The Occurrence of Methylated Guanines in Ribonucleic Acids from Several Sources

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A number of additional purines and pyrimidines have recently been described as minor constituents of ribonucleic acids. Cohn (1957, 1958, 1959) and Davis & Allen (1957) have described an unusual pyrimidine nucleotide from yeast ribonucleic acid, thymine, 2-methyladenine, 6-methylaminopurine and 6-dimethylaminopurine have been identified as components of ribonucleic acid from several sources (Littlefield & Dunn, 1958*a, b*), and 5-methylcytosine has been detected in ribonucleic acid from *Escherichia coli* (Amos & Korn, 1958) and from wheat embryo, liver and *Aerobacter aerogenes* (Dunn, unpublished work). The occurrence of methylated adenines in ribonucleic acid led us to look for methyl-substituted guanines. We have found 1-methylguanine, 6-hydroxy-2-methylaminopurine and 2-dimethylamino-6-hydroxypurine to be constituents of ribonucleic acid from several different biological sources, by identification of the free bases, ribonucleosides and ribonucleotides from the ribonucleic acids. While this work was in progress Adler, Weissmann & Gutman (1958) reported the isolation of two of these bases, 1-methylguanine and 6-hydroxy-2-methylaminopurine from an acid hydrolysate of yeast ribonucleic acid.

Preliminary results of our work have been reported (Dunn & Smith, 1959*a, b*).

MATERIALS AND METHODS

Ribonucleic acid preparations

The isolation of ribonucleic acid (RNA) from *A. aerogenes*, wheat-embryo and rat-liver microsome particles has been described previously (Littlefield & Dunn, 1958*b*). The soluble RNA fraction from rat liver (Hoagland, Stephenson, Scott, Hecht & Zamecnik, 1958) was a gift from Dr M. B. Hoagland.

Plant leaf ribonucleic acid. RNA was prepared from *Nicotiana glutinosa* leaves according to Markham (1955). *Beta vulgaris* leaf RNA was given by Dr A. R. Trim. After extraction of the leaves with acetone, the RNA had been extracted with aqueous NaCl soln. at 100°. It was purified by precipitation with Al^{3+} ions (A. R. Trim, unpublished work).

Virus ribonucleic acid. Tobacco-mosaic virus was grown in *Nicotiana tabacum* var. white Burley and was purified by differential centrifuging after treatment with 0.1 M-sodium citrate (pH 7.0) to remove any non-viral nucleoprotein (Ginoza, Atkinson & Wildman, 1954). The RNA was isolated according to Markham & Smith (1950). Turnip-yellow-mosaic virus was grown in Chinese cabbage (*Brassica chinensis*) and purified by the method of Markham

& K. M. Smith (1949). The RNA was isolated after denaturation of the virus with ethanol (Markham & Smith, 1951).

Hydrolysis of nucleic acids

RNA was hydrolysed to nucleoside 2'- and 3'-phosphates by treatment with *n*-KOH at 30° for 18 hr. followed by precipitation of potassium with perchloric acid, as described by Littlefield & Dunn (1958*b*). Hydrolysis of RNA to nucleosides by Russell-viper venom, and the conversion of nucleotides into nucleosides with prostate phosphomonoesterase were both also carried out under the conditions described by Littlefield & Dunn (1958*b*). Purines were liberated from their nucleotides and nucleosides by hydrolysis in *n*-HCl at 100° for 1 hr. (Smith & Markham, 1950).

Paper chromatography and electrophoresis

The following solvent systems were used in chromatography: (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). (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) Water saturated with (NH₄)₂SO₄ (800 ml.), *m*-sodium acetate (180 ml.), propan-2-ol (20 ml.) (Markham & Smith, 1952).

Paper electrophoresis was carried out according to Markham & Smith (1952).

Buffers

Phosphate buffers (0.05*M*; pH 2.1-2.5) were prepared from 0.05*M*-KH₂PO₄ and 0.05*M*-H₃PO₄. Glycine buffer (0.05*M*; pH 10) was made by adding KOH to an aqueous glycine solution. Borate buffer (pH 9.2) was 0.05*M*-sodium tetraborate. The buffer solutions were adjusted to the correct pH by using a glass-electrode pH meter.

Spectrophotometry

Compounds located according to Markham & J. D. Smith (1949) were eluted in 0.1*N*-HCl and the spectra measured against those of eluates from appropriate paper blanks in a Unicam SP. 500 spectrophotometer. Alkaline spectra were measured on the same solutions after addition of 2*N*-KOH to give a final concentration of 0.1*N*-KOH.

The molar extinction coefficients of the methylated guanosines were not available. For the approximate estimation of the compounds we used the following values at pH 1.0: 6-hydroxy-2-methylaminopurine riboside, 13.1×10^3 at 258 $m\mu$; 2-dimethylamino-6-hydroxypurine riboside, 15.1×10^3 at 265 $m\mu$. These were estimated from the molar extinction coefficients of the bases (Elion, Lange & Hitchings, 1956) and the relative difference between those of guanine and guanosine. The value for 1-methylguanosine was taken as 12.2×10^3 at 258 $m\mu$, which is the molar extinction coefficient of guanosine. The molar extinction coefficient of uridine at pH 1.0 was taken as 10.1×10^3 at 262 $m\mu$.

Methylated guanines and their ribosides

Synthetic samples of 1-methylguanine, 6-hydroxy-2-methylaminopurine, 2-dimethylamino-6-hydroxypurine and 2-ethylamino-6-hydroxypurine were gifts from Dr

G. H. Hitchings of the Wellcome Laboratories, Tuckahoe, N.Y., U.S.A. Another sample of 1-methylguanine was obtained from Professor D. Keilin, F.R.S. The methylamino- and dimethylamino-guanine derivatives contained traces of ultraviolet-absorbing impurities which were removed by chromatography before measurement of their ultraviolet-absorption spectra. Small quantities of guanine were identified as an impurity in the samples of 1-methylguanine. This was removed by chromatography in solvent 2 before the material was used for spectroscopy and preparation of the nucleoside.

The ribonucleosides of the methylated guanines were synthesized enzymically with an enzyme preparation from *E. coli* (Paegle & Schlenk, 1952) and conditions similar to those of Littlefield & Dunn (1958*b*). To obtain sufficiently high concentrations of the methylated guanines it was necessary to dissolve them in 0.1*N*-HCl before adding them to the solution of inosine in phosphate buffer (Littlefield & Dunn, 1958*b*) and to adjust the solution to pH 7.0 immediately before adding the enzyme. As previously described, the products were separated in solvent 4 but the methylated guanosines did not separate readily from hypoxanthine. The material in this band was therefore chromatographed in solvent 3 where hypoxanthine moved ahead of the methylated guanosines.

RESULTS

Separation of mononucleotides

The presence of the methyl groups in 1-methylguanine, 6-hydroxy-2-methylaminopurine and 2-dimethylamino-6-hydroxypurine gave *R_F* values for these compounds, their nucleosides and nucleotides which were greater than those of the corresponding guanine derivatives, when chromatographed in solvent mixtures containing propan-2-ol and butan-1-ol. This behaviour, which was also observed with methylated adenines (Littlefield & Dunn, 1958*b*), was used in their separation. In addition, like guanine, the methylated guanines and their derivatives showed a blue fluorescence in ultraviolet light (260 $m\mu$) at acid pH which aided their detection by the method of Markham & Smith (1950). 2-Dimethylamino-6-hydroxypurine and its derivatives showed a stronger fluorescence than the other two compounds.

The RNA was hydrolysed in *n*-KOH to give the 2'- and 3'-mononucleotides and the hydrolysate placed in a band across a sheet of Whatman no. 3 MM. filter paper and chromatographed in solvent 2. This solvent separates the four major nucleotides into two bands, one containing guanylic acid, and the other, a faster-moving band of adenylic acid, cytidylic acid and uridylic acid. The nucleotides of the three methylated guanines are found in the latter band. The material from this was eluted in water and separated by paper electrophoresis in 0.05*M*-phosphate buffer, pH 2.1 or, in some cases, pH 2.5. In this pH range adenylic acid and cytidylic acid have almost zero net charge and

move only slightly towards the anode, whereas uridylic acid has one negative charge. When an alkaline hydrolysate of wheat-embryo RNA was separated in this way two additional ultraviolet-absorbing bands, 1 and 2 in order of increasing mobilities, were observed (Table 1). The mobilities of these were less than that of guanylic acid. Bands 1 and 2 were eluted and re-chromatographed in solvent 2 to remove phosphate.

Identification of the methylated guanine derivatives

2-Dimethylamino-6-hydroxypurine. Band 1 contained a single substance which was identified as the ribonucleotide of 2-dimethylamino-6-hydroxypurine. After treatment with prostate phosphomonoesterase it was converted into a substance with an increased R_F value in solvent 2 corresponding to that of a nucleoside. On paper chromatography in solvents 1-4 (Table 2) and on electrophoresis in 0.05M-borate, pH 9.2, the dephosphorylated product migrated in the same position as the enzymically synthesized 2-dimethylamino-6-hydroxypurine riboside (Table 3). Its ultraviolet-absorption spectra at pH 1.0 and pH 13.0 were the same as those of the reference nucleoside (Fig. 1; Table 4).

On hydrolysis in N-HCl at 100° for 1 hr. the nucleoside obtained from band 1 yielded a substance identified as 2-dimethylamino-6-hydroxy-

purine by chromatography in solvents 1-4 (Table 2) and from its ultraviolet-absorption spectra (Fig. 2; Table 4).

6-Hydroxy-2-methylaminopurine and 1-methylguanine. Band 2 from the electrophoretic separation of the nucleotides at pH 2.1 was found to be a mixture of the nucleotides of 6-hydroxy-2-methylaminopurine and 1-methylguanine. These were separated after conversion into the nucleosides since we failed to find a system for resolving the two nucleotides. The material from band 2 was incubated with prostate phosphomonoesterase. On electrophoresis in 0.05M-borate, pH 9.2, the digest gave two ultraviolet-absorbing spots. The more

Table 1. *Electrophoretic mobilities of methylated guanine nucleotides in 0.05M-phosphate buffer, pH 2.1*

	Mobility (cm./hr.) towards the anode at 20 v/cm.
Adenosine 3'-phosphate	1.0
Cytidine 3'-phosphate	1.0
2-Dimethylamino-6-hydroxypurine riboside 2'- and 3'-phosphates	2.6
6-Hydroxy-2-methylaminopurine riboside 2'- and 3'-phosphates	2.9
1-Methylguanosine 2'- and 3'-phosphates	2.9
Guanosine 3'-phosphate	3.4
Uridine 3'-phosphate	7.4

Table 2. *R_F values of methylated guanines and their ribosides*

Solvents: (1) propan-2-ol (680 ml.), 11.6N-HCl (176 ml.), water to 1 l.; (2) propan-2-ol (700 ml.), water (300 ml.) with NH_3 in vapour phase; (3) butan-1-ol (770 ml.), water (130 ml.), 98% formic acid (100 ml.); (4) butan-1-ol (860 ml.), water (140 ml.) with NH_3 in vapour phase.

Solvent	R_F			
	1	2	3	4
Guanine	0.26	0.49	0.09	0.08
1-Methylguanine	0.26	0.62	0.19	0.13
6-Hydroxy-2-methylaminopurine	0.50	0.66	0.25	0.18
2-Dimethylamino-6-hydroxypurine	0.43	0.69	0.28	0.20
2-Ethylamino-6-hydroxypurine	0.61	0.82	—	—
Guanosine	0.37	0.42	0.10	0.02
1-Methylguanosine	0.45	0.64	0.16	0.11
6-Hydroxy-2-methylaminopurine riboside	0.55	0.63	0.18	0.08
2-Dimethylamino-6-hydroxypurine riboside	0.49	0.66	0.23	0.10
Uracil	0.69	0.72	0.38	0.20
Uridine	0.70	0.70	0.21	0.08

Table 3. *Electrophoretic mobilities of the methylated guanosines*

Buffer	Mobility (cm./hr.) towards the anode at 20 v/cm.		
	0.05M-borate (pH 9.2)	0.05M-glycine (pH 10.0)	0.05M-phosphate (pH 2.2)
Guanosine	+7.0	—	—
1-Methylguanosine	+5.0	-0.4	—
6-Hydroxy-2-methylaminopurine riboside	+5.9	+0.6	-4.5
2-Dimethylamino-6-hydroxypurine riboside	+5.9	—	-5.1
Cytidine	+5.4	—	-8.6

slowly migrating substance was identified as 1-methylguanosine and the faster-moving spot as 6-hydroxy-2-methylaminopurine riboside. Separation of the two nucleosides could also be obtained by chromatography in solvent 4 if the ammonia vapour concentration in the tank was sufficiently high. 1-Methylguanosine had the smaller R_f (Table 2). The following criteria were used in the identification of the two nucleosides. (i) Both had chromatographic properties in solvent systems 1-4 and electrophoretic mobilities in 0.05 M-borate, pH 9.2, and 0.05 M-glycine buffer, pH 10.0, identical with those of the corresponding enzymically synthesized nucleosides. Both nucleosides form borate

complexes and in borate, pH 9.2, migrate towards the anode, but 6-hydroxy-2-methylaminopurine riboside has an additional charge due to the ionization of the 6-hydroxy group. This dissociation is absent in 1-methylguanosine since the hydrogen atom on $N_{(1)}$ is replaced by a methyl group, and the nucleoside migrates more slowly than the monomethylamino- and dimethylamino-guanosine derivatives. In glycine at pH 10.0, where the only charges are those due to the 6-hydroxy group, 1-methylguanosine remains stationary and the 6-hydroxy-2-methylaminopurine riboside migrates towards the anode (Table 3).

Fig. 1. Ultraviolet-absorption spectra of enzymically prepared 2-dimethylamino-6-hydroxypurine riboside in 0.1 N-HCl (—) and in 0.1 N-KOH (---). Points are for a sample isolated from the RNA of wheat embryo in 0.1 N-HCl (●) and in 0.1 N-KOH (○). For each sample the spectra in acid and alkali are for solutions of equal concentration, the spectrum in acid being plotted to give E 1.0 at 265 $m\mu$.

Fig. 2. Ultraviolet-absorption spectra of synthetically prepared 2-dimethylamino-6-hydroxypurine in 0.1 N-HCl (—) and in 0.1 N-KOH (---). Points are for a sample isolated from the RNA of wheat embryo in 0.1 N-HCl (●) and in 0.1 N-KOH (○). For each sample the spectra in acid and alkali are for solutions of equal concentration, the spectrum in acid being plotted to give E 1.0 at 265 $m\mu$.

Table 4. Principal ultraviolet-absorption maxima of the methylated guanines and their ribosides

Inflexion points and subsidiary peaks are not given.

pH	...	...	...	...	$\lambda_{max.}$ (m μ)			
					Base		Riboside	
					1.0	13.0	1.0	13.0
Guanine*					248.5	274	256.5	258-266
1-Methylguanine					250	277	258	256
6-Hydroxy-2-methylaminopurine					251	278	258	258
2-Dimethylamino-6-hydroxypurine					256	283	265	262
2-Ethylamino-6-hydroxypurine†					253	—	—	—

* Data from Beaven, Holiday & Johnson (1955).

† Data from Elion, Lange & Hitchings (1956).

(ii) Comparison of the ultraviolet-absorption spectra of the natural and reference nucleosides at pH 1.0 and pH 13.0 (Figs. 3, 4; Table 4).

(iii) On hydrolysis in *N*-HCl the bases liberated from the nucleosides were identified as 1-methyl-

guanine and 6-hydroxy-2-methylaminopurine respectively by comparison of their chromatographic properties in solvents 1-4 (Table 2) and their ultraviolet-absorption spectra (Figs. 5, 6; Table 4) with those of the synthetic purines.

Fig. 3. Ultraviolet-absorption spectra of enzymically prepared 1-methylguanosine in 0.1*N*-HCl (—) and in 0.1*N*-KOH (---). Points are for a sample isolated from the RNA of *A. aerogenes* in 0.1*N*-HCl (●) and in 0.1*N*-KOH (○). For each sample the spectra in acid and alkali are for solutions of equal concentration, the spectrum in acid being plotted to give *E* 1.0 at 258 *mμ*.

Fig. 5. Ultraviolet-absorption spectra of synthetically prepared 1-methylguanine in 0.1*N*-HCl (—) and in 0.1*N*-KOH (---). Points are for a sample isolated from the soluble fraction of rat-liver RNA in 0.1*N*-HCl (●) and 0.1*N*-KOH (○). For each sample the spectra in acid and alkali are for solutions of equal concentration, the spectrum in acid being plotted to give *E* 1.0 at 250 *mμ*.

Fig. 4. Ultraviolet-absorption spectra of enzymically prepared 6-hydroxy-2-methylaminopurine riboside in 0.1*N*-HCl (—) and in 0.1*N*-KOH (---). Points are for a sample isolated from the soluble fraction of rat-liver RNA in 0.1*N*-HCl (●) and in 0.1*N*-KOH (○). For each sample the spectra in acid and alkali are for solutions of equal concentration, the spectrum in acid being plotted to give *E* 1.0 at 258 *mμ*.

Fig. 6. Ultraviolet-absorption spectra of synthetically prepared 6-hydroxy-2-methylaminopurine in 0.1*N*-HCl (—) and in 0.1*N*-KOH (---). Points are for a sample isolated from the soluble fraction of rat-liver RNA in 0.1*N*-HCl (●) and in 0.1*N*-KOH (○). For each sample the spectra in acid and alkali are for solutions of equal concentration, the spectrum in acid being plotted to give *E* 1.0 at 251 *mμ*.

Stability of 1-methylguanosine. Dr P. D. Lawley (personal communication) has recently suggested that 1-methyladenylic acid is unstable at alkaline pH and undergoes a re-arrangement to yield 6-methylaminopurine ribonucleotide. Although no such interconversions have been described in guanine derivatives, we investigated the stability of 1-methylguanosine in alkali to eliminate the possibility that the nucleotide of 6-hydroxy-2-methylaminopurine had been derived from 1-methylguanylic acid by a similar methyl migration during alkaline hydrolysis of the RNA. A solution of 1-methylguanosine in 0.1N-KOH was incubated at 37° for 18 hr. After neutralization with perchloric acid it was chromatographed in solvent 2 and then placed on electrophoresis in borate, pH 9.2, where the two nucleosides separate. No 6-hydroxy-2-methylaminopurine riboside was detected. Likewise the nucleoside of 6-hydroxy-2-methylaminopurine was unchanged by treatment in 0.1N-KOH.

Although the sugar has not yet been isolated from any of the three nucleosides, their identity as ribosides is shown by the comparison of their chromatographic and electrophoretic properties with enzymically synthesized ribosides. Particularly, their electrophoretic mobilities in borate buffer, pH 9.2, were those expected from the formation of borate complexes on the 2'- and 3'-*cis*-hydroxyl groups. In the nucleosides of 1-methylguanine and 6-hydroxy-2-methylaminopurine, comparison of their electrophoretic mobilities in borate, pH 9.2, and glycine buffer, pH 10.0, more directly demonstrated borate-complex formation.

Separation of nucleoside 2'- and 3'-phosphates

From the mechanism of alkaline hydrolysis of RNA involving phosphoryl migration (Brown & Todd, 1952) the mononucleotides so produced should be a mixture of the nucleoside 2'- and 3'-phosphates. Paper chromatography in solvent 5 resolves the 2'- and 3'-phosphates of adenosine and guanosine. In this solvent the nucleotide of 2-dimethylamino-6-hydroxypurine, isolated from alkaline hydrolysates of wheat-embryo RNA, also separated into two substances migrating just behind guanosine 2'- and 3'-phosphates respectively. These had ultraviolet-absorption spectra at pH 1.0 which were both similar to those of the nucleoside.

Isolation of nucleosides from snake-venom digests of ribonucleic acid

The isolation of 1-methylguanosine and 2-dimethylamino-6-hydroxypurine riboside after hydrolysis of wheat-embryo RNA with the phosphodiesterase and 5'-nucleotidase present in crude snake venom provided confirmation that these

nucleosides were bound by 3':5'-phosphodiester linkages in the polynucleotide chain. Wheat-embryo RNA (50 mg.) was incubated with Russell viper venom at pH 9.0. The hydrolysate was chromatographed in solvent 2, where it separated into two major bands, one containing guanosine and a faster band which contained adenosine, cytidine and uridine. The latter material was eluted and separated by electrophoresis in 0.05M-phosphate buffer, pH 2.2. Uridine, being uncharged, remained at the starting position whereas adenosine and cytidine migrated towards the negative electrode. A third band migrated behind adenosine with a mobility 1.1 times that of guanosine. This was re-chromatographed in solvent 2 to remove phosphate. The single ultraviolet-absorbing band was transferred to a second chromatogram and run in solvent 3, where it separated into two substances, one moving with a marker of 1-methylguanosine and a faster-moving substance migrating with 2-dimethylamino-6-hydroxypurine riboside. The identities of these were confirmed by their electrophoretic mobilities in borate buffer, pH 9.2, and their ultraviolet spectra at pH 1.0 and pH 13.0. On electrophoresis in borate the slower-moving substance in solvent 3 gave, in addition to 1-methylguanosine, a small spot with the mobility of 6-hydroxy-2-methylaminopurine riboside. There was insufficient of this material for its identification.

Distribution of methylated guanines in ribonucleic acids

RNA preparations from a variety of sources were examined for the presence of the three methylated guanines. As these compounds, like guanine, are very insoluble in water and tend to trail in most chromatographic systems it was easier to separate and estimate them as the more soluble ribosides. The nucleotides were isolated from alkaline hydrolysates as described above, dephosphorylated with prostate phosphomonoesterase, and the nucleosides isolated by chromatography in solvent 3 followed by electrophoresis in borate buffer, pH 9.2. They were identified by the criteria already described and estimated spectrophotometrically. The amounts were determined relative to uridine. The uridylic acid separated on the paper electrophoresis at pH 2.1 was re-chromatographed in solvent 2. It was then converted into uridine with prostate phosphomonoesterase, chromatographed in solvent 3 and estimated spectrophotometrically. The results, which are necessarily very approximate, are given in Table 5.

DISCUSSION

1-Methylguanine and 6-hydroxy-2-methylaminopurine have been found in the urine of normal human subjects, together with three other

Table 5. *Approximate molar proportions of methylated guanines in ribonucleic acids from several sources*

Source of ribonucleic acid	— Indicates less than 0.01 %.		
	1-Methylguanine	6-Hydroxy-2-methylaminopurine (moles/100 moles of uracil)	2-Dimethylamino-6-hydroxypurine
<i>Aerobacter aerogenes</i>	0.1	0.05	—
Yeast†	0.02	0.02	*
Wheat embryo	0.6	0.2	0.5
<i>Nicotiana glutinosa</i> leaves	0.2	*	0.06
Sugar-beet leaves	0.2	*	0.1
Rat-liver microsomes	0.09	0.08	0.09
Rat-liver soluble ribonucleic acid fraction	4.0	2.2	2.9
Turnip-yellow-mosaic virus	—	—	—
Tobacco-mosaic virus	—	—	*

* Not examined.

† Data of Adler, Weissmann & Gutman (1958).

methylated purines, 1-methylhypoxanthine, 7-methylguanine and 8-hydroxy-7-methylguanine (Weissmann, Bromberg & Gutman, 1957). 2-Dimethylamino-6-hydroxypurine, however, has not previously been reported to occur naturally. 1-Methylguanine and 6-hydroxy-2-methylaminopurine have been isolated as the bases from acid hydrolysates of yeast RNA by Adler *et al.* (1958). The isolation of the ribonucleosides and nucleotides from RNA described in this paper provides more conclusive evidence that these two purines and 2-dimethylamino-6-hydroxypurine are present in the polynucleotide chain as nucleotide residues bound by 3'- and 5'-phosphodiester linkages.

The distribution of these purines in RNA preparations from different biological sources shows many similarities with that of the methylated adenines (Littlefield & Dunn, 1958*a, b*). The methylated guanines were not detected in the ribonucleic acids of turnip-yellow-mosaic and tobacco-mosaic viruses. If present they would represent less than one residue per virus particle. Of other RNA preparations examined only in that from *A. aerogenes* was one of the three methylated guanines (2-dimethylamino-6-hydroxypurine) definitely shown to be absent.

The soluble RNA fraction from rat liver differed from the other nucleic acids in having unusually high proportions of the methylated guanines and also of 6-methylaminopurine and the additional pyrimidine nucleotide described by Cohn and Davis & Allen (Dunn & Smith, 1959*a*). The RNA from plant tissues and micro-organisms was prepared by methods which isolate almost all the RNA in the cell (in the isolation of wheat-embryo RNA the nuclear RNA is possibly discarded). It is thus quite possible that the methylated purines found in these preparations may be largely or exclusively present in a low-molecular-weight RNA similar to the rat-liver soluble RNA.

SUMMARY

1. 6-Hydroxy-2-methylaminopurine, 2-dimethylamino-6-hydroxypurine and 1-methylguanine have been detected in ribonucleic acid from several sources in amounts varying from 0.05 to 4 % of the uracil.

2. The compounds were isolated as the ribosides and identified by chromatographic and spectroscopic comparison with synthetic samples. Each of the ribosides was converted into the base, which was similarly identified. The ribonucleoside 2'- and 3'-phosphates have also been isolated, and other evidence indicates that the three nucleosides were originally present in 3':5'-phosphodiester linkage in the ribonucleic acid.

3. None of the methylated guanines was found in the ribonucleic acids of tobacco-mosaic and turnip-yellow-mosaic viruses. 2-Dimethylamino-6-hydroxypurine was absent from the ribonucleic acid of *Aerobacter aerogenes*. The highest proportions of the methylated guanines were found in the soluble ribonucleic acid fraction of rat liver.

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Degradation of the Hyaluronic acid Complex of Synovial Fluid by Proteolytic Enzymes and by Ethylenediaminetetra-acetic acid

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Blumberg & Ogston (1957*a, b*) observed that papain in the presence of ethylenediaminetetra-acetic acid hydrolysed the protein present in the hyaluronic acid complex of ox-synovial fluid, strongly reduced the non-Newtonian viscosity of the complex and caused a considerable drop in its molecular weight. They concluded that the protein of this complex is essential to the integrity of its macromolecular structure and to its physicochemical properties in solution and, consequently, that the effect of papain on the viscosity of hyaluronic acid from other sources may provide evidence of its existence as a complex with protein.

Experiments done in collaboration with Dr B. I. Aldrich (Aldrich, 1958) in which ethylenediaminetetra-acetic acid was used in an attempt to remove calcium from the complex, showed that buffered solutions of complex containing ethylenediaminetetra-acetic acid suffered loss of viscosity during measurements in the stainless-steel Couette viscometer, but that this did not occur when an analogue of ethylenediaminetetra-acetic acid was used instead, nor when ethylenediaminetetra-acetic acid was used in a glass viscometer. These results suggested that ethylenediaminetetra-acetic acid might be concerned in catalytic degradation of the carbohydrate component of the complex; they thus also cast doubts upon the conclusions of Blumberg & Ogston, whose work with papain involved the use of ethylenediaminetetra-acetic acid as an activator and of the stainless-steel viscometer.

Further investigations on the effects of ethylenediaminetetra-acetic acid and of proteolytic enzymes on the complex were therefore undertaken.

EXPERIMENTAL

Materials

Hyaluronic acid complex. (Complex.) Synovial fluid was collected from the astragalotibial joints of oxen soon after death, as described by Ogston & Stanier (1950). The fluid was centrifuged in a Spinco model L ultracentrifuge at 73 000 *g* for 60 min. to remove particulate matter, and the complex was separated by filtration, with 9–11 washings, at 4° on a 5-on-3 sintered-glass filter of 1 μ (or less) average pore diameter (Baird and Tatlock Ltd.). The buffer used for washing was 0.2 M-NaCl–0.0077 M-Na₂HPO₄–0.0023 M-KH₂PO₄ (pH 7.3). The complex used for experiments with trypsin, chymotrypsin and pepsin was finally dissolved in this same buffer to approximately the volume of the original fluid; that used for experiments with papain and cysteine was dissolved in 0.0453 M-Na₂HPO₄–0.0213 M-KH₂PO₄ buffer (pH 7.0) to about half the volume of the original fluid. In all cases, the complex was then centrifuged at 73 000 *g* for 45 min. and dialysed against the buffer in which it was finally dissolved. Complex that was used for the determination of proteolysis by the method of Anson (1938) was further centrifuged at 105 000 *g* for 180 min. to remove any 'fast component' (Blumberg & Ogston, 1957*a*; Johnston, 1955).

Trypsin. This was Armour salt-free material of stated activity 1800 units/mg.

Chymotrypsin. This was Armour salt-free material of stated activity 975 units/mg.