THE SUGAR COMPONENT OF DEOXYRIBONUCLEOSIDES[1]

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ABSTRACT

From highly polymerized deoxyribonucleate the nucleosides have been isolated by an improved method. The sugar obtained on hydrolysis of the individual nucleosides has been shown to be D-2-deoxyribose in each case.

INTRODUCTION

Although it is assumed that the only carbohydrate constituent of deoxyribonucleate (DNA) is D-2-deoxyribose, the sugar has not been isolated from all of the five nucleosides known to be present in most samples of DNA. Levene and Mori (16) obtained a guanine nucleoside after digestion of calf thymus DNA and concluded that it was a glycoside of D-2-deoxyribose (15). Subsequently other workers found that the same sugar was liberated on acid hydrolysis of the mixed purine deoxynucleosides (6, 10, 12). Similar studies of the pyrimidine deoxynucleosides have been unsuccessful because of the difficulty of cleaving the glycosidic linkages without destroying the sugar.

The results obtained by MacNutt (19) and Friedkin and Roberts (8) in their experiments with nucleoside phosphorylase have provided good evidence that the same sugar is present in the purine—and pyrimidine—deoxynucleosides.

Burke (3) reported, without giving experimental details, that reduction with sodium and ethanol in liquid ammonia rendered pyrimidine nucleosides easily hydrolyzable by Dowex 50 cation exchange resin. The sugars liberated from thymidine and deoxycytidine were identified with D-2-deoxyribose by chromatography and ionophoresis on paper.

In spite of all this evidence we considered that it would be worth while to isolate and to identify in the conventional way the sugar from each of the nucleosides of DNA.

Highly polymerized DNA (20) was hydrolyzed by the combined actions of pancreatic deoxyribonuclease (18) and the phosphodiesterase and 5′-nucleotidase of water moccasin venom. The resulting nucleoside mixture was resolved by anion exchange chromatography (1). Two difficulties were encountered at this stage. The first of these was the finding that the nucleosides of cytosine and 5-methylcytosine did not separate during the chromatography. Therefore, to prepare each of these free of the other the corresponding nucleotides were separated and isolated and from them the nucleosides obtained by hydrolysis with phosphomonoesterase. The second difficulty was that of isolating crystals of the other nucleosides from the large volumes of salt solutions in which they were eluted from the ion exchange column. This problem was solved by our discovery, in common with others (4, 17), that nucleosides and nucleotides were readily adsorbed from aqueous solutions on passage through a column of activated charcoal. When this procedure was applied to the eluates from

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the ion exchange columns the nucleosides were retained quantitatively on the charcoal column and the salt passed through. The nucleoside (or nucleotide) could be eluted from the charcoal easily with ammoniacal alcohol. Good yields of the nucleosides were obtained and their physical constants agreed well with the most recently reported values (1, 7, 19).

The purine nucleosides deoxyadenosine and deoxyguanosine were most conveniently hydrolyzed by an aqueous suspension of a sulphonic acid cation exchange resin in the hydrogen form (11), the times and temperatures being selected to give a maximum color development when the quantitative Schiff's test (5) was applied to the supernatant solution.

The pyrimidine nucleosides were reduced prior to hydrolysis since Levene and La Forge (13) had demonstrated that ribose could be obtained from uridine by mild acid hydrolysis following catalytic hydrogenation of the nucleoside. Levene and London (14), however, reported that their efforts to hydrogenate thymidine, in the same manner as uridine, were unsuccessful. In our experiments deoxycytidine readily absorbed one mole of hydrogen in the presence of palladium black; the product, on acid hydrolysis, yielded a reducing sugar and dihydrouracil. This was expected since Brown and Johnson (2) had found that cytosine was deaminated on catalytic hydrogenation. Under the same conditions thymidine absorbed two moles of hydrogen and was converted to a substance that did not liberate any reducing or Schiff-positive material on heating with 0.1 *N* or 1.0 *N* sulphuric acid at 100° for periods up to 90 min. This experience made it necessary to use other methods of reducing thymidine, and sodium amalgam was found to be suitable for the purpose. The reduction product in this case was readily amenable to acid hydrolysis.

The hydrolytic conditions needed to free the pyrimidine-bound sugars were harsher than those used for the purine nucleosides. In order to avoid possible chemical changes brought about by this treatment and the preliminary reduction, thymidine and deoxycytidine (after deamination to deoxyuridine) were hydrolyzed with the thymidine phosphorylase system of Friedkin and Roberts (8).

The sugars, liberated by the procedures described above from each of the four nucleosides, were compared with each other and with synthetic L-2-deoxyribose (21) by paper chromatography in two different solvent systems and by the melting points, mixed melting points, and rotations of their crystalline benzylphenylhydrazones. In each of the four cases it was concluded that the sugar was D-2-deoxyribose.

EXPERIMENTAL

All distillations at reduced pressure were carried out with a bath temperature not exceeding 40°.

Isolation and Characterization of the Deoxynucleosides

Twenty grams of DNA, prepared by the method of Marko and Butler (20), was dispersed in 500 ml. of water and hydrolyzed with 150 mgm. of deoxyribonuclease (18). When hydrolysis was complete, the pH of the solution was

adjusted to 9.0 with conc. ammonium hydroxide, and 500 mgm. of lyophilized water moccasin venom,* dissolved in 10 ml. of water, was added. The digest was incubated at 37° and maintained at pH 9.0 by the periodic addition of 2 *N* ammonium hydroxide. When the pH of the digest ceased to fall (4–6 hr.) the addition of ammonium hydroxide was stopped and the digest stored at 37° overnight. After this treatment, analyses usually showed that 70–80% of the total phosphorus of the digest had become inorganic phosphate. In order to carry the release of nucleosides nearer to completion, magnesium formate (equivalent to the phosphate content) was added and the precipitated magnesium ammonium phosphate removed by centrifugation. More of the venom (200 mgm.) was added to the supernatant solution which had been readjusted to pH 9.0, and the mixture was stored at 37° for 15 hr.

The resulting solution was reduced to a volume of 200 ml. by distillation *in vacuo*, brought to pH 10.4 with conc. ammonium hydroxide, and poured onto a column (17 cm. height × 6.8 cm. diameter) of Dowex 2 (200–400 mesh) in the formate form. The flow rate throughout the chromatography was 150 ml. per hr. Each fraction was collected for 30 min. and its nucleoside content measured by ultraviolet spectrophotometry. The procedure and results may be summarized as follows:

Fraction	Nucleoside of	Eluent	Volume (l.)
I	Adenine and cytosine	0.02 *M* formate, pH 9.8	2
II	Thymine	0.05 *M* formate, pH 8.5	2
III	Guanine	0.05 *M* formate, pH 4.5	3

The first of these eluents was prepared by dissolving 0.02 mole of formic acid in 750 ml. of water, adding ammonium hydroxide until the pH was 9.8, adjusting the volume to one liter, and finally readjusting the pH to 9.8 with ammonium hydroxide. The other eluents were prepared by a similar procedure.

After reduction to a volume of 50 ml. by vacuum distillation Fraction I was stored at 5° for 24 hr. Most of the deoxyadenosine crystallized and yields of 1.9–2.0 gm. were obtained. When it was recrystallized from water this material had: nitrogen content, 23.1% (calculated for $C_{10}H_{13}O_3N_5.3H_2O$, 23.0%); m.p. 187.5–189°; $[\alpha]_D^{21}$, −32° (c = 1.11 in water at pH 7.0).

The mother liquors and washings of the deoxyadenosine crystals were combined, adjusted to pH 4.5 with formic acid, and chromatographed on a column of carboxyl cation exchange resin (Amberlite IRC 50, 500 mesh). The column measured 20 cm. in height by 3.4 cm. in diameter and was prepared by washing it with a 0.5 *M* solution of ammonium formate of pH 4.5 followed by water to remove the buffer. After the nucleoside solution had passed into the resin, elution was carried out with 0.05 *M* ammonium formate (pH 4.5) at the rate of 80 ml. per hr. By collecting 60 ml. samples it was found that deoxycytidine was eluted in a volume of 700 ml. followed closely by deoxyadenosine.

**Purchased from Ross Allen's Reptile Institute, Silver Springs, Florida.*

The deoxycytidine solution was passed through a column containing 10 gm. of activated charcoal* and the column washed with 50 ml. of water to remove salt. Elution of the nucleoside from the charcoal was effected with 200 ml. of ammoniacal alcohol (prepared by mixing 500 ml. of 95% ethanol, 430 ml. of water, and 17 ml. of conc. ammonium hydroxide). The resulting filtrate was distilled to dryness under reduced pressure and the residue dried to a fluffy white solid by the repeated vacuum distillation of 10 ml. portions of absolute ethanol. In order to crystallize the deoxycytidine it was converted to the hydrochloride by dissolving the dry residue in 7 ml. of methanol and adding a solution of dry hydrogen chloride in methanol (15 ml. of 3 *N*). Crystallization began almost immediately and after storing at 5° overnight 780 mgm. of crystals were collected. This hydrochloride was recrystallized by dissolving it in 1 ml. of hot water and slowly adding 3 ml. of ethanol. In a melting-point determination the crystals turned brown at 168° and melted with decomposition at 174°. The nitrogen content was 15.8%; calculated for $C_9H_{14}O_4N_3.HCl$, 15.9%. The optical rotation was $[\alpha]_D^{24} = +54.3°$ (in water at pH 7.0, $c = 5.51$ calculated as the free nucleoside).

Fraction II was distilled *in vacuo* to a volume of about 10 ml. On storing this solution at 5°, thymidine crystallized in yields of 1.6 to 2.0 gm. After recrystallization from water the thymidine had: nitrogen content, 10.2% (calculated for $C_{10}H_{15}O_5N_2.2H_2O$, 10.1%); m.p. 189–190°; $[\alpha]_D^{21}$, +18° ($c = 1.16$ in water at pH 7.0).

Fraction III was passed through 15 gm. of charcoal in a column 3.4 cm. in diameter and the column washed with water. Deoxyguanosine was eluted with 1 liter of ammoniacal alcohol. After the volume of the filtrate was reduced to 400 ml. by vacuum distillation and it was stored at 5°, 1.4 gm. of nucleoside crystallized as fine needles. Concentration of the mother liquors yielded another 0.6 gm. After recrystallization from water the deoxyguanosine had: N, 24.3% (calculated for $C_{10}H_{13}O_4N_5.1\frac{1}{4}\ H_2O$, 24.2%); no m.p. up to 250°; $[\alpha]_D^{24}$, −40° ($c = 0.924$ in 0.1 *N* NaOH, pH 12.64).

In order to prepare deoxycytidine free of the 5-methylcytosine nucleoside it was found convenient to dephosphorylate chromatographically pure deoxycytidine-5′-phosphate. Deoxycytidylic acid (310 mgm.) was dissolved in 35 ml. of water and the pH of the solution adjusted to 9.0 with sodium hydroxide. On treatment with 10 mgm. of water moccasin venom for 30 hr. at 38°, all of the phosphorus in the solution had become inorganic. The digest was acidified to pH 5.0 with glacial acetic acid and passed through 3 gm. of charcoal in a column of 2.5 cm. diameter. After the column was washed with 10 ml. of water the nucleoside was eluted with 100 ml. of the ammoniacal ethanol. This eluate was evaporated to dryness and the residue dried by distilling from it 20 ml. portions of absolute ethanol. The residue was dissolved in 5 ml. of methanol and the solution clarified by centrifugation. After 3 ml. of 3 *N* hydrogen chloride in methanol was added to the clear supernatant solution crystals began to appear immediately and 180 mgm. were collected following storage at 5° for a few hours. An additional 35 mgm. of crystals was obtained from the mother

**Darco G–60, Atlas Powder Co.*

liquors. The combined product was recrystallized by dissolving it in 0.15 ml. of hot water, slowly adding 2 ml. of absolute ethanol, and chilling. The resulting crystals showed browning at 167° and melted with decomposition at 171°.

When the procedure just described was applied to 110 mgm. of chromatographically pure 5-methyldeoxycytidylic acid, 59 mgm. of 5-methyldeoxycytidine hydrochloride (m.p. 157–158°) was obtained.

Isolation of the Sugar Component of the Purine Deoxynucleosides

Deoxyguanosine (600 mgm.), 2.4 gm. of dry Nalcite HCR*, and 11 ml. of water were mixed in a 15 ml. centrifuge tube and heated at 80° for 10 min. with stirring. The resin was collected by centrifuging and washed three times with small portions of water. Traces of sulphuric acid were removed from the combined supernatant and wash solutions by treatment successively with two drops of 3 *N* barium hydroxide and a small pellet of dry ice. The filtrate obtained from this mixture was concentrated to a sirup *in vacuo* at room temperature.

The sirup was transferred to a 30 ml. beaker with 2 ml. of water and to it were added 300 mgm. of sodium acetate (trihydrate) and 528 mgm. of recrystallized benzylphenylhydrazine hydrochloride dissolved in 4 ml. of β-methoxy-ethanol (methyl cellosolve). The solution was thoroughly mixed and water added to it dropwise with mixing until an opalescence appeared. After storage in the cold the yellow crystals were collected by filtration and washed with methyl cellosolve – water (2 : 1). A final washing with ether removed much of the yellow color leaving 323 mgm. of light yellow needles with m.p. 118–124°. The crude product was purified by dissolving it in the minimum volume of methyl cellosolve, adding two volumes of ether, and storing the solution in the cold. After collection by filtering the white needles that separated melted at 127–128°.

Treatment of deoxyadenosine (600 mgm.) in a similar manner yielded 400 mgm. of crude benzylphenylhydrazone which on recrystallization from methyl cellosolve – ether gave 215 mgm. of white crystals with m.p. 126–128°.

Isolation of the Sugar Component of Deoxycytidine

Deoxycytidine hydrochloride (645 mgm.) was dissolved in 50 ml. of water and the solution neutralized with sodium hydroxide solution. Palladium black (500 mgm.) was added and the hydrogenation allowed to proceed for $3\frac{1}{2}$ hr. at 75° with a hydrogen pressure slightly above atmospheric. The uptake of hydrogen ceased at one mole per mole of nucleoside. After removal of catalyst the solution, now alkaline because of the ammonia released on deamination(2), was neutralized with hydrochloric acid. The nucleoside was adsorbed from the solution by passing it through 9 gm. of charcoal in a column 3.4 cm. in diameter. The column was washed with water to remove salts and the nucleoside eluted with 200 ml. of water: ethanol (1: 1). Solvent was distilled from the eluate, the sirupy residue was dissolved in 20 ml. of 0.1 *N* sulphuric acid, and

**Cation exchange resin converted to the hydrogen form with sulphuric acid, washed with water, and dried in air.*

the solution heated under reflux in a boiling water bath for 30 min. The solution was cooled, neutralized with barium hydroxide, filtered with the aid of a little charcoal, and distilled to dryness *in vacuo*. The residue was dissolved in methanol to give a solution which on slow evaporation deposited crystalline dihydrouracil of m.p. 272° (Lit. (9) 272–274°). From the sugar remaining in the methanol a benzylphenylhydrazone was prepared, two such experiments yielding 180 mgm. of crude product which had m.p. 127–129° after recrystallization from methyl cellosolve – ether.

Isolation of the Sugar Component of Thymidine

Thymidine (400 mgm.) in 10 ml. of water was stirred for two hours with 3 gm. of sodium–mercury amalgam (2.5% sodium). A further 3 gm. of amalgam was added and the stirring continued until all the sodium had reacted. After a further treatment with 3 gm. of fresh amalgam the solution had a low optical density at 2670 Å indicating that reduction of the pyrimidine ring was almost complete. After being filtered the solution was neutralized with 1 *N* sulphuric acid and poured into 10 times its volume of ethanol: ether (4: 1). The precipitated sodium sulphate was removed by filtration and ethanol and ether removed from the filtrate by vacuum distillation. The aqueous solution remaining was adjusted to 20 ml., made $\frac{1}{10}$ *N* with sulphuric acid, and heated at 100° under reflux for 30 min. The sirupy residue left after removal of sulphuric acid and water yielded 135 mgm. of crude benzylphenylhydrazone which on recrystallization from methyl cellosolve – ether had m.p. 127–129°. No dihydrothymine was isolated in these preparations and subsequent experiments showed that it was destroyed in alkaline solution.

Enzymatic Hydrolysis of Deoxycytidine and Thymidine

The enzyme thymidine phosphorylase was extracted from fresh rat liver as described by Friedkin and Roberts (8) but the purification was not carried beyond the first fractionation with ammonium sulphate.

Thymidine (1.8 mM.) was dissolved in 50 ml. of 0.1 *M* arsenate – 0.1 *M* succinate buffer of pH 5.9 and added to 100 ml. of enzyme solution. After incubation of the mixture at 38° for 16 hr. spectrophotometric assay (8) indicated complete arsenolysis of the nucleoside. A protein precipitate was removed by centrifugation and the remainder of the protein in solution was precipitated by the addition of one-tenth volume of 3% perchloric acid and centrifuging. The combined supernatant solution and water washings of the precipitate were reduced to a volume of 50 ml. by vacuum distillation and desalted by passage in turn through a 50 cc. bed of Nalcite HCR (H form) and 50 cc. of Dowex 1 (Acetate form). The dry residue, obtained from the effluent solution by distillation, was converted to the benzylphenylhydrazone in the usual manner. The recrystallized product weighed 140 mgm. and had m.p. 126–128°.

Thymidine phosphorylase acts upon thymidine and deoxyuridine but not upon deoxycytidine. Consequently, to obtain the sugar from this last nucleoside it was deaminated by the method of MacNutt (19) before treatment with the enzyme. Thus 1.6 mM. of deoxyuridine yielded 83 mgm. of recrystallized sugar benzylphenylhydrazone with m.p. 126–128°.

Specific Rotations of the Benzylphenylhydrazones

The optical rotations of the benzylphenylhydrazones, prepared as described in the foregoing, were measured in pyridine solution and compared with that of the same derivative of synthetic L-2-deoxyribose (21) which derivative had m.p. 126–128°. The results are to be found in Table I.

TABLE I

A COMPARISON OF THE OPTICAL ROTATIONS OF THE BENZYLPHENYLHYDRAZONES DERIVED FROM THE DEOXYNUCLEOSIDE SUGARS AND SYNTHETIC L-2-DEOXYRIBOSE

Benzylphenylhydrazone derived from	Method of preparation of sugar	Specific rotation in pyridine		
		Temp. (°C.)	Concn. (%)	$[\alpha]_D^t$
Deoxyguanosine	Resin hydrolysis	20	3.5	−19°
Deoxyguanosine	Resin hydrolysis	18	3.0	−18°
Deoxyguanosine	Resin hydrolysis	23	2.5	−16°
Deoxyadenosine	Resin hydrolysis	24	4.2	−20°
Deoxyadenosine	Resin hydrolysis	20	3.5	−17°
Deoxycytidine	Catalytic hydrogenation and acid hydrolysis	27	4.6	−17°
Deoxycytidine	Enzymatic hydrolysis	28	4.2	−16°
Thymidine	Amalgam reduction and acid hydrolysis	24	4.7	−19°
Thymidine	Amalgam reduction and acid hydrolysis	20	2.3	−15°
Thymidine	Enzymatic hydrolysis	25	5.4	−18°
Synthetic L-2-deoxyribose		18	4.0	+21°
Synthetic L-2-deoxyribose		21	3.0	+20°

Comparison of Sugars by Paper Chromatography

Two solvent systems were used: (*a*) butanol, pyridine, water, 8:2:1, and (*b*) butanol, ethanol, water, 4:1:1. Chromatograms on Whatman No. 3 paper were developed upwards until the solvent front had reached a height of 15 cm. This needed 1.5 hr. for system (*a*) and 2 hr. for system (*b*). After development the papers were dried in a warm oven, sprayed with a 2% solution of *m*-phenylenediamine hydrochloride in 75% ethanol (6), and heated at 100° for 5 min. The positions of the sugars were indicated by spots fluorescing under ultraviolet light.

The following sugars were compared by simultaneous chromatography:

1. Two samples obtained from the hot acid (resin) hydrolysis of deoxyadenosine and deoxyguanosine.
2. Two samples from the enzymatic arsenolysis of thymidine and deoxyuridine.
3. One sample from the hot acid hydrolysis of reduced thymidine.
4. Glucose.
5. Arabinose.
6. L-2-Deoxyribose.

The R_f values so obtained were:

With system (*a*): All nucleoside sugars and L-2-deoxyribose, 0.43; glucose, 0.14; arabinose, 0.22.

With system (*b*): All nucleoside sugars and L-2-deoxyribose, 0.43; glucose, 0.23; arabinose, 0.34.

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