides

Using a nucleoside phosphorylase isolated from *E. coli*, Lampen (1952) synthesized a riboside of thymine, which has since been identified as the β -ribofuranoside (Fox *et al.* 1956). We have used similar conditions to prepare the ribosides of thymine, 2-methyladenine, 6-methylaminopurine and 6-dimethylaminopurine. An aqueous phosphate extract made from *E. coli* B/r, after being ground with Pyrex glass powder, was fractionated twice with (NH₄)₂SO₄ as in the method of Paege & Schlenk (1952) and the crude enzyme thus isolated was used without further purification. About 1 mg. of the methylated base and 3 mg. of inosine were incubated in 1 ml. of 0.01*M*-phosphate buffer (pH 7) at 37° for 2 hr. with 0.2 mg. of the enzyme preparation. The reaction mixture was concentrated in a stream of air at 50° and chromatographed as a line in solvent 4. In 18-24 hr. the riboside of the methylated base had separated from any free base and also from the slower-running inosine and hypoxanthine.

RESULTS

Isolation of ribosides

In chromatographic systems with propan-2-ol and butan-1-ol the methyl groups of thymine, 2-methyladenine, 6-methylaminopurine and 6-dimethylaminopurine give these bases, their ribosides and their ribotides greater mobility than that of uracil and adenine and their corresponding derivatives. By using this property, small amounts (one per 10 000 bases) of the methylated compounds can be separated conveniently from digests of RNA. The following method was used both for detecting these additional bases in RNA preparations from a range of organisms and for determining the proportions of these compounds present. After alkaline hydrolysis of the RNA, we purified the mixture of 2'- and 3'-phosphates as bands by chromatography for 18-24 hr. with solvent 2. The area of the paper which contained uridylic acid, adenylic acid and cytidylic acid was then eluted together with the area ahead up to the traces of ribosides present, even when no clear-cut ultraviolet-absorbing bands were apparent in this area. The eluted nucleotides were dephosphorylated with prostatic phosphomonoesterase and rechromatographed for 18 hr. in solvent 2. Only the front third of the main riboside band, plus again the area ahead almost up to the solvent front (with or without visible ultraviolet-absorbing bands), was eluted and chromatographed in two dimensions with solvent 3 followed by solvent 4. Fig. 1 shows

the positions of the ribosides of thymine, 2-methyladenine, 6-methylaminopurine and 6-dimethylaminopurine relative to the small amounts of adenosine and uridine included in this two-dimensional chromatogram. The only free base which has chromatographic behaviour similar to that of any of these four compounds is adenine. Small amounts of this compound have been observed in several preparations, and in the absence of markers it may be confused with 6-methylaminopurine riboside, as the two compounds do not differ markedly in their R_f in solvents 3 and 4 (Table 1) and have the same spectral maxima (Beaven, Holiday & Johnson, 1955; Table 2). Where both compounds were present on the same chromatogram they were distinguished by the difference in R_f ; except where only very small amounts were detected, the higher spectral minimum in alkali was used to identify adenine.

Identification of ribosides

As only small amounts of these four ribosides were isolated, their identification rests mainly on a comparison of their spectra and chromatographic behaviour with ribosides synthesized enzymically from known bases. More recently we have been able to confirm that these ribosides had the expected structures by direct comparison with specimens of chemically prepared ribofuranosides. In Figs. 2-5 the ultraviolet-absorption spectra at pH 1 and 13 of one or two naturally occurring compounds are superimposed on those of the corresponding known ribosides. The complete spectra of the naturally occurring compounds agreed closely with those of the known ribosides when reasonably large amounts were present, and in all instances the two maxima of the naturally occurring compounds were the same as those of the known compounds (Table 2). These maxima were in agreement with published data on the chemically prepared ribosides (Fox *et al.* 1956; Davoll & Lowy, 1952; Kissman *et al.* 1955). The enzymically and chemically prepared ribosides had the same R_f in four solvent systems (Table 1). In these four solvents the movement of each of the naturally occurring compounds corresponded with that of a synthetic riboside marker. Thymidine and 6-methylaminopurine deoxyriboside (Dunn & Smith, 1958*a*), available for comparison, ran ahead of thymine riboside and 6-methylaminopurine riboside in solvents 2, 3 and 4 (Table 1). The 2-methyladenosine isolated from RNA agreed both spectroscopically and chromatographically with the 9β -riboside, and not with the 7α -compound isolated from vitamin B₁₂-like Factor A (Friedrich & Bernhauer, 1957).

On electrophoresis in glycine buffer (pH 9.2) the purine ribosides failed to move appreciably, whereas thymine riboside showed a small mobility, due presumably to the ionization of the 6-hydroxyl group of the pyrimidine. In borate buffer at the

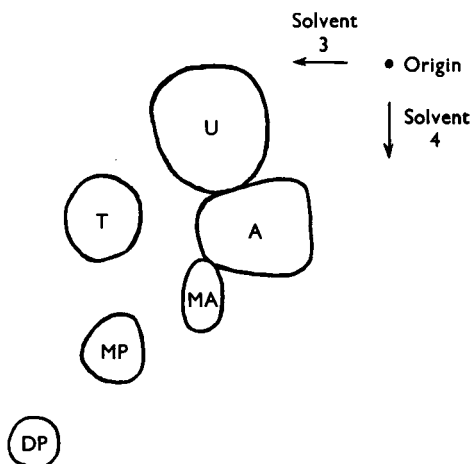


Fig. 1. Positions of the ribosides of thymine (T), 2-methyladenine (MA), 6-methylaminopurine (MP) and 6-dimethylaminopurine (DP) relative to those of uridine (U) and adenosine (A) on a chromatogram run two-dimensionally in solvents 3 and 4.

Table 1. R_f values for methylated bases, their ribosides and related compounds

For composition of solvents, see text.

	Solvent 1	Solvent 2	Solvent 3	Solvent 4
2-Methyladenine 9β -riboside	0.51	0.63	0.23	0.32
6-Methylaminopurine 9β -riboside	0.55	0.71	0.29	0.44
6-Dimethylaminopurine 9β -riboside	0.64	0.76	0.38	0.56
Thymine 3β -riboside	0.76	0.66	0.29	0.23
Adenosine	0.41	0.57	0.17	0.19
2-Methyladenine 7α -riboside	0.45	0.56	0.20	0.14
6-Methylaminopurine deoxyriboside	—	0.78	0.41	0.59
Thymidine	—	0.74	0.46	0.41
2-Methyladenine	0.48	0.64	0.37	0.46
6-Methylaminopurine	0.49	0.72	0.42	0.58
6-Dimethylaminopurine	0.52	0.78	0.44	0.70
Thymine	0.77	0.70	0.51	0.45
Adenine	0.37	0.61	0.28	0.35

same pH all the ribosides showed marked mobilities, the purine compounds moving with adenosine and thymine riboside between adenosine and uridine (Table 3). The different mobilities in these

two buffer systems indicated that the four compounds formed negatively-charged complexes with the borate ion and presumably contained the *cis*-2' and -3' hydroxyl groups of ribose (Jaenicke & Vollbrechtshausen, 1952). The formation of this

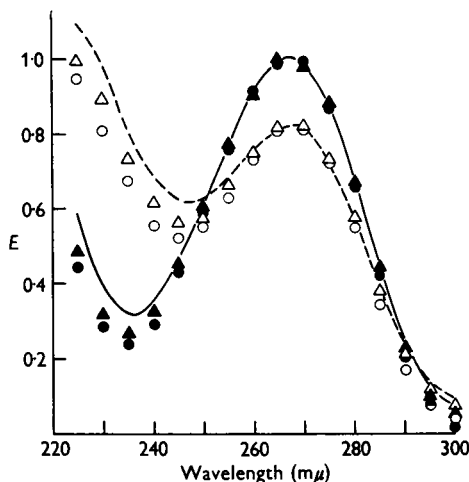


Fig. 2. Ultraviolet-absorption spectra of enzymically prepared thymine riboside in 0.1N-HCl (—) and in 0.1N-KOH (---). Points are for samples isolated from the RNA of wheat germ in 0.1N-HCl (●) and 0.1N-KOH (○), and of *E. coli* 15T⁻ in 0.1N-HCl (▲) and 0.1N-KOH (△). For each sample the spectra in acid and alkali are for solutions of the same concentration, the spectrum in acid being plotted to give a value for *E* of 1.0 at 267 mμ.

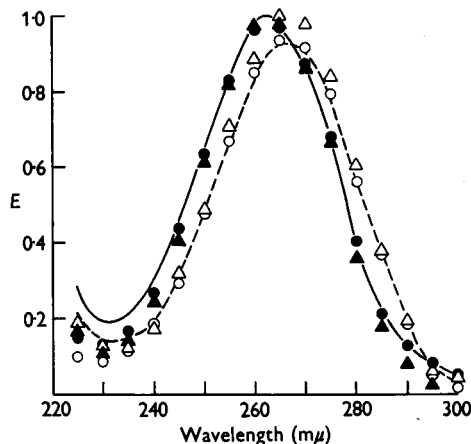


Fig. 4. Ultraviolet-absorption spectra of enzymically prepared 6-methylaminopurine riboside in 0.1N-HCl (—) and in 0.1N-KOH (---). Points are for samples isolated from the RNA of *A. aerogenes* in 0.1N-HCl (●) and 0.1N-KOH (○), and of yeast in 0.1N-HCl (▲) and 0.1N-KOH (△). For each sample the spectra in acid and alkali are for solutions of the same concentration, the spectrum in acid being plotted to give a value for *E* of 1.0 at 262 mμ.

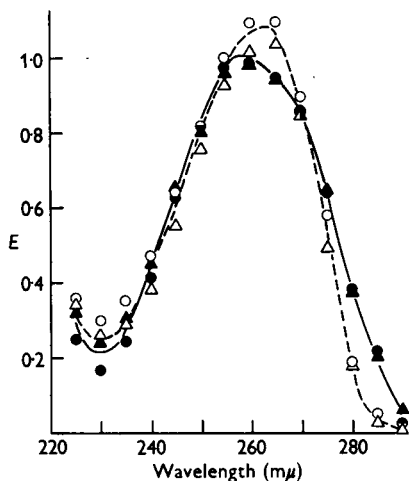


Fig. 3. Ultraviolet-absorption spectra of enzymically prepared 2-methyladenosine in 0.1N-HCl (—) and in 0.1N-KOH (---). Points are for samples isolated from the RNA of *E. coli* B/r in 0.1N-HCl (●) and 0.1N-KOH (○), and of *A. aerogenes* in 0.1N-HCl (▲) and 0.1N-KOH (△). For each sample the spectra in acid and alkali are for solutions of the same concentration, the spectrum in acid being plotted to give a value for *E* of 1.0 at 258 mμ.

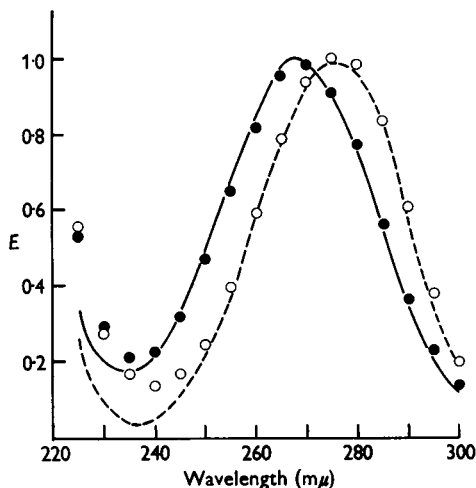


Fig. 5. Ultraviolet-absorption spectra of enzymically prepared 6-dimethylaminopurine riboside in 0.1N-HCl (—) and in 0.1N-KOH (---). Points are for a sample isolated from the RNA of *A. aerogenes* in 0.1N-HCl (●) and 0.1N-KOH (○). Spectra in acid and alkali are for solutions of the same concentration, the spectrum in acid being plotted to give a value for *E* of 1.0 at 268 mμ.

complex was also indicated by a markedly reduced R_f of these four ribosides in solvent 4 in the presence of borate.

Ribose, identified by its mobility on chromatography with solvent 5 and by the colour given on heating with aniline hydrogen phthalate (Partridge, 1949), was the only sugar detected in acid hydrolysates of the ribosides of 2-methyladenine and 6-methylaminopurine. Similarly, using the reaction of thymine riboside with hydrazine, we demonstrated the presence of ribose in this compound. The R_f in solvent 5 and the colour given with aniline hydrogen phthalate would distinguish ribose from deoxyribose, xylose, lyxose, and other pentoses and hexoses (Bernstein, 1953). Owing to the small amounts of material available we have not demonstrated the presence of ribose in 6-dimethylaminopurine riboside by this method.

Further evidence that these compounds were not deoxyribosides was provided by the conditions required for their acid hydrolysis. Unlike purine deoxyribosides, the purine ribosides were stable in 0.1N-HCl at 20°, although they were hydrolysed in N-HCl at 100°. The riboside of thymine was unchanged by this latter treatment but was converted into thymine by heating with perchloric acid, or by treatment with periodate followed by alkali. Thymidine would not have been converted into thymine by the latter method as it depends on the

presence of two adjacent hydroxyl groups in the sugar.

By the above methods the four ribosides in two preparations of RNA from *E. coli* and one from *A. aerogenes* were converted into the bases. In addition 6-methylaminopurine has been isolated from the riboside prepared from the RNA of liver and wheat germ. In all instances the bases released corresponded in chromatographic behaviour with known samples of thymine, 2-methyladenine, 6-methylaminopurine and 6-dimethylaminopurine (Table 1) and had the same ultraviolet-absorption maxima (Table 2). In Figs. 6-9 the spectra of the naturally occurring bases are superimposed on those of the known bases.

Isolation of ribotides

Since the RNA preparations were purified by reprecipitation, it seemed unlikely that these four methylated bases were present in the RNA preparations as free nucleotides. To exclude this possibility more conclusively, we have isolated from the RNA of *E. coli* substances that we presume are the riboside 2'- and 3'-phosphates of the four bases. As described above, we chromatographed an alkaline digest of RNA in solvent 2 and eluted the area between guanylic acid and the small amounts of ribosides present. This material was rechromatographed in solvent 2 for 48 hr. By this time 2 or 3 indistinct bands had separated ahead of uridylic acid; these were eluted together and run on paper electrophoresis in ammonium formate buffer at pH 3.5. We found that in this system the mobilities of the ribotides of thymine, 6-methylaminopurine, 6-dimethylaminopurine and 2-methyladenine were 0.90, 0.46, 0.42 and 0.19 of that of a uridine 2'- and 3'-phosphate marker. Although these compounds did not always give distinct bands, thymine riboside was thus separated from the mixture of ribotides of 6-methylaminopurine and 6-dimethylaminopurine, which were then distinguished by chromatography with solvent 2. The ribotide of 2-methyladenine was sometimes present on this electrophoresis paper, but was usually found by similar electrophoresis of the band containing uridylic acid. It was separated from any cytidylic acid by subsequent chromatography with solvent 2.

These ribotides were present in approximately the same amounts relative to uridylic acid as was predicted by the proportions of the ribosides isolated from the same RNA. They were dephosphorylated with prostatic phosphomonoesterase but not by rattlesnake venom containing a specific 5'-nucleotidase (Gulland & Jackson, 1938), and since they were formed by alkaline hydrolysis of the RNA they were regarded as riboside 2'- and 3'-phosphates. The ribosides produced by prostatic

Table 2. Ultraviolet-absorption maxima for methylated bases and their ribosides

	$\lambda_{\max.}$ (m μ)	
	pH 1	pH 13
Thymine	265	290
2-Methyladenine	266	271
6-Methylaminopurine	267	273
6-Dimethylaminopurine	277	281
Thymine 3 β -riboside	267	268
2-Methyladenine 9 β -riboside	258	263
2-Methyladenine 7 α -riboside*	273	276
6-Methylaminopurine 9 β -riboside	262	266
6-Dimethylaminopurine 9 β -riboside	268	276

* Data from Friedrich & Bernhauer (1957); the value in alkali is for pH 12.

Table 3. Electrophoretic mobilities of ribosides in borate and glycine at pH 9.2

Mobilities are expressed as distance (cm.) moved towards anode in 2 hr. with 20 v/cm.

	In borate buffer	In glycine buffer
Thymine riboside	12.7	2.7
2-Methyladenine riboside	8.9	0.2
6-Methylaminopurine riboside	9.5	0.5
6-Dimethylaminopurine riboside	9.7	0.4
Uridine	15.7	4.8
Adenosine	9.2	0.3

phosphomonoesterase, as well as the bases released on subsequent acid hydrolysis, were identical with those described above. The ultraviolet-absorption maxima of the ribotides were essentially the same as those of the ribosides given in Table 2. In solvent 2 the ribotides of 6-dimethylaminopurine,

6-methylaminopurine, thymine and 2-methyladenine had R_f values of 0.52, 0.44, 0.33, and 0.23, compared with 0.20 for uridine 2'- and 3'-phosphates. In the presence of large amounts of other ribotides the R_f values for these four compounds were reduced, and they frequently did not form

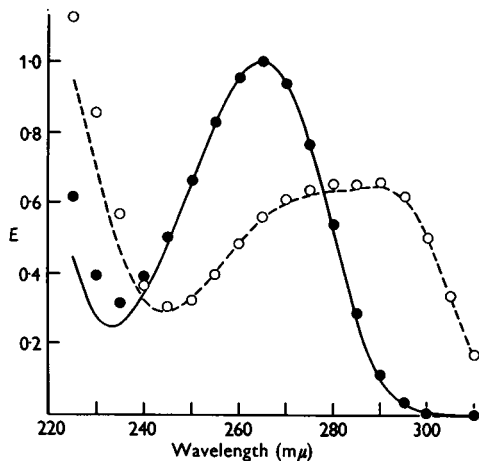


Fig. 6. Ultraviolet-absorption spectra of thymine. Smooth curves are for a synthetically prepared sample, and points for a sample isolated from the RNA of *E. coli* B/r, in 0.1N-HCl (●-●) and 0.1N-KOH (○-○). For each sample spectra in acid and alkali are for solutions of the same concentration, the spectrum in acid being plotted to give a value for E of 1.0 at 265 m μ .

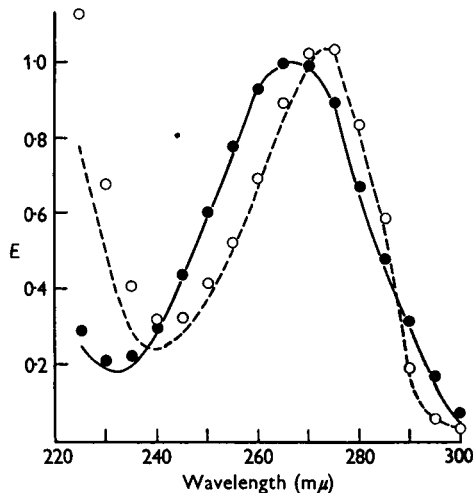


Fig. 8. Ultraviolet-absorption spectra of 6-methylaminopurine. Smooth curves are for a synthetically prepared sample, and points for a sample isolated from the RNA of *A. aerogenes*, in 0.1N-HCl (●-●) and 0.1N-KOH (○-○). For each sample spectra in acid and alkali are for solutions of the same concentration, the spectrum in acid being plotted to give a value for E of 1.0 at 267 m μ .

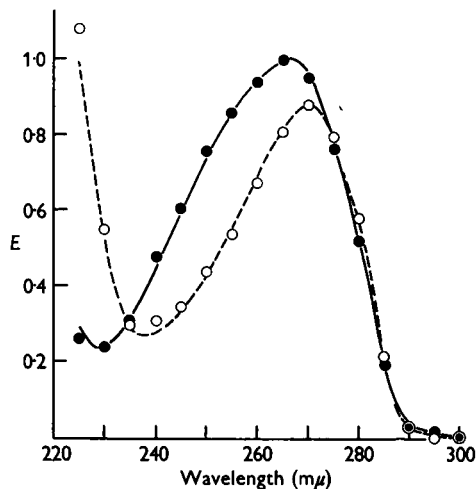


Fig. 7. Ultraviolet-absorption spectra of 2-methyladenine. Smooth curves are for a synthetically prepared sample, and points for a sample isolated from the RNA of *A. aerogenes*, in 0.1N-HCl (●-●) and 0.1N-KOH (○-○). For each sample spectra in acid and alkali are for solutions of the same concentration, the spectrum in acid being plotted to give a value for E of 1.0 at 266 m μ .

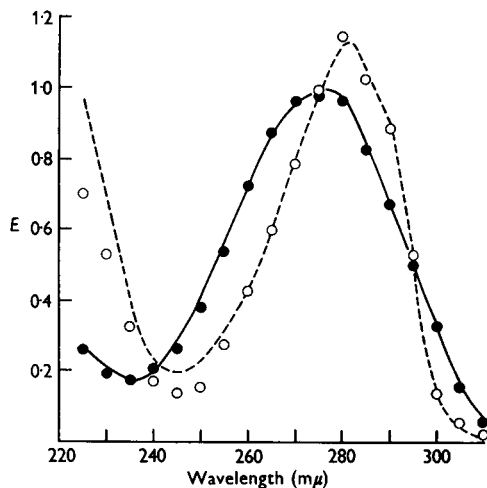


Fig. 9. Ultraviolet-absorption spectra of 6-dimethylaminopurine. Smooth curves are for a synthetically prepared sample, and points for a sample isolated from the RNA of *E. coli* B/r, in 0.1N-HCl (●-●) and 0.1N-KOH (○-○). For each sample spectra in acid and alkali are for solutions of the same concentration, the spectrum in acid being plotted to give a value for E of 1.0 at 277 m μ .

clear-cut bands ahead of uridylic acid. However, when present in large quantities, as in wheat-germ RNA, they may be separated by chromatography alone.

This evidence that the riboside 2'- and 3'-phosphates of thymine, 2-methyladenine, 6-methylaminopurine and 6-dimethylaminopurine can be prepared from the RNA of *E. coli* makes it unlikely that these compounds were present as free contaminants of our RNA preparations, since it would be unusual for the latter to occur in the 2'- and 3'-phosphate form (Schmitz, Hurlbert & Potter, 1954; Schmitz, 1954a, b; Manson, 1956; Ballio, Casinovi & Serlupi-Crescenzi, 1956; Dr J. T. Park, personal communication). Moreover, we have searched for and failed to find the ribotides or ribosides of thymine and 2-methyladenine in the solution obtained on extraction of *E. coli* with 60% ethanol at pH 5. (As this work was carried out before the isolation of 6-methylaminopurine and 6-dimethylaminopurine from the RNA, we did not examine this material for the nucleosides and nucleotides of these compounds.)

From the RNA of *E. coli* digested with rattlesnake venom we have isolated the ribosides of thymine, 2-methyladenine and 6-methylaminopurine, and material with the chromatographic behaviour of the riboside of 6-dimethylaminopurine. This last compound has been detected in two such digests, but in both instances the ultraviolet absorption of this material has been greater than was expected and the acid and alkaline maxima have been 3-4 m μ below those expected, suggesting that it might be contaminated by another compound. As the nucleotidase in rattlesnake venom would be expected to hydrolyse 5'-nucleotides, but not 3'-nucleotides (Gulland & Jackson, 1938), the formation of the ribosides by this method indicates that the 5'-ribotides were formed as intermediates. This provides evidence that some, if not all, of these four compounds are present in the RNA in 3':5'-phosphodiester linkages.

*Amounts of methylated bases in RNA
from different sources*

In Table 4 are shown the molar proportions of thymine, 2-methyladenine, 6-methylaminopurine and 6-dimethylaminopurine relative to uracil in RNA from various sources. The values were actually determined from the ultraviolet absorption of the isolated ribosides, a known portion of the total ribosides being chromatographed in solvent 1 to isolate the uridine for estimation. Most of the figures were based on a single analysis, but where two analyses were carried out the results varied by 10-70%, the smaller variations being obtained where larger amounts of the riboside were isolated. In Table 4 all multiple analyses have been averaged.

Each microbial RNA contained all four bases in similar proportions, except that 6-dimethylaminopurine appeared to be absent from the RNA of *S. aureus*. *E. coli*, a known source of a compound containing 2-methyladenine (Dion *et al.* 1954; Brown *et al.* 1955), had a relatively large proportion of this base in the RNA. In wheat-germ RNA thymine and 6-methylaminopurine were particularly prominent, the ribotides of these two bases in an alkaline digest forming clear-cut bands ahead of uridylic acid with solvent 2.

In contrast with the results with RNA extracted from plant and microbial sources, thymine and 2-methyladenine were absent from the RNA of both whole rabbit liver and of rat-liver microsomes. The RNA of turnip yellow mosaic virus was essentially free (less than one per 7000 nucleotides) from all four bases and the RNA of tobacco mosaic virus contained only a small amount of 6-methylaminopurine riboside (one per 10 000 nucleotides). Moreover, it is possible that this material was either derived from contaminating leaf RNA in the virus preparation or, as was explained above, was actually adenine. As the RNA in a single particle of each of these viruses has a molecular weight of

Table 4. *Molar proportions of methylated bases in ribonucleic acids from several sources*

Source of ribonucleic acid	Moles/100 moles of uracil				No. of analyses
	Thymine	2-Methyladenine	6-Methylaminopurine	6-Dimethylaminopurine	
<i>E. coli</i> B/r	0.9	0.5	0.4	0.3	3
<i>E. coli</i> 15T ⁻	1.0	0.3	*	*	1
<i>A. aerogenes</i>	1.2	0.3	0.3	0.1	2
<i>S. aureus</i>	0.9	0.05	0.2	—	1
Yeast	0.6	0.1	0.2	0.05	1
Wheat germ	3.7	0.1	3.7	0.06	1
Whole rabbit liver	—	—	0.3	—	1
Rat-liver microsomes	—	—	0.5	0.1	3
Turnip yellow mosaic virus	—	—	—	—	1
Tobacco mosaic virus	—	—	—?	—	1

* Not examined.

— Indicates less than 0.05%.

2×10^6 (Markham, 1951; Schramm, 1954), it seems unlikely that any of the four methylated bases is a regular component of the virus particles.

DISCUSSION

We have presented evidence that thymine, 2-methyladenine, 6-methylaminopurine and 6-dimethylaminopurine occur naturally in ribonucleic acids derived from several microbial, plant and mammalian sources. Particularly have we tried to establish that these compounds occur as ribotides in the polyribonucleotide chains and not as deoxyribotides or free ribotides accompanying our ribonucleic acid preparations. We have not so far examined whether these bases are present mainly in short or long polynucleotides. None of the ribotides we isolated was the same as the additional nucleotide which Cohn (1957) and Davis & Allen (1957) isolated from yeast ribonucleic acid and possibly from that of other sources. However, our chromatographic methods might be expected to reveal only such methylated derivatives of uracil and adenine as we have found. Other additional bases may well be discovered by different methods. The results of Cohn and Davis & Allen and the isolation of three methylated derivatives of guanine from ribonucleic acids of several sources (Adler *et al.* 1958; Dunn & Smith, 1958*b*) support such a viewpoint.

Although there is increasing evidence that cells contain ribonucleic acids of different types and functions (Smellie, 1955), the precise role of the different ribonucleotides in any type of ribonucleic acid is unknown. Consequently, the presence in ribonucleic acid of the small amounts of the four bases we have studied is open to many interpretations. For example, in ribonucleic acid which functions as a template for protein synthesis (Brachet, 1955), 6-methylaminopurine might participate in the coding of an uncommon amino acid such as cysteine or tryptophan, or in some way it might act to produce a correct termination of the polypeptide chain. Though such possibilities cannot be excluded, the ribonucleic acid from plant viruses, which might be considered most representative of 'template' ribonucleic acid, appears to be devoid of these four methylated bases. In addition, liver microsomes, which have been shown to be involved in protein synthesis (Brachet, 1955), contain 6-methylaminopurine but no thymine or 2-methyladenine.

Cells contain some ribonucleic acid which turns over rapidly, and it has been suggested that this may in part serve to store bases or their nucleotides for future use (Soodak, 1956). Such ribonucleic acid may occur in the soluble fraction of liver cells (Smellie, 1955) and its synthesis may involve

enzymes such as the polynucleotide phosphorylase, found in many bacteria, which appears to synthesize a ribonucleic acid of rather unspecific composition (Ochoa & Heppel, 1957). The four bases we have studied may be stored in this type of ribonucleic acid and used for more important functions elsewhere in the cell. The thymine present in ribonucleic acid may thus be used for synthesis of deoxyribonucleic acid, but the small quantities of this compound in ribonucleic acid are unlikely to contribute significantly in this way. It also seems unlikely that the ribotide of thymine represents the direct precursor of the deoxyribotide, since the thymine-requiring organism, *E. coli* 15T⁻, is unable to use thymine riboside directly for growth (Cohen & Barner, 1956). It seems quite possible from studies with unnatural purines and pyrimidines (Markham, 1958) that these four methylated bases, rather than being stored for future use, replace uracil or adenine in ribonucleic acid merely because they are present in the cell for other purposes. The occurrence of thymine in the ribonucleic acid of bacteria and yeast and its absence from that of liver may thus simply reflect the much lower rate of its synthesis for deoxyribonucleic acid in the latter. The presence of the three methylated adenines in ribonucleic acid may similarly result from their formation for other purposes. The occurrence of 6-methylaminopurine in deoxyribonucleic acid (Dunn & Smith, 1958*a*), 2-methyladenine in the vitamin B₁₂-like Factor A (Dion *et al.* 1954; Brown *et al.* 1955), and 6-dimethylaminopurine in puromycin (Waller *et al.* 1953), all support this view. However, the limited range of micro-organisms in which such compounds containing these methylated adenines have been found previously contrasts with the wide range of organisms which have these bases in their ribonucleic acids. It thus seems possible that the methylated adenines may have functions in cell metabolism other than those that have so far been described.

SUMMARY

1. The ribonucleic acid from several microbial, plant and mammalian sources has been shown to contain thymine, 2-methyladenine, 6-methylaminopurine and 6-dimethylaminopurine in amounts varying from 0.05 to 3.7% of the uracil.

2. The compounds were isolated mainly as ribosides and identified by chromatographic and spectroscopic comparison with known samples. Each of the ribosides was converted into the corresponding base. In addition the riboside 2'- and 3'-phosphates have been isolated; this and other evidence indicates that the four bases were originally present in 3':5'-phosphodiester linkage in the ribonucleic acid.

3. Although all four methylated bases were detected in the ribonucleic acid from most of the micro-organisms examined, thymine and 2-methyladenine were not found in liver ribonucleic acid and all these bases appeared to be absent from the ribonucleic acid of two plant viruses.

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