The Occurrence of 6-Methylaminopurine in Deoxyribonucleic Acids

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While investigating the effects of structural analogues of thymine on the thymine-requiring strain of *Escherichia coli* 15 T⁻ we noticed the presence in the bacterial deoxyribonucleic acid of a new base which we have identified as 6-methylaminopurine. Although 6-methylaminopurine is usually present in small amounts (2 % of the adenine) in the deoxyribonucleic acid of *E. coli* 15 T⁻ its relative proportion increases considerably during growth of the bacteria in thymine-deficient conditions, obtained either by maintaining the thymine concentration in the medium at a low level, or by the addition of thymine antagonists such as 5-aminouracil or 2-thiothymine. Examination of a number of deoxyribonucleic acids has shown that 6-methylaminopurine also occurs in small proportions as a constituent of deoxyribonucleic acid from several bacteria and bacterial viruses. This paper describes the identification of 6-methylaminopurine, its deoxyribonucleoside and deoxyribonucleotide from deoxyribonucleic acid of *E. coli* 15 T⁻, and the occurrence of the base in other deoxyribonucleic acids. Brief accounts of this work have appeared previously (Dunn & Smith, 1955*a, b*).

MATERIALS

5-Aminouracil was obtained as a gift from Imperial Chemical Industries Ltd. and 2-thiothymine from Genatosan Products. 5-Methylaminouracil was prepared according to Johnson & Matsuo (1919) and 5-dimethylaminouracil according to Wheeler & Jamieson (1904). 6-Methylaminopurine, 6-methylpurine, and 8-methylpurine were obtained from Dr D. J. Brown, 2-methyladenine from Dr D. M. Brown and 6-dimethylaminopurine from the American Cyanamid Co.

5-*Amino-4-methyluracil.* This was prepared from 4-methyluracil (L. Light and Co.) according to Behrend (1885). The product gave a single ultraviolet-absorbing spot in solvents 1–5.

5-*Amino-1-methyluracil.* 1-Methyluracil was prepared by essentially the method which has since been described by Brown, Hoerger & Mason (1955*a*). It was crystallized from acetone and its spectrum was identical with that of 1-methyluracil (Shugar & Fox, 1952). The 1-methyluracil was converted into the 5-bromo derivative by treatment with Br₂ in CS₂ (Wheeler & Merriam, 1903), and this was crystallized from water. 5-Bromo-1-methyluracil was converted into 5-*amino-1-methyluracil* by heating with excess of NH₃ in a sealed tube at 150° for 2 hr. (Wheeler &

Johnson, 1904). This was not crystallized, but paper chromatography showed that there was only one major ultraviolet-absorbing product. Spectral data were obtained from chromatographically purified samples.

4-*Amino-5-methyluracil.* This was prepared by a method essentially the same as that described by Bergmann & Johnson (1933), but the intermediate methylcyanacetyl urea was not isolated. Instead, the conditions used by Conrad (1905) for preparing 4-aminouracil were applied. 4-Amino-5-methyluracil separated out as the red sodium salt. After washing this with ethanol the free base was released by the addition of HCl and was crystallized from water at pH 7. As the product contained a small proportion of 4-aminouracil the spectral data were obtained from material further purified by paper chromatography.

Commercial trypsin was purified by the method previously described (Dunn & Smith, 1957). Deoxyribonuclease was purified according to Kunitz (1950). *Crotalus adamanteus* venom diesterase was prepared as described by Sinsheimer & Koerner (1952).

METHODS

Growth of bacteria and bacteriophages

The general conditions of bacterial growth have been described previously (Dunn & Smith, 1957).

Escherichia coli strains B/r and K 12 were grown in medium *A* (KH₂PO₄, 13·6 g.; (NH₄)₂SO₄, 3·3 g.; MgSO₄,7H₂O, 0·4 g.; CaCl₂, 11 mg.; FeSO₄,7H₂O, 0·56 mg.; glucose, 10 g.; adjusted to pH 7 with NaOH and water to 1 l.).

For the preparation of *E. coli* 15 T⁻ grown in the presence of 5-aminouracil, the bacteria were grown in medium *A* containing 1·5 or 2·5 μg. of thymine/ml. to a cell density of 1–4 × 10⁸/ml. and 5-aminouracil was added to mм-concentration. Growth was continued until maximum cell density (about 2 × 10⁹/ml.) was reached, usually after 18–24 hr. Cultures were also grown in the presence of 2 mм-5-aminouracil, which was added at a higher bacterial density.

In the growth of bacteria inhibited by 2-thiothymine this was added at a bacterial cell density of 1·5 × 10⁸/ml. to give 2 mм-2-thiothymine in the presence of 2·5 μg. of thymine/ml.

Only one satisfactory preparation of deoxyribonucleic acid (DNA) has so far been made from bacteria grown on limiting amounts of thymine. The medium contained 0·5 μg. of thymine/ml. and the bacteria were harvested soon after growth ceased. In other cultures, where incubation of the bacteria was continued for several hours after growths had ceased, the yields of DNA were exceptionally low. The optimum conditions for this type of growth have not yet been determined.

For normal growth the bacteria were originally grown in medium A containing $2 \cdot 5 \mu g$. of thymine/ml. for 18 hr. However, as the DNA from these cells was found to contain small amounts of 6-methylaminopurine, it seemed possible that this could result from a thymine deficiency or that the 6-methylaminopurine might be present only in the late stages of growth. Cultures of $E.\ coli$ 15T⁻ were therefore grown in the presence of $25 \mu g$. of thymine/ml. and harvested in the logarithmic growth phase. These were used for the analyses of normal $E.\ coli$ 15T⁻ DNA.

Aerobacter aerogenes was cultivated at 30° in a medium consisting of: glucose, 20 g.; peptone, 4 g.; K_2HPO_4, 8 g.; tap water, 100 ml.; distilled water, 900 ml.; and adjusted to pH 7 with NaOH.

Staphylococcus aureus (Copenhagen strain) was obtained from Dr J. L. Strominger and grown at 37° in the following medium: yeast extract (Difco), 5 g.; peptone (Oxo Ltd. bacterial), 5 g.; K_2HPO_4, 1 g.; distilled water 1 l.; and adjusted to pH 7 with NaOH (J. L. Strominger, unpublished work).

Staphylococcus albus was grown at 37° on nutrient agar in Roux flasks.

Streptomyces griseus (strain C20) was obtained from Glaxo Laboratories Ltd. and grown in the following medium: sodium lactate, 11·2 g.; casein hydrolysate (casamino acids; Difco), 2 g.; KCl, 4·47 g.; Na_2HPO_4, 0·28 g.; KH_2PO_4, 0·23 g.; $(NH_4)_2SO_4$, 3·3 g.; $MgSO_4,7H_2O$, 1·23 g.; $ZnSO_4,7H_2O$, 14·4 mg.; $FeSO_4,7H_2O$, 13·9 mg.; $MnSO_4,7H_2O$, 84·5 mg.; distilled water to 1 l. After sterilization in the autoclave the medium was adjusted to pH 7·3 with NaOH and sterile glucose solution added to give 2 % (w/v) of glucose.

Spores were removed from nutrient-agar slopes with a platinum loop and suspended in Ringer's solution diluted in water (1:3, v/v). Shaking with glass beads aided the formation of smooth suspensions. The spore suspensions retained their viability for at least 12 months at 5°. The medium (50 ml.) was inoculated with 1 ml. of a suspension containing 4×10^8 viable spores and incubated on the rotary shaker at 28° for 48–72 hr. These cultures were used to inoculate 300 ml. batches of medium, which were similarly shaken for 24 hr.

Bacillus cereus (strain 569 H) was grown as described by Smith & Matthews (1957).

Mycobacterium tuberculosis (bovine strain) was supplied as dried cells by the Wellcome Research Laboratories.

T_2 bacteriophage was prepared from lysates of $E.\ coli$ B/r in medium A by the method of Herriott & Barlow (1952).

Salmonella C bacteriophage (Lwoff, Kaplan & Ritz, 1954) was prepared from lysates of *Salmonella typhimurium* grown aerobically at 37° in medium A. Phage (10^6 particles/ml.) was added at a bacterial density of 5×10^8 cells/ml. and incubation continued for 18 hr. The phage was purified by differential centrifuging and treated with deoxyribonuclease and ribonuclease to remove any adhering host nucleic acid.

Streptomyces C 20 bacteriophage, which lyses *S. griseus* C 20, was obtained from Glaxo Laboratories Ltd. The lactate medium used for the host failed to support multiplication of the phage. Like other phages which lyse *S. griseus* (Perlman, Langlykke & Rothberg, 1951) it appears to require calcium for its multiplication, and lactate probably prevents phage multiplication by chelation with

Ca^{2+}. For phage growth the *Streptomyces* medium was modified by the replacement of lactate by 0·5 M-glycine and the addition of mM-$CaCl_2$. As this medium did not always give satisfactory mycelium growth from spores a culture of *Streptomyces* grown in the lactate medium was used as inoculum in the proportion of 1 part in 50. When the mycelium density reached about 0·5 mg./ml., phage was added to give 10^7 phage particles/ml., and aeration continued at 28° for 18–24 hr. The culture was then kept at 4° for 24 hr. and the phage purified by differential centrifuging and treatment with deoxyribonuclease.

Isolation of deoxyribonucleic acids

Animal and plant tissues. Calf-thymus and horse-spleen DNA were prepared according to Mirsky & Pollister (1946). DNA was isolated from baker's yeast by the method of Chargaff & Zamenhof (1948) and from wheat germ according to Wyatt (1951). Salmon-sperm DNA was obtained from the California Foundation for Biochemical Research.

E. coli, Aerobacter aerogenes and Mycobacterium tuberculosis. DNA was prepared from these bacteria by the method of Smith & Wyatt (1951). This method involves extraction with N-KOH and gives a product completely free of ribonucleic acid (RNA) but considerably depolymerized. Polymerized DNA was prepared from $E.\ coli$ B/r and $E.\ coli$ 15T⁻ according to Gandelman, Zamenhof & Chargaff (1952). DNA was also prepared from $E.\ coli$ and *Aerobacter aerogenes* by a modification of the trypsin method of Dunn & Smith (1957). Products obtained by this method contain 90 % or more of the DNA from the bacteria. The bacteria were washed and defatted as in the method of Smith & Wyatt (1951). They were then heated at 100° for 10 min. before the RNA was hydrolysed with N-KOH at 30° for 18 hr. Acetic acid to give pH 4 and 2 vol. of ethanol were then added. The precipitate was collected by centrifuging and was washed with 60 % (v/v) ethanol before incubation with 0·1 M-sodium citrate, pH 7·5, containing purified trypsin for 18 hr. at 30°. The mixture was centrifuged and the residue extracted with 0·1 N-KOH, the extract being added to the citrate solution. The DNA was precipitated from this by bringing to pH 4 with acetic acid, and adding 0·05 vol. of M-$MgSO_4$ and 2 vol. of ethanol. The precipitate was collected by centrifuging, washed with 60 % (v/v) ethanol, ethanol and acetone and dried in air.

B. cereus. The above methods yielded only small amounts of DNA from *B. cereus*; other procedures, including incubation with M-glycine at 37°, or with 2 % (w/v) sodium choleate at 50°, also failed to extract DNA in appreciable amounts. If before incubation with the trypsin, the bacterial residues were ground with Pyrex glass powder, substantial amounts of DNA were extracted from this organism by the trypsin procedure described above.

Staphylococcus. DNA was extracted from defatted cells with N-KOH and precipitated in acid–ethanol. As this precipitate was insoluble in M-NaCl solution it was not possible to deproteinize the material by the chloroform–octanol method of Sevag, Lackman & Smolens (1938). Incubation with trypsin also failed to remove significant amounts of protein or increase its solubility. The material was consequently used for analysis without further purification.

Streptomyces griseus. Only very small amounts of the DNA present in *S. griseus* were removed by extraction

with N-KOH or M-NaCl, even after the cells were disintegrated by grinding with glass powder. Trypsin digestion also failed to remove an appreciable proportion of the cell DNA. The residues from these extractions and the small amounts of DNA extracted were analysed separately.

Pneumococcus *deoxyribonucleic acid.* This was obtained from Dr Harriett Ephrussi-Taylor. Any traces of RNA in the preparation were removed by treatment with N-KOH and subsequent reprecipitation of the nucleic acid.

Bacteriophages. T_2 DNA was isolated by the method of Mayers & Spizizen (1954). In the analysis of *Salmonella* and *Streptomyces* bacteriophage nucleic acids the intact viruses were hydrolysed.

Analysis of nucleic acids

Hydrolysis to purines and pyrimidines. DNA was hydrolysed in 72% (w/w) aq. $HClO_4$ soln. for 1 hr. at 100° (Marshak & Vogel, 1950) or in 98% (v/v) aq. formic acid soln. at 175° for 30 min. (Wyatt & Cohen, 1953).

Liberation of purines from deoxyribonucleic acid. DNA was heated in a stoppered tube at 55° for 30 min. with approximately four times its weight of N-HCl. This released the purines quantitatively, leaving the pyrimidines as polynucleotides. Insoluble material was removed by centrifuging and the supernatant containing the purines concentrated in a stream of air at 50° and applied to paper chromatograms

Hydrolysis to nucleosides. An aqueous solution containing 20 mg. of DNA/ml. and 2 mM-$MgSO_4$ was adjusted to pH 7·4 and deoxyribonuclease added to give 10 μg./ml. The solution was incubated at 37° for 6 hr. and the pH maintained at 7·4 by the periodic addition of dilute aq. NH_3 soln. After the addition of glycine buffer, pH 9·6, to give 0·02 M-glycine, and 100 μg. of *Crotalus adamanteus* venom/ml. incubation was continued for 5 hr. with periodic adjustment of the pH to 9–9·6.

Hydrolysis to nucleotides. In the above procedure *Crotalus adamanteus* venom phosphodiesterase, free of 5′-nucleotidase activity (Sinsheimer & Koerner, 1952), was substituted for the venom.

Paper chromatography. The following solvent systems were used: 1, propan-2-ol–conc. HCl–water (680:176:1, by vol.) (Wyatt, 1951); 2, propan-2-ol–water (7:3, v/v), with NH_3 in the vapour phase (Markham & Smith, 1952*b*); 3, butanol–water–98% (v/v) aq. formic acid soln. (77:13: 10, by vol.), (Markham & Smith, 1949); 4, butanol–water (43:7, v/v), with NH_3 in the vapour phase (Markham & Smith, 1949); 5, saturated $(NH_4)_2SO_4$–M-sodium acetate–propan-2-ol (40:9:1, by vol.) (Markham & Smith, 1952*b*).

Purines, pyrimidines and their derivatives were estimated as previously described (Dunn & Smith, 1957). The molar-extinction coefficient of 6-methylaminopurine was found to be $15·3 \times 10^3$ at 267 mμ in 0·1 N-HCl. This is slightly higher than the value of $15·1 \times 10^3$ given by Mason (1954) and that of $14·9 \times 10^3$ reported by Elion, Burgi & Hitchings (1952). A mean value of $15·1 \times 10^3$ was used in calculating our results.

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

Phosphorus. The estimation was according to Allen (1940).

RESULTS

Isolation of the new substance from the deoxyribonucleic acid of Escherichia coli 15 T^-

5-Aminouracil and 2-thiothymine, which may be considered as structural analogues of thymine, both inhibit growth of *E. coli* 15 T^-. Although neither of these substances was found to be incorporated into the bacterial DNA, examination by paper chromatography of perchloric or formic acid hydrolysates of the DNA showed, in addition to the four normal bases, a fifth ultraviolet-absorbing substance (X). This substance was also found in hydrolysates of DNA from bacteria grown under conditions of thymine deficiency and in much smaller but constant amounts in DNA from control bacteria grown in medium A containing 2·5 or 25 μg. of thymine/ml. in the absence of any inhibitor. X was present in DNA isolated by any of the three methods described.

X moves in the same position as cytosine in solvent 1 and was first observed on two-dimensional chromatograms run in solvents 1 and 5 as a substance separating from cytosine, with a smaller R_F value in solvent 5. This method of separation was inconvenient for its isolation because of the high salt content of the latter solvent. However, X has an R_F value in solvent 2 slightly greater than that of thymine (Table 1) and is thus easily separated from cytosine. Consequently for the isolation of X we used two-dimensional paper chromatography in solvents 1 and 2.

Comparison of substance X with 6-methylaminopurine

At first the fact that X accumulated in the DNA of *E. coli* 15 T^- under conditions of thymine deficiency led us to believe that it was a pyrimidine, possibly a precursor of thymine, and we compared its properties with a number of pyrimidine derivatives. However, X was found to be quantitatively liberated from DNA by hydrolysis with N-HCl for 30 min. at 55°, a property characteristic of the purines. In general the pyrimidine deoxyribonucleotide linkage is stable to acid under these conditions with perhaps the exception of some pyrimidine deoxyribosides substituted in the 4-position, a possibility which seemed unlikely from a comparison of X with pyrimidines of this type. X also possesses other characteristics of purines and on comparison with a number of purine derivatives its properties were found to be identical with those of 6-methylaminopurine.

Formation of copper and silver salts. The formation of an insoluble cuprous salt of X was demonstrated on paper chromatograms by the method of Dalby & Holdsworth (1956). As suggested by

Table 1. R_F *values of* X, *the purines and pyrimidines with which it was compared and some derivatives*

Substance	R_F in solvent				
	1	2	3	4	5
X	0·53	0·75	0·36	0·54	0·18
Purines*					
6-Methylaminopurine	0·53	0·75	0·36	0·54	0·18
2-Methyladenine	0·50	0·67	0·23	0·50	0·19
6-Methylpurine	0·54	0·74	0·56	0·40	0·40
8 Methylpurine	0·56	0·76	0·38	0·46	0·40
Pyrimidines*					
5-Aminouracil	0·27	0·45	0·15	0·16	0·56
5-Methylaminouracil	0·44	0·61	—	—	0·43
5-Dimethylaminouracil	0·52	0·71	—	—	0·50
5-Amino-1-methyluracil	0·53	0·70	—	—	0·50
5-Amino-4-methyluracil	0·82	0·68	0·37	0·33	0·50
4-Amino-5-methyluracil	0·57	0·45	—	—	0·36
DNA bases					
Guanine	0·30	0·43	0·10	0·08	0·28
Adenine	0·41	0·60	0·21	0·32	0·19
Cytosine	0·53	0·61	0·14	0·21	0·61
Thymine	0·78	0·70	0·42	0·42	0·45
Derivatives					
X nitroso compound	—	—	0·68	—	—
6-Methylaminopurine nitroso compound	—	—	0·68	0·49	—
X deoxyriboside	—	0·80	0·34	0·55	0·11
Thymidine	—	0·75	0·38	0·38	0·39
Deoxyadenosine	—	0·64	0·21	0·33	0·15

* Nomenclature of purines and pyrimidines is that used by Bendich (1955).

Table 2. *Electrophoretic mobilities of* X
and its nucleoside and nucleotide

	Mobility towards the cathode in cm./hr. at 20v/cm.	
	pH 2·5	pH 3·5
X	+10·1	+6·7
X Deoxyriboside	—	+3·1
X Deoxyribonucleotide	—	−2·9
6-Methylaminopurine	+10·4	+6·9
Adenine	+10·8	+9·0
Adenosine 3′-phosphate	—	−3·2

Dr E. Lester Smith (personal communication) the washing time was extended to 1 hr. With chromatograms which had been run in alkaline solvents it was found necessary to add a few drops of acetic acid to the copper acetate solution. This prevented the formation of copper hydroxide which otherwise obscured the result. X resembled adenine and 6-methylaminopurine in giving a brown spot on the paper. Purines but not pyrimidines react in this way. The silver reaction for purines described by Reguera & Asimov (1950) was found to give a good positive reaction with adenine, but guanine and 6-methylaminopurine, especially when present in small amounts, gave spots lighter than the background. As pyrimidines gave no effect at all with this method, a lighter area formed by X was taken as an indication that it was a purine.

Conversion into glycine. On hydrolysis with strong HCl, the purine ring is known to break down, giving glycine, carbon dioxide, carbon monoxide and ammonia (Strecker, 1868). On heating X, 6-methylaminopurine and adenine at 120° in 6n-HCl for 18 hr., they were all converted into substances which did not absorb ultraviolet light. The products chromatographed on solvents 1–3 were found to contain a ninhydrin-reacting compound corresponding to glycine on each of the solvents. On solvents 1 and 3, the products from X and 6-methylaminopurine but not that from adenine were found to contain a second ninhydrin-reacting compound, the nature of which has not been investigated.

Paper chromatography and electrophoresis. The high R_F value of X relative to adenine in solvents 1–4 suggested that it carried an alkyl group, and its electrophoretic mobilities at pH 2·5 and 3·5 showed the presence of a basic dissociation. Comparison of the R_F values and electrophoretic mobilities of X and a number of purines or pyrimidines showed that in all systems the positions of X and 6-methylaminopurine were identical (Tables 1 and 2).

Ultraviolet-absorption spectra. The ultraviolet-absorption spectra of X at pH 1 has a maximum at 267 mμ and a minimum at 232 mμ. At pH 13 the maximum and minimum are shifted to 273 and

239 mμ respectively. These spectra are the same as those of 6-methylaminopurine (Fig. 1) and distinct from those of other purines and pyrimidines examined (Table 3). From the change in spectrum over the range pH 0–14 it is possible, by the method of Stenström & Goldsmith (1926), to determine the number of dissociating groups and their approximate pK values. Such measurements
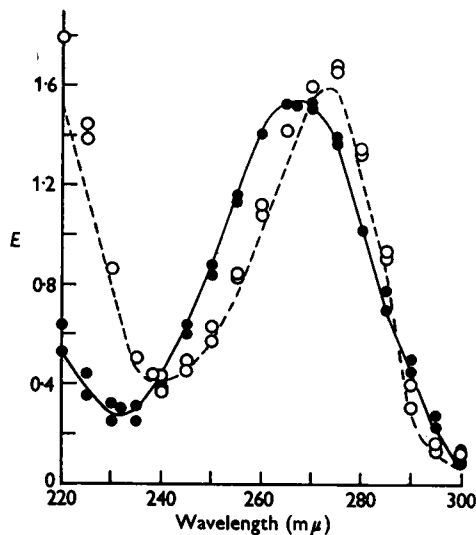

Fig. 1. Ultraviolet-absorption spectra of synthetic 6-methylaminopurine at pH 1 (—) and pH 13 (- - -). Points are for two samples of X from E. coli 15T⁻ DNA: at pH 1 (●) and pH 13 (○). Spectra at pH 1 and pH 13 are for solutions at the same concentration. The spectra are plotted to give the same value of E at 265 mμ at pH 1.

Table 3. *Spectral characteristics of X and compounds with which it was compared*

| | Wavelength (mμ) | | | |
| | Maximum | | Minimum | |
Substance	pH 1	pH 13	pH 1	pH 13
X	267	273	233	239
Purines				
6-Methylaminopurine	267	273	232	239
6-Ethylaminopurine*	270	273	—	—
6-Dimethylaminopurine	277	281	236	245
2-Methyladenine	266	271	229	238
6-Methylpurine	264	271	232	236
8-Methylpurine	264	273	231	236
Pyrimidines				
5-Aminouracil	260	290	228	259
5-Methylaminouracil	262	282	230	256
5-Dimethylaminouracil	260	290	228	264
5-Amino-1-methyluracil	262	292	230	258
4-Amino-5-methyluracil	273	273	243	246

* Data from Elion *et al.* (1952).

showed that X carried two dissociating groups with pK values of approx. 3·6 and 10·3 respectively. The relative rates of migration of X on paper electrophoresis at pH 2·5 and 3·5 showed the value of the basic dissociation to be about pK 4. These pK values are similar to those determined for 6-methylaminopurine (4·18 and 9·99; Mason, 1954).

Treatment with nitrous acid. On treatment with nitrous acid most purines and pyrimidines bearing primary amino groups are deaminated whereas those carrying secondary amino groups may be expected to yield characteristic derivatives. Dry samples of X and 6-methylaminopurine were each incubated at room temperature for 3 hr. with one drop of acetic acid and one drop of saturated aqueous sodium nitrite solution (conditions found satisfactory in our Laboratory for the deamination of cytosine or adenine). The products were separated on paper chromatograms in solvent 3. X and 6-methylaminopurine each yielded a single ultra-violet-absorbing derivative. These had the same R_F value (Table 1), which was greater than that of 6-methylaminopurine. The ultraviolet-absorption spectra at pH 1 and 13 of the isolated derivatives from X and 6-methylaminopurine were similar (Fig. 2). We presume the substance to be the nitroso derivative of 6-methylaminopurine.

6-Methylaminopurine deoxyribonucleoside. A substance with the expected properties of 6-methylaminopurine deoxyriboside was isolated from enzymic hydrolysates of DNA from E. coli 15 T⁻
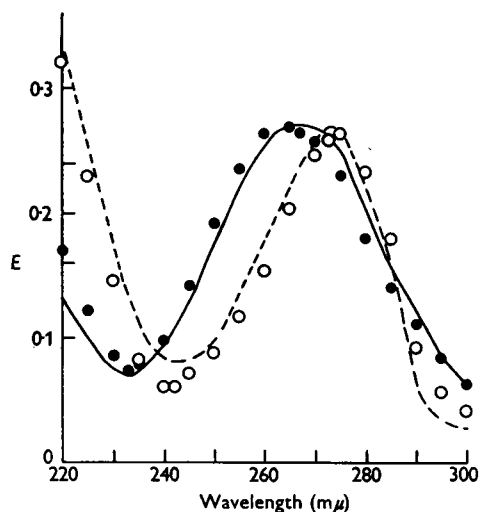

Fig. 2. Ultraviolet-absorption spectra of the assumed nitroso derivative of 6-methylaminopurine at pH 1 (—), λ_max. 267 mμ, λ_min. 233 mμ, pH 13 (- - -), λ_max. 273 mμ, λ_min. 241 mμ, and of X at pH 1 (●) and pH 13 (○). Spectra at pH 1 and 13 are for solutions of the same concentration. The spectra are plotted to give the same value of E at 265 mμ at pH 1.

grown in the presence of either 5-aminouracil or 2-thiothymine. This substance, which has the R_F values given in Table 1, could be separated by two-dimensional chromatography in solvent 3 and solvent 4. Its separation was best achieved, however, by a combination of paper chromatography and electrophoresis. The hydrolysate was applied as a band across a sheet of Whatman no. 3 MM paper and the chromatogram run in solvent 2. 6-Methylaminopurine deoxyriboside moves slightly ahead of thymidine, which in turn has a higher R_F value than the other nucleosides. The band containing 6-methylaminopurine deoxyriboside and thymidine was eluted in water and examined by paper electrophoresis at pH 2·5, where thymidine, having no charge, remains stationary and 6-methylaminopurine deoxyriboside migrates towards the cathode. The electrophoretic mobility of the nucleoside was less than that of 6-methylaminopurine (Table 2). Its ultraviolet-absorption spectrum (Fig. 3) was similar to that of a sample of 6-methylaminopurine riboside prepared enzymically in this Laboratory by Dr J. W. Littlefield, and differed from that of 6-methylaminopurine in not showing a shift in maximum towards the longer wavelengths in alkaline solution. On hydrolysis in N-HCl at 55° it yielded a substance identical with 6-methylaminopurine in its ultraviolet-absorption spectrum and chromatographic properties.

6-*Methylaminopurine deoxyribonucleotide*. Digestion of the DNA successively with pancreatic deoxyribonuclease and snake-venom phosphodiesterase yielded a substance with the expected properties of 6-methylaminopurine deoxyribonucleotide. This separated from the other deoxyribonucleotides on chromatography in solvent 2 where it moved ahead of thymidylic acid. (Separation was not complete until the thymidylic acid had moved 20 cm.) It was further purified by paper electrophoresis at pH 3·5 (Table 2). The ultraviolet-absorption spectra of the nucleotide at pH 4 and 13 were similar to those of nucleoside (Fig. 4). On hydrolysis with N-HCl at 55° it yielded 6-methylaminopurine and a phosphorus determination gave a molar ratio of 6-methylaminopurine/P of 1·2.

Purine and pyrimidine composition of
Escherichia coli 15 T^- deoxyribonucleic acid

The content of 6-methylaminopurine in the DNA from *E. coli* 15 T$^-$ grown in 2·5 or 25 μg. of thymine/ml. is constant and about 0·4% of the total bases (molar ratio). Within experimental error the proportions of adenine and thymine are equal, as are those of cytosine and guanine. DNA from 5-aminouracil-inhibited, 2-thiothymine-inhibited or thymine-deficient bacteria contained a

much increased proportion of 6-methylaminopurine, ranging to 4% or more of the total bases. A smaller increase was also found in the DNA of *E. coli* 15 T$^-$ inhibited by 5-chloro- or 5-bromouracil (Dunn & Smith, 1957). The purine and pyrimidine compositions of DNA from 5-aminouracil-inhibited and thymine-deficient bacteria (Table 4) show a decrease in the relative proportion of thymine, which is not generally quantitatively related to the increase in 6-methylaminopurine. The proportion of adenine in these nucleic acids is also slightly decreased.

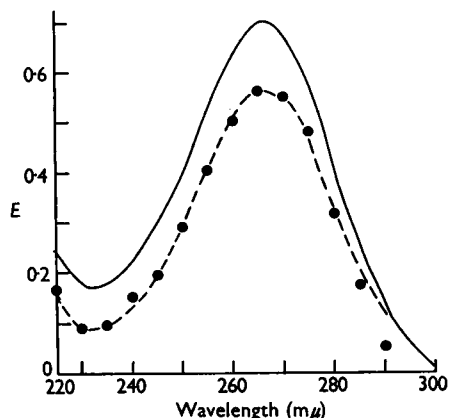The increase in 6-methylaminopurine during growth with low thymine concentrations occurs only
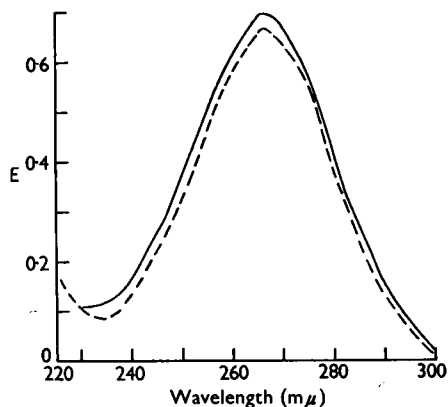

Fig. 3. Ultraviolet-absorption spectra of 6-methylaminopurine deoxyriboside isolated from *E. coli* 15T$^-$ DNA: at pH 7 (——), $\lambda_{max.}$ 266 mμ, $\lambda_{min.}$ 232 mμ; at pH 13 (●), $\lambda_{max.}$ 266 mμ, $\lambda_{min.}$ 232 mμ, and synthetic 6-methylaminopurine riboside at pH 13 (– – –), plotted to give the same value of E at 265 mμ.

Fig. 4. Ultraviolet-absorption spectra of 6-methylaminopurine deoxyribonucleotide isolated from *E. coli* 15T$^-$ DNA: at pH 4 (——), $\lambda_{max.}$ 266 mμ, and at pH 13 (– – –), $\lambda_{max.}$ 266 mμ.

Table 4. *Purine and pyrimidine compositions of deoxyribonucleic acid from control, 5-aminouracil-inhibited,*
5-chlorouracil-inhibited and thymine-deficient Escherichia coli 15 T^-

	Proportion of bases in DNA (moles/100 moles of total recovered bases)					
Bacterial preparation	Adenine	Guanine	Cytosine	Thymine	5-Chloro-uracil	6-Methyl-aminopurine
Control	23·6	27·5	26·1	22·4	—	0·42
5-Aminouracil-inhibited	22·4	27·8	26·7	19·1	—	4·1
5-Chlorouracil-inhibited*	23·4	27·3	26·0	7·9	14·2	1·4
Thymine-deficient	22·0	28·6	27·5	18·9	—	3·1

* Dunn & Smith (1957).

when there is sufficient thymine to allow growth and DNA synthesis. Bacteria which were washed and resuspended in medium *A* without thymine, conditions under which the cells rapidly lose their viability (Barner & Cohen, 1954), showed no detectable increase in the 6-methylaminopurine content of their DNA even after incubation for several hours.

Identification and estimation of 6-methylaminopurine in other deoxyribonucleic acids

We examined DNA preparations from a number of other organisms for the presence of 6-methyl-aminopurine. Liberation of the purines by hydrolysis in N-HCl at 55°, followed by two-dimensional chromatography in solvents 1 and 2, proved the simplest technique for its estimation. Whereas solvent 1 alone separates 6-methylaminopurine from the other purines, solvent 2 separates the former from traces of ultraviolet-absorbing materials coinciding with its position in solvent 1. The adenine and 6-methylaminopurine spots were eluted and estimated.

Where larger amounts of DNA were available 6-methylaminopurine was also estimated in perchloric or formic acid hydrolysates. The material was placed as a band on Whatman no. 1 paper and run in solvent 1. The area containing cytosine and 6-methylaminopurine was eluted in water, the material concentrated in a stream of air at 50° and run on paper chromatograms in solvent 2, where 6-methylaminopurine moves in front of cytosine, and the ratio 6-methylaminopurine:cytosine was thus obtained. We consider the values from this method to be the more reliable owing to variability in the readings from the 'blank' areas from the two-dimensional paper chromatograms.

The criteria used in the identification of 6-methylaminopurine were: (1) the correspondence of its R_F value with that of the synthetic 6-methylaminopurine 'marker'; (2) its ultraviolet-absorption spectra at pH 1 and 13 (Fig. 1). The base was identified as a constituent of the DNA from three strains of *E. coli*, *Aerobacter aerogenes*, *Mycobacterium tuberculosis*, *Pneumococcus*, *E. coli* bacteriophages T_{2r} and T_{2r+} and *Salmonella c*

Table 5. *Relative proportions of 6-methylamino-*
purine and adenine in deoxyribonucleic acids from
bacteria and bacteriophages

Values are expressed as moles of 6-methylaminopurine/
100 moles of adenine.

	Method of hydrolysis		
Source of DNA	N-HCl at 55°	HClO₄ at 100°	H·CO₂H at 175°
E. coli B/r	2·4*	1·8	—
E. coli K 12	1·7*	—	—
E. coli 15 T⁻	2·4*	1·8	—
Aerobacter aerogenes	2·7	2·6	—
Pneumococcus	0·44*	—	—
Mycobacterium tuberculosis (bovine strain)	0·5*	—	—
T_{2+} bacteriophage	0·44*	—	0·46
T_{2r} bacteriophage	0·51	—	—
Salmonella c bacteriophage	0·43	—	—

* Results based on single estimations.

bacteriophage. In all the nucleic acids the proportion of 6-methylaminopurine was low, varying from 0·4 to 2·5 moles/100 moles of adenine (Table 5). The complete purine and pyrimidine compositions of some of the nucleic acids are given in Table 6.

To exclude the possibility that the small amount of 6-methylaminopurine found in T_2 DNA (0·44% of the adenine) was due to contaminating host DNA (molar proportion of 6-methylaminopurine/adenine, 1·8–2·4%) we estimated the cytosine content of the phage DNA. (The cytosine contents of T_2 and *E. coli* DNA are 0 and 25% of the total bases respectively.) Examination of formic acid hydrolysates by two-dimensional chromatography in solvents 1 and 4 showed no cytosine. Amounts greater than 5% of the 5-hydroxymethyl cytosine would have been detected.

In those nucleic acids where 6-methylaminopurine was not found we have given in Table 7 estimates of the proportions which would have remained undetected by our methods. These are based on the optical densities of eluates from the appropriate areas of the chromatograms which failed to give an absorption maximum over the range 230–300 mμ.

Table 6. *Purine and pyrimidine compositions of deoxyribonucleic acids from strains of*
Escherichia coli *and* Aerobacter aerogenes

DNA of *Aerobacter aerogenes* was prepared by (a) the method of Smith & Wyatt (1951), (b) the trypsin method.

Source of DNA	Proportions of bases in DNA (moles/100 moles of total recovered bases)				
	Adenine	Guanine	Cytosine	Thymine	6-Methyl-aminopurine
E. coli 15 T⁻	23·6	27·5	26·1	22·4	0·42
E. coli B/r	23·1	26·4	25·1	25·0	0·42
Aerobacter aerogenes (a)	19·8	28·1	27·5	19·2	0·55
Aerobacter aerogenes (b)	20·3	28·2	27·0	19·1	0·53

Table 7. *Maximum possible amounts of 6-methylaminopurine in deoxyribonucleic acids
where it was not detected*

Source of DNA	Maximal proportion of 6-methylaminopurine (moles/100 moles of adenine)	Method of hydrolysis
Calf thymus	0·15	HClO₄
Horse spleen	0·28	
Wheat germ	0·26	
Baker's yeast	1·0*	
Bacillus cereus	0·08	N-HCl
Staphylococcus albus	0·10	
S. aureus	0·21	
Streptomyces griseus	0·28	
Streptomyces c 20 bacteriophage	2·0*	H·CO₂H

* Estimates based on visual observations of photographic prints of chromatograms.

DISCUSSION

The methylated derivatives of adenine, 2-methyl-adenine and 6-dimethylaminopurine occur naturally as components of vitamin B_{12} and puromycin respectively (Brown, Cain, Gant, Parker & Smith, 1955b; Dion, Calkins & Pfiffner, 1954; Waller, Fryth, Hutchings & Williams, 1953). Recently, both these purines together with 6-methylaminopurine have been found to occur in small amounts in ribonucleic acids from several organisms (Littlefield & Dunn, 1958a, b). The occurrence of these derivatives raises the question of their significance in nucleic acid structure.

With the exception of *E. coli* 15 T⁻ grown under conditions causing thymine deficiency, 6-methylaminopurine occurs in DNA in small proportions. Taking the value for the molecular weight of *E. coli* DNA as $9·1 \times 10^6$ (Brown, M'Ewen & Pratt, 1955c) and assuming a random distribution of 6-methylaminopurine, the base would constitute 120 residues/DNA molecule in *E. coli* B/r. T_2 bacteriophage would contain 500 residues/virus particle. In calf-thymus DNA, where we were unable to detect 6-methylaminopurine, it must represent less than nine residues/molecule (assuming a molecular weight of 6×10^6, Brown et al. 1955c). Equally small amounts of 6-methylaminopurine

could have remained undetected in the other DNA which we investigated.

The amounts of 6-methylaminopurine in bacterial DNA are of the same order of magnitude as those of 5-methylcytosine in some animal DNA (Wyatt, 1951). However, the biological distribution of 6-methylaminopurine in DNA is quite different from that of 5-methylcytosine which occurs in plant and animal DNA but is absent from microbial DNA.

The molar proportions adenine/thymine and cytosine/guanine in deoxyribonucleic acid are generally close to 1, in agreement with the double helical structure proposed by Watson & Crick (1953). In those DNA's containing 5-methylcytosine the molar ratio (cytosine and 5-methylcytosine)/guanine also equals 1, suggesting that in the Watson–Crick structure 5-methylcytosine, like cytosine, pairs with guanine. Similarly, it can be concluded that the 5-halogenated uracils, when incorporated into DNA, must replace thymine residues (Dunn & Smith, 1957). In the normal DNA we have examined 6-methylaminopurine comprises only 0·6 % or less of the total purine and pyrimidine residues. This corresponds to less than the deviations from unity of the molar ratios adenine/thymine and guanine/cytosine generally observed in analyses of DNA. In these nucleic

acids it is not possible from analytical data to determine the position that 6-methylaminopurine might occupy in the Watson–Crick structure. After growth of *E. coli* 15 T⁻ in the presence of 5-aminouracil, 2-thiothymine or limiting concentrations of thymine, the proportion of 6-methylaminopurine in the bacterial DNA increases up to 15% or more of the adenine. This is usually accompanied by a decrease in the proportion of thymine. In several preparations the decrease in the proportion of thymine residues was very nearly equalled by the increase in that of 6-methylaminopurine, suggesting the latter base replaced thymine in the nucleic acid structure. This correspondence was fortuitous, as in many analyses of DNA from 5-aminouracil-inhibited bacteria the decrease in the proportion of DNA thymine is not related to the increase in 6-methylaminopurine. In addition, an increase in the proportion of 6-methylaminopurine in the DNA of *E. coli* 15 T⁻ was noted where 5-chlorouracil was apparently completely replacing thymine residues (Table 4). From their similarity in structure it seems more likely that 6-methylaminopurine was replacing adenine residues, and our analytical data so far do not eliminate this possibility.

The causes of increase of 6-methylaminopurine in *E. coli* 15 T⁻ DNA are not clear. The formation of the abnormal DNA appears to be associated with growth in a limited supply of thymine. Some thymine analogues which are not incorporated into the DNA appear to limit the utilization of thymine by the bacteria, but even with analogues which apparently replace thymine residues in the DNA, an increase in the proportion of 6-methylaminopurine has been noted. Growth in the presence of 6-methylaminopurine, which is inhibitory to the bacteria, does not result in an increase in the proportion of the base in the DNA (Dunn & Smith, 1955 b). With 5-aminouracil-inhibited bacteria we have shown that growth is accompanied by a loss in viable cell count. In the complete absence of thymine, when there is little or no DNA synthesis, no increase in DNA 6-methylaminopurine can be detected. Thus loss in viability of *E. coli* 15 T⁻, characteristic of incubation in a medium devoid of thymine (Barner & Cohen, 1954), appears to be unassociated with changes in the 6-methylaminopurine content of the DNA.

SUMMARY

1. 6-Methylaminopurine has been identified as a constituent of deoxyribonucleic acid from *Escherichia coli* 15 T⁻ when grown in the presence of 5-aminouracil or 2-thiothymine or in low concentrations of thymine. During growth under these conditions it increases in amount from 2 to 15% or more of the adenine. The purine and pyrimidine compositions of these nucleic acids are given.

2. The isolation of 6-methylaminopurine deoxyriboside and deoxyribonucleotide from *E. coli* 15 T⁻ deoxyribonucleic acid is described.

3. 6-Methylaminopurine also occurs normally in deoxyribonucleic acid of *E. coli* strains B/r, K 12 and 15 T⁻, *Aerobacter aerogenes*, amounting to about 2% of the adenine, and in smaller amounts in the deoxyribonucleic acids of *Mycobacterium tuberculosis*, *Pneumococcus*, *E. coli* bacteriophage T₂ and *Salmonella* c bacteriophage.

4. No 6-methylaminopurine could be detected in deoxyribonucleic acid from calf thymus, horse spleen, wheat germ, salmon sperm, yeast, *Staphylococcus*, *Bacillus cereus*, *Streptomyces griseus* and *Streptomyces* bacteriophage. Values are given for the maximum proportion of 6-methylaminopurine which would have remained undetected.

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Comparison and Combination of the Starch-Gel and Filter-Paper Electrophoretic Methods Applied to Human Sera: Two-Dimensional Electrophoresis

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Recently a method of zone electrophoresis using starch gel as the supporting medium has been described (Smithies, 1955a, b), which enables a number of serum-protein components to be demonstrated which are not seen with the classical electrophoretic methods. A series of experiments has therefore been carried out in order to correlate the results obtained with starch gels with those obtained with filter-paper electrophoresis. The results of these experiments suggested that a suitable combination of the two methods in a single method would lead to a more complete resolution of the serum-protein components than is possible by any other single method. A system involving electrophoresis in two dimensions, first on filter paper and secondly at right angles in starch gel, has been developed. This two-dimensional system has been briefly described in a preliminary report (Smithies & Poulik, 1956). A full account of the method is given below, and the results obtained with it are discussed.

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A small number of abnormal sera have been studied by the starch-gel and filter-paper electrophoretic methods in order to confirm the findings with normal sera, and to illustrate the potentialities of starch-gel electrophoresis in the study of abnormal sera.

EXPERIMENTAL

Starch-gel electrophoresis

The method of starch-gel electrophoresis described by Smithies (1955b) was used. No important changes in the procedure have been made, but the method of preparing a starch suitable for serum electrophoresis has been studied in more detail. The source of unhydrolysed potato starch appears to be of considerable importance. Of the many varieties of potato starch tested, starch obtained from Denmark gives a product closest to that originally used. Starch from Idaho was also found to be suitable. In testing a given starch, hydrolysis is carried out with acetone–hydrochloric acid (1 vol. of conc. hydrochloric acid to 100 vol. of reagent-grade acetone). Several small samples of the starch and the acetone–hydrochloric acid are equilibrated overnight in a thermostatically controlled room at 37°. The hydrolysis is started by adding the acetone–hydrochloric acid to the starch samples (approx.