637. *The Isolation of 5-Methylcytosine Deoxyriboside from Wheat-germ Deoxyribonucleic Acid.*

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5-Methylcytosine deoxyriboside has been isolated as its hydrochloride and picrate from enzymic hydrolysates of wheat-germ deoxyribonucleic acid by the combined techniques of ion exchange and partition chromatography. Physical data for this compound are recorded.

The general adherence of investigators in the nucleic acid field to the "tetranucleotide" theory, and lack, until recently, of suitable microanalytical methods, hindered the search for new components of the ribo- and deoxyribo-nucleic acids. Development of the chromatographic-separation and the spectral-analysis technique by Hotchkiss (*J. Biol. Chem.*, 1948, **175**, 315) and by Vischer and Chargaff (*ibid.*, 1948, **176**, 715) has now eliminated the latter obstacle and has provided elegant means for the quantitative determination of the purine and the pyrimidine degradation products of nucleic acids. Employing a relatively simple procedure, which combined a modification of the Vischer–Chargaff method for degradation and separation and the Markham–Smith (*Biochem. J.*, 1949, **45**, 294) photographic method for detection, Wyatt (*ibid.*, 1950, **47**, vii; *Nature*, 1950, **166**, 237) has demonstrated the presence of 5-methylcytosine in a variety of deoxypentose nucleic acids derived from both plant and animal sources. This pyrimidine base had very early been described by Johnson and Coghill (*J. Amer. Chem. Soc.*, 1925, **47**, 2838) as a constituent of tuberculinic acid, but the failure of Vischer, Zamenhof, and Chargaff (*J. Biol. Chem.*, 1949, **177**, 429), and of Wyatt (*locc. cit.*), to locate 5-methylcytosine in the hydrolysates of the deoxypentose nucleic acids of avian, human, and bovine tubercle bacilli, makes the validity of this early claim extremely doubtful. Furthermore, Wyatt was unable to confirm Rosedale's claim (*J. Ent. Soc. S. Africa*, 1948, **11**, 34) that in the nucleic acids of locusts and certain other insects thymine is quantitatively replaced by 5-methylcytosine. Pertinent to Wyatt's discovery, it should be mentioned that Hotchkiss (*loc. cit.*) and Daly, Allfrey, and Mirsky (*J. Gen. Physiol.*, 1950, **33**, 497), while examining the purine and pyrimidine base content of nucleic acids from various sources, obtained chromatographic evidence for the presence of a substance which had properties similar to those of cytosine. Although positive identification was not achieved, it now seems possible, in view of the chromatographic and spectral properties, that in each case the new base was 5-methylcytosine. Further evidence for the existence of this pyrimidine as an integral component of certain nucleic acids has come from ion-exchange studies by Cohn (*J. Amer. Chem. Soc.*, 1950, **72**, 2811). In an investigation of the nucleotides formed by enzymic degradation of a deoxypentose nucleic acid, a small quantity of a substance was isolated from which a base was derived, whose elution position and spectral properties suggested that it might be 5-methylcytosine. The existence of this new nucleotide has been more firmly established in a very recent paper (Cohn, *J. Amer. Chem. Soc.*, 1951, **73**, 1539).

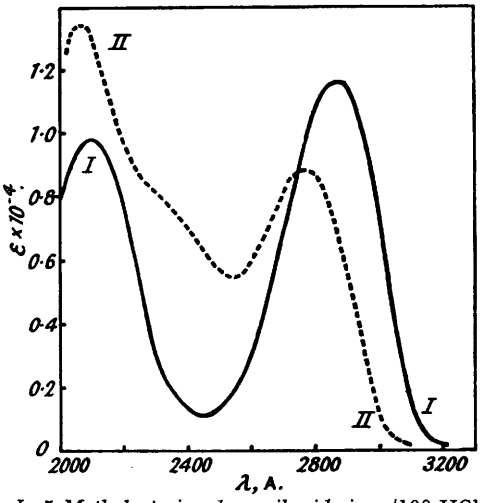
In view of the foregoing observations it was considered desirable to isolate and unequivocally identify 5-methylcytosine deoxyribonucleoside. Although this substance has not heretofore been isolated, evidence for its enzymic synthesis has been obtained by MacNutt (*Nature*, 1950, **166**, 444) in exchange reactions of 5-methylcytosine with purine deoxyribonucleosides. It was decided that the deoxyribonucleic acid isolated from wheat germ, which contains more 5-methylcytosine than any other nucleic acid so far investigated, would be the best source of the desired nucleoside. The deoxyribonucleic acid obtained was impure and contained up to 7% of ribonucleic acid, but removal of the latter was considered unnecessary for the present work. The presence of 5-methylcytosine was confirmed by paper-chromatographic examination of the formic acid hydrolysate (Vischer and Chargaff, *loc. cit.*; Wyatt, *locc. cit.*).

The nucleic acid was subjected to enzymic degradation, initially by deoxyribonuclease from beef pancreas (McCarty, *J. Gen. Physiol.*, 1946, **29**, 123), and then by the alkaline phosphatase of calf intestinal mucosa (Bielschowsky and Klein, *Z. physiol. Chem.*, 1932, **207**, 202; Klein, *ibid.*, 1938, **255**, 82). No attempt was made to inhibit the adenine deoxyriboside deaminase which is also present in calf intestinal mucosa. The amount of phosphorus split off as inorganic phosphate varied from 86 to 98%.

Nucleotides and most of the neutral nucleosides were removed by passing the hydrolysate down a column of "Zeo-Karb 215" in the H^+ cycle (Elmore, *Nature*, 1948, **161**, 931; *J.*, 1950,

2084; Harris and Thomas, *Nature*, 1948, **161**, 931; *J.*, 1948, 1936). The percolate appeared to contain deoxyribose as shown by paper chromatography, and it was concluded that purine deoxyribonucleosides were hydrolysed to the free bases during this process; this was considered desirable since it facilitated the isolation of the required nucleoside. Paper chromatography in *iso*propanol–2N-hydrochloric acid revealed that cytosine deoxyriboside, 5-methylcytosine deoxyriboside, guanine, hypoxanthine, and traces of thymidine and adenine were eluted from the column with aqueous ammonia. The purine bases were partly precipitated by alcohol; residual guanine and hypoxanthine and part of the adenine and thymidine were removed by chromatography on a cellulose column with *iso*propanol–2N-hydrochloric acid (65 : 35) as the solvent. Spectral analysis indicated that 5-methylcytosine deoxyriboside was present in the foremost fractions of those containing cytosine deoxyriboside. The absorption characteristics for 5-methylcytosine deoxyriboside were estimated from the data for the free pyrimidine (Hitchings, Elion, Falco, and Russell, *J. Biol. Chem.*, 1949, **177**, 357; Wyatt, *Nature*, 1950, **166**, 237), on the assumption that the carbohydrate on $N_{(3)}$ would produce a shift of $\lambda_{max.}$ towards the visible region of 20—50 Å in neutral or acid solution as has been observed for cytosine and cytidine (Ploeser and Loring, *J. Biol. Chem.*, 1949, **178**, 431). Adenine

FIG. 1.



I. 5-*Methylcytosine deoxyriboside in* N/100-HCl.
II. 5-*Methylcytosine deoxyriboside in* N/90-NaOH.

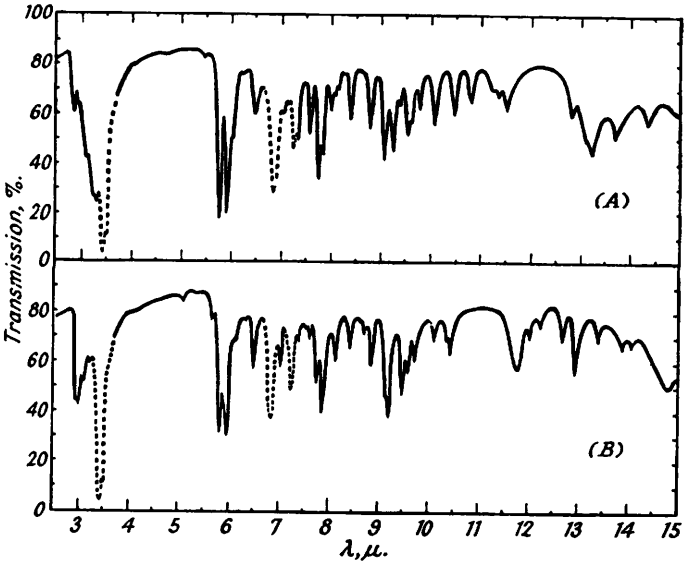
and thymidine were completely removed, and partial separation of cytosine deoxyriboside and 5-methylcytosine deoxyriboside was achieved by chromatography on a cellulose column with *n*-butanol saturated with water as the solvent. From those fractions, which were shown by paper chromatography to contain only 5-methylcytosine deoxyriboside, the nucleoside picrate, m. p. 175—178° (decomp.), was isolated. Most of the picrate was converted into the free nucleoside by percolation of an aqueous solution through a column of " Amberlite IRA-400." The product was somewhat hygroscopic and proved difficult to crystallise on account of its high solubility; the majority was therefore converted into the hydrochloride for ease of characterisation. An additional quantity of the hydrochloride was obtained from chromatographic fractions, which contained both cytosine deoxyriboside and 5-methylcytosine deoxyriboside, by further separation on a column of powdered cellulose, the upper layer of *n*-butanol–aqueous ammonia (*d* 0·88)–water (60 : 15 : 25) being used as the solvent system.

The ultra-violet and the infra-red absorption spectra are given in Figs. 1 and 2. The former confirms the prediction that the presence of a substituent on $N_{(3)}$ of 5-methylcytosine produces a shift of $\lambda_{max.}$ similar to that observed in the case of the cytosine nucleosides. Data are also given in Fig. 2 for cytosine deoxyriboside hydrochloride for comparison.

During an earlier attempt to isolate cytosine deoxyriboside and 5-methylcytosine deoxyriboside from an enzymic hydrolysate, neither could be detected by paper chromatography; instead, uracil deoxyriboside (Dekker and Todd, *Nature*, 1950, **166**, 557) and an increased amount of thymidine were present. These must have resulted from the adventitious action

of a deaminase. Since calf intestinal mucosa apparently does not contain a cytosine deoxyriboside deaminase (Klein, in Bamann and Myrbäck, " Die Methoden der Fermentforschung," New York, 1945), it must be concluded that bacterial contamination occurred at some stage. Certain strains of *Escherichia coli* are known to possess a powerful cytosine nucleoside deaminase (Wang, Sable, and Lampen, *J. Biol. Chem.*, 1950, **184**, 17). It is not known, however, if the same enzyme is responsible for the deamination of both cytosine and 5-methylcytosine deoxyribosides. In later experiments, protection of hydrolysates by toluene overcame this difficulty.

FIG. 2.



A. 5-Methylcytosine deoxyriboside hydrochloride in " Nujol."
B. Cytosine deoxyriboside hydrochloride in " Nujol."

Additional evidence for the formulation of this new nucleoside as 5-methylcytosine deoxyriboside was derived from its deamination with nitrous acid to thymidine, identified by its R_F on a paper chromatogram and its ultra-violet absorption spectrum. Furthermore, the new nucleoside gave a positive Dische reaction (*Mikrochemie*, **1930**, **8**, 4).

EXPERIMENTAL.

All m.-p. determinations were made on the Kofler hot stage. Paper chromatograms were examined by means of a " Mineralite " lamp and subsequently photographed on Ilford Reflex Document Paper No. 50 in ultra-violet light (Markham and Smith, *loc. cit.*); fractions from cellulose columns were spotted on filter paper and examined under the " Mineralite " lamp. Ultra-violet absorption spectra were determined with a " Unicam " quartz spectrophotometer, Model S.P. 500. Infra-red absorption spectra were determined with a Perkin Elmer Instrument, Model 21, with rock salt optics.

Wheat-germ Deoxyribonucleic Acid.—Several batches of commercial wheat germ (" Froment ") were extracted by the method of Daly, Allfrey, and Mirsky (*loc. cit.*) and the nucleoprotein deproteinised by the method of Sevag, Lackmann, and Smolens (*J. Biol. Chem.*, 1938, **124**, 425). The product (2·0—5·2 g. per 36 oz. of " Froment ") was usually fibrous in nature (Found, in typical sample: C, 35·8; H, 5·3; N, 15·3%). The ribonucleic acid content was determined by Schneider's method (*ibid.*, 1945, **161**, 293) and shown to be not more than 7%.

Chromatographic Examination of Base Content.—Wheat-germ deoxyribonucleic acid (15 mg.) was heated with formic acid (1 c.c.) at 175° for 30—40 minutes. The hydrolysate was evaporated to dryness and dissolved in N-hydrochloric acid (0·2 c.c.). The filtered solution was qualitatively analysed by paper chromatography in *iso*propanol–5·7N-hydrochloric acid (65 : 35). The major constituents were guanine (R_F 0·31—0·34), adenine (R_F 0·45—0·48), cytosine (R_F 0·56—0·58), and thymine (R_F 0·81—0·83); 5-methylcytosine (R_F 0·64—0·66) and uracil (R_F = 0·72—0·74) were present in small quantity.

Enzymic Degradation.—Wheat-germ deoxyribonucleic acid (9·8 g.) was dissolved in distilled water (980 c.c.) and the pH adjusted to 7·0 with dilute aqueous ammonia. The solution was covered with toluene during all subsequent operations. Magnesium sulphate (2·27 g.) and crude deoxyribonuclease (50 mg.) were added and the solution incubated at 37—38° for 22 hours. The pH was then adjusted to 8·0 by the addition of N-aqueous ammonia (9 c.c.). N-Ammonia–N-ammonium sulphate buffer

(1 : 1; 100 c.c.) and calf intestinal mucosa extract (100 c.c.), prepared according to Klein's directions (*loc. cit.*), were introduced and the solution was incubated at 37—38° overnight. Magnesium sulphate (4 g.) and sufficient ammonia to bring the pH to 9·0 were added and the incubation was continued for a total of 47 hours. Magnesium ammonium phosphate was filtered off and sufficient barium hydroxide was added to precipitate sulphate ions quantitatively. Analysis of the filtrate for total phosphorus (Allen, *Biochem. J.*, 1940, **34**, 858) indicated that approx. 98% of the organic phosphate had been split off during the enzymic hydrolysis. The filtrate was evaporated under reduced pressure to 100 c.c. and then freeze-dried.

Isolation of Pyrimidine Deoxyribosides.—The dry residue was extracted with 8 portions (100 c.c.) of 95% ethanol. The filtered extracts were combined and analysed by paper chromatography in *iso*propanol–2N-hydrochloric acid (65 : 35). Purine deoxyribosides were hydrolysed to the free bases on the paper. The following substances were observed: guanine (R_F 0·26—0·31), hypoxanthine (R_F 0·36—0·42), adenine (R_F 0·40—0·47), cytosine deoxyriboside (R_F 0·58—0·63), 5-methylcytosine deoxyriboside (R_F 0·65—0·70), and thymidine (R_F 0·86—0·89). After evaporation of the ethanolic solution to dryness, the residue was dissolved in water (250 c.c.) and passed down a column (11·4 × 1·7 cm.) of " Zeo-Karb 215 " (12 g.; 30—40 mesh). The column was washed with water (800 c.c.). From the combined percolate and washings, after evaporation under reduced pressure and addition of ethanol, crystalline thymidine (0·80 g.) was obtained, having m. p. 184—186° after one recrystallisation. The column was eluted with 0·5N-aqueous ammonia (3600 c.c.), and the eluate evaporated *in vacuo* to 75 c.c. Paper chromatography with *iso*propanol–2N-hydrochloric acid (65 : 35) revealed that most of the thymidine had been removed. Ethanol (150 c.c.) was added and the solution was placed in the refrigerator overnight. The precipitated purines were removed by filtration and washed with 66% aqueous ethanol. The combined filtrate and washings were evaporated under reduced pressure to 30 c.c. Precipitation with ethanol (170 c.c.) was repeated and a further small quantity of purines was removed. Paper chromatographic analysis in *iso*propanol–2N-hydrochloric acid (65 : 35) showed that the purine precipitate contained guanine and hypoxanthine, and the ethanolic filtrate was considerably enriched with respect to cytosine deoxyriboside and 5-methylcytosine deoxyriboside.

The ethanolic solution was evaporated under reduced pressure and freeze-dried. The residue (1·883 g.) was dissolved in *iso*propanol–2N-hydrochloric acid (65 : 35) and subjected to chromatography on a column (34 × 6 cm.) of powdered cellulose (350 g.) with the same solvent system, the automatic fraction collector described by Gilson (*Chem. and Ind.*, 1951, 185) being used. Paper chromatography of individual fractions revealed that, although separation was incomplete, the bulk of the purines had been removed. All fractions containing 5-methylcytosine deoxyriboside were combined, neutralised with aqueous sodium hydroxide, and evaporated to dryness. The residue was extracted with six portions (30 c.c. each) of *iso*propanol, and the combined extracts were evaporated to dryness. The residue (1·430 g.) was dissolved in *n*-butanol saturated with water (70 ml.). One fifth of the solution was subjected to chromatography on a column (29·4 × 3·3 cm.) of powdered cellulose (85 g.) with *n*-butanol saturated with water as the solvent. Thymidine and remaining traces of adenine were removed and partial separation of cytosine deoxyriboside and 5-methylcytosine deoxyriboside was effected, as revealed by spectroscopic examination and paper chromatography of relevant fractions. The remaining four-fifths of the mixture were then separated as above on a column (32·3 × 6·0 cm.) of powdered cellulose (350 g.) with a similar degree of fractionation.

Fractions from both separations containing only 5-methylcytosine deoxyriboside were combined and evaporated to small bulk. Ethanol (5 c.c.) and aqueous picric acid (7 c.c.) were added and the solution set aside in the refrigerator. *5-Methylcytosine deoxyriboside picrate* crystallised in fine needles and was recrystallised from ethanol. The yield was 3·0 mg. and the m. p. 175—177° (decomp.). A further quantity crystallised on storage in the refrigerator for several days. This was also recrystallised from ethanol [yield, 4·8 mg.; m. p. 175—178° (decomp.)] (Found: C, 41·0; H, 4·0. $C_{16}H_{15}O_{11}N_6$ requires C, 40·85; H, 3·9%).

The mother liquors from 5-methylcytosine deoxyriboside picrate were evaporated to dryness and the residue recrystallised from ethanol. The product (33·0 mg.) was passed in water (5 c.c.) down a column (2·0 × 0·6 cm.) of " Amberlite IRA-400." The column was washed thoroughly with water and the combined percolate and washings were evaporated under reduced pressure and freeze-dried. The residue was crystallised from ethanol–ether and afforded somewhat hygroscopic needles (8·2 mg.) of the free nucleoside. The mother-liquors were concentrated and saturated with dry hydrogen chloride. *5-Methylcytosine deoxyriboside hydrochloride* (8·0 mg.) crystallised in stout prisms, m. p. 156°.

All fractions from chromatographic separations containing both cytosine deoxyriboside and 5-methylcytosine deoxyriboside were combined, evaporated under reduced pressure, and freeze-dried. The residue (104·8 mg.) was dissolved in the upper layer of the solvent system *n*-butanol–aqueous ammonia (*d* 0·880)–water (60 : 15 : 25) and subjected to chromatography on a column (28·0 × 3·3 cm.) of powdered cellulose (80 g.), the same solvent being used. Paper chromatography of individual fractions revealed that a considerable degree of separation had been achieved. Fractions containing only 5-methylcytosine deoxyriboside were combined, concentrated, and freeze-dried. The residue (23·4 mg.) was converted into the picrate as above. The product (45 mg.) was converted into the free nucleoside on a column of " Amberlite IRA-400." A solution of the freeze-dried nucleoside (19·7 mg.) in ethanol–ether was saturated with dry hydrogen chloride, giving *5-methylcytosine deoxyriboside hydrochloride* (16·3 mg.), m. p. 156°, $[\alpha]_D^{14}$ +65° ±4° (*c*, 0·461 in N-sodium hydroxide) (Found: C, 42·1, 42·1; H, 5·7, 5·7; N, 13·6; Cl, 12·6. $C_{10}H_{16}O_4N_3Cl,\frac{1}{2}H_2O$ requires C, 41·9; H, 6·0; N, 14·6; Cl, 12·4%). Although the single nitrogen analysis is unsatisfactory, insufficiency of material precluded a duplicate determination. It is felt, however, in view of the other acceptable analyses and further evidence (crystallinity, sharp m. p., ultra-violet absorption spectra, and chromatographic homogeneity), that the substance is pure and has the structure designated. Ultra-violet light absorption: (i) in

N/100-hydrochloric acid : Maxima at 2860 and 2090 Å, $\epsilon_{max.}$ 11,610 and 9780; minimum at 2450 Å, $\epsilon_{min.}$ 1050; (ii) in N/90-sodium hydroxide : Maxima at 2770 and 2060 Å, $\epsilon_{max.}$ 8810 and 13,380; minimum at 2550 Å, $\epsilon_{min.}$ 5430.

All fractions from chromatographic separations containing only cytosine deoxyriboside were combined and evaporated under reduced pressure and the picrate isolated in the usual manner, the yield being 0·22 g. and the m. p. 208° (decomp.).

Deamination of 5-Methylcytosine Deoxyriboside.—The solution used for determination of optical rotation was acidified with acetic acid, and sodium nitrite was added. After several hours at room temperature, the solution was evaporated to dryness and the residue extracted with ethanol. Paper chromatography of the ethanol extract in *n*-butanol saturated with water revealed the presence of a considerable quantity of thymidine. The thymidine was extracted from the chromatogram with water, and the ultra-violet absorption spectrum determined : $\lambda_{max.} = 2680$, $\lambda_{min.} = 2350$ Å in neutral solution.

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