ACTA CHEMICA SCANDINAVICA 8 (1954) 192—198

The Isolation of 2-Deoxy-D-ribose from Deoxypentosenucleic Acids

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The sugar component of deoxypentosenucleic acids (DNA) isolated from various sources is generally assumed to be 2-deoxy-D-ribose, and in recent years Chargaff and his collaborators [1] have provided evidence by their chromatography studies, to support this assumption. The most satisfactory way of establishing this point would be to develop a method for the isolation in good yield, of the sugar in crystalline form, from hydrolysates of various samples of DNA. In the past the isolation of 2-deoxy-D-ribose from DNA has proved to be very difficult. It was usual to degrade the nucleic acid by chemical methods to the constituent nucleosides, separate these, and then hydrolyse the sugar-base glycosidic linkage, and isolate the sugar portion. Separation of the nucleosides was a tedious procedure, and since acidic treatment for hydrolysis results in some conversion of the deoxypentose to levulinic acid [2], yields were poor. Following attempts by Thannhauser and Ottenstein [3], who employed picric acid for the hydrolysis of thymus nucleic acid and obtained diphosphoric esters of pyrimidine deoxyribosides, Levene and London [4] resorted to enzymic methods. A solution of thymus nucleic acid was passed through a segment of the gastro-intestinal tract of a dog and collected from an intestinal fistula. After incubation for several days the deoxyribonucleosides of guanine, hypoxanthine (arising from deamination of adenine), cytosine and thymine were isolated. Very mild hydrolysis of the guanine nucleoside gave the deoxy-sugar in crystalline form [5,6]. Similar mild acidic hydrolysis of hypoxanthine deoxyriboside gave a solution with the same optical rotation as an equivalent amount of 2-deoxy-D-ribose [4]. Attempts to isolate the sugar from the pyrimidine deoxyribonucleosides were unsuccessful, as the sugar was immediately converted into levulinic acid under the more drastic hydrolytic conditions necessary to cleave the linkage between the pyrimidine base and the deoxysugar [4].

Although methods of enzymic hydrolysis afforded the possibility of preparing with comparative ease, quantities of mixed nucleotides and nucleosides from DNA, the problem remained of obtaining the individual compounds in pure form by separation of the mixtures. The development of chromatographic

methods and of ion-exchange resins has solved this problem and it is now possible to isolate from enzymic digests of DNA's, deoxypentose-nucleotides and -nucleosides in greatly improved yield and in a high degree of purity. For example, by chromatographic separation on a column of alumina, Schindler [7] was able to obtain the deoxyribosides of guanine, hypoxanthine, thymine and cytosine, from an enzymic hydrolysate of sodium thymonucleate. Chromatography on Dowex 50 and starch was also effective [8]. Volkin, Khym and Cohn[9] have separated deoxyribonucleotides in good yield, on ion exchange resins and other workers [10], have described similar experiences with this method. Ion-exchange fractionation of enzymic digests of DNA isolated from thymus gland and wheat germ has resulted in the isolation of the deoxyribonucleoside [11] and -nucleotide [12] of 5-methylcytosine. Recently a method suitable for the large scale preparation of deoxyribonucleosides from DNA has been described [13]. In the present communication we describe a further extension of the use of ion-exchange resins, since we have been able to use acidic resins to cleave the glycosidic linkage in purine deoxyribosides.* The liberated deoxy-sugar has been freed from purine bases by the ion-exchange resin technique, and has been obtained in crystalline form and characterised. It is our belief that the procedure now reported is the most satisfactory method so far described for obtaining 2-deoxy-D-ribose from DNA.

EXPERIMENTAL

Materials

DNA from soft cod roe was prepared essentially according to the method already described by us [14] for the preparation of DNA from soft herring roe. The material was dialysed at +3° C against distilled water and was finally obtained by evaporation of its solution in the frozen state. [Found: P 8.76; N 14.63 %, *i. e.* N/P = 1.67 (After drying at 110° C/15 mm Hg over phosphoric oxide)].

The herring roe DNA used in this investigation was a purified preparation of a commercial product supplied by Isaac Spencer and Co. Ltd., Aberdeen. Purification was effected as follows: the commercial material (10 g) was dissolved in 1 *M* sodium chloride solution (800 ml) and "Teepol" (100 ml) and 50 % (by volume) acetic acid (800 ml) was added. The solution was treated twice with chloroform-amyl alcohol mixture (75 : 25 respectively). The nucleic acid was precipitated by the addition of ethanol (2 vols.) and the precipitate was collected at the centrifuge. The solid obtained was redissolved in water (200 ml) and the solution was dialysed against water for 48 hours and then dried in the frozen state. The solid obtained (4 g) was essentially free from protein material.

Deoxyribonuclease was prepared from ox-pancreas according to the procedure of McCarty [15] as modified by Overend and Webb [16].

Intestinal phosphatase was isolated by the method described by Klein [17]. Both enzymes were stored in the freeze-dried state.

Methods

Phosphorus analyses were carried out by the colorimetric method of Allen [18] as modified by Jones, Lee and Peacocke [19] and nitrogen values were estimated by means of a micro-Kjeldahl method using the apparatus described by Markham [20].

* When this work was completed it came to our notice that Khym *et al.* (*J. Am. Chem. Soc.* **75** (1953) 1262) have cleaved the glycosidic linkage in purine ribonucleotides with an acidic type of resin.

Enzymic Hydrolysis of Cod-roe DNA

Cod-roe DNA (3 g, undried), hydrated magnesium sulphate (2.35 g) and deoxyribonuclease (0.03 g) were dissolved in water (32.5 ml) and thereafter 10 % ammonium hydroxide was added cautiously to give a pH value of 7. Toluene (20 ml) was layered on the solution which was incubated at 37° C for 12 hours. The pH value was maintained constant by addition at intervals of ammonium hydroxide. The phosphorus content was determined on an aliquot (1 ml) of the digest suitably diluted (2 ml of the digest was diluted to 25 ml) and hence the total amount of phosphorus in the digest was calculated (0.165 g) and from this the dry weight (1.88 g) of nucleic acid used was determined. (Control analyses showed that the amount of phosphorus in the digest arising from the enzyme was negligible.)

Ammonium sulphate (2.0 g) and intestinal phosphatase (0.12 g) were added to the digest and the pH value of the mixture was adjusted to 9 by addition of 10 % ammonium hydroxide. Digestion was continued at 37° C for a further 48 hours. At intervals the pH value was checked and maintained at a constant value (9). The solid precipitate (magnesium ammonium phosphate) was removed by filtration and analyses showed that dephosphorylation of the nucleic acid was essentially quantitative (98 %). A solution in hot water (100 ml) of the theoretical amount of barium hydroxide (4.8 g) required to precipitate quantitatively the sulphate ions in solution, was added to the digest and the barium sulphate which separated was removed by filtration and well washed with water (4 × 50 ml). The washings and filtrate were combined and concentrated to small volume (A) (10 ml) by heating at 40° C under diminished pressure.

Passage of the DNA-Hydrolysate Through a Bed of Ion-exchange Resins

The resins employed were Amberlite IR—120 (H) and IR—4B (OH) (40—60 mesh). The former was prepared for use by treatment with 10 % hydrochloric acid (520 ml per 100 g of resin) followed by washing with water (2—3 litres) until free from chloride ions, whereas the latter was treated with 2 % sodium carbonate solution (3.6 litres per 100 g of resin) and subsequent washing with water until free from carbonate ions.

The regenerated resins (57 ml of each in the wet state) were shaken together and then packed into a glass column (4 cm diameter). The concentrated mixture of nucleosides (A) (10 ml) was poured onto the column and washed through with water (8 ml per minute), until the effluent contained negligible amounts of material exhibiting a positive Dische [21] test or absorption at 2 600 Å. [The Dische test was carried out as follows: Effluent (0.5 ml), water (1.5 ml) and Dische diphenylamine reagent (5 ml) were heated together at 100° for 15 minutes, thereafter immediately cooled to room temperature, and the solution examined in the Colman spectrophotometer at 5 900 Å]. When 600 ml of effluent had been collected, negative results were obtained in the above tests. As will be seen from the subsequent account the effluent contained 2-deoxy-D-ribose and thymidine and Table 1 shows the approximate amounts of the deoxy-sugar in various fractions collected from the resincolumn. It is obvious that the major portion of the sugar is concentrated in fraction "b" (*i. e.* 100—300 ml of effluent).

Table 1

Fraction	*Vol. of effluent (ml)*	*Amount of 2-deoxy-D-ribose (mg)*
a	0—100	0
b	100—300	256
c	300—400	17
d	400—500	11
e	500—600	0.8
	Total weight of 2- deoxy-D-ribose	284.8

(The amounts of deoxy-sugar in the fractions of effluent were determined by the Dische method using synthetic 2-deoxy-D-ribose as standard. It was necessary to dilute the aliquot of fraction "b" before carrying out the analysis.)

The presence of thymidine affects the determination of 2- deoxy-D-ribose only very slightly. For example it was demonstrated that the presence of 100 μg of thymidine in the Dische mixture during a determination of 2-deoxy-D-ribose gave a "Spekker" reading corresponding to only 3 μg of the sugar, compared with control tests. Since the maximum amount of thymidine present in a Dische analysis was of the order of 100 μg, and the corresponding amount of 2- deoxy-D-ribose was 40 μg it was clear that the presence of thymidine could be ignored in comparing the results in Table 1.

The ultra-violet absorption spectrum of the combined fractions of the effluent (a neutral solution) after suitable dilution (2 ml diluted to 100 ml) was examined in the Beckmann spectrophotometer. Results obtained were as follows:

Wavelength ($m\mu$)	240	250	260	267	270	280	290
Optical density of the effluate (expressed relative to the maximum value measured)	0.28	0.57	0.92	1.00	0.98	0.66	0.20
Optical density of a solution of the thymidine in water (expressed relative to the maximum value measured) as reported by Hotchkiss [22]	0.30	0.60	0.91	1.00	0.97	0.64	0.20

It is clear that the ultra-violet absorption spectrum of the effluate closely ressembled that reported by Hotchkiss [22] for thymidine. When an aliquot of the solution was subjected to chromatography on a paper strip [the solvent system employed was prepared by shaking together *n*-butanol, ethanol, water and concentrated aqueous ammonium hydroxide (40 : 10 : 49 : 1, *v/v/v/v*) and separating for use the upper phase] only one fast-moving spot was detected by photography in ultra-violet light [14,23].

Isolation of 2-Deoxy-D-ribose and Thymidine from the Effluent

The combined fractions (a—e) were concentrated at 40° C under diminished pressure to small volume and then finally dried in the frozen state. Absolute acetone (10 ml) was added to the residue and the mixture was stored at 0° C for 24 hours. A crystalline precipitate (0.27 g) was formed and was collected by filtration. (The filtrate [B] was set aside for further use.) The solid showed m. p. 170—183° and its extinction coefficient at 2 600 Å in aqueous solution (1 mg/ml) was 33.5. Hotchkiss [22] reports a value of 36 for thymidine, when measured under these conditions. Recrystallisation from acetone afforded pure thymidine (0.2 g), m. p. 183—185°, $[\alpha]_D^{20}$ + 62.5° (*c*, 1.54 in water) (Found: C 49.8; H 6.2. Calc. for $C_{10}H_{14}O_5N_2$: C 49.6; H 5.8. Brady [24] reports m. p. 185—186° for thymidine and Todd *et al.* [13] state m. p. 182—183°. The acetone-filtrate (B) obtained after removal of the precipitated thymidine, and which contained 2- deoxy-D-ribose and some residual thymidine was evaporated to dryness under diminished pressure and the residue was redissolved in water (10 ml). The amount of residual thymidine in solution was estimated by ultra-violet absorption measurements to amount approximately to a 10 % contamination of the 2- deoxy-D-ribose. The solution was reconcentrated and the syrupy residue (0.27 g) obtained was induced to crystallise. It showed m. p. 70—80° C [this m. p. was not depressed when the substance was mixed with synthetic 2- deoxy-D-ribose having m. p. 78—80° C] and $[\alpha]_D^{20}$ —56° (equilibrium; *c*, 0.44 in H_2O) (corrected for 10 % impurity of thymidine with $[\alpha]_D^{20}$ + 62.5°). It was demonstrated by chromatographic methods that the substance contained no other sugars than 2- deoxy-D-ribose, and that the R_F value of the sugar isolated was identical with that of authentic 2- deoxy-D-ribose. The sugar (0.025 g) isolated, was added to a solution of freshly distilled aniline (0.08 ml) in absolute ethanol (5 ml) and the mixture was heated for 2 hours at 100° C. Thereafter the solution was concentrated until crystals appeared which were collected and recrystallised from

ethanol. Shining crystals of aniline deoxy-D-riboside (0.014 g) were obtained, m.p. 163–164° alone or in admixture with an authentic specimen, $[\alpha]_D^{20}$ +19° (*c*, 0.525 in EtOH). Deriaz *et al.*[25] report m.p. 168° and $[\alpha]_D^{20}$ + 19.5° (in EtOH) and Kent, Stacey and Wiggins[26] quote m.p. 165–166° for this compound.

An Alternative Method for the Isolation of Thymidine and 2-Deoxy-D-ribose

A solution (10 ml) of a mixture of nucleosides from an enzymic digest of DNA (prepared as described above) was diluted with water (40 ml) and shaken for 30 minutes with Amberlite resin IR–120 (H) (20 ml of wet regenerated resin). The resin was filtered off and washed with water (100 ml). The filtrate and washings were combined and shaken for a further 30 minutes with Amberlite resin IR–4B (OH), (20 ml of wet regenerated resin). This resin was removed and washed as before. By methods already described the combined filtrate and washings were shown to contain 2- deoxy-D-ribose and thymidine, and these were separated as previously outlined. The yield of thymidine was 0.26 g (*cf.* Todd *et al.*[13] who obtained 4.2 g of thymidine from 36 g of nucleic acid). The impure 2-deoxy-D-ribose was recrystallised from ethyl acetate according to the procedure of Deriaz *et al.*[13] and small nodules of crystals (0.2 g) were obtained, m.p. 78–80° C alone or on admixture with an authentic specimen.

A control experiment was carried out to determine whether any 2- deoxy-D-ribose had been retained by the resins. A column (5 cm × 4 cm diam.) of mixed resins was prepared as already described and a solution of 2- deoxy-D-ribose (0.0027 g) in water (5 ml) was poured on the column and washed through with water. The amount of deoxy-sugar in the effluent was determined by the aforementioned Dische method. When 100 ml of effluent had been collected no more 2-deoxyribose appeared to be eluted from the column, and recovery of the sugar was quantitative.

Isolation of 2-Deoxy-D-ribose from the DNA Isolated from Soft Herring Roes

By application of the above methods to DNA from soft herring roes, 2- deoxy-D-ribose and thymidine were obtained in crystalline form in comparable yields with those reported when using DNA from cod roes. In earlier experiments an alternative procedure had been used, involving acidic hydrolysis with dilute hydrochloric acid of the isolated purine nucleosides from enzymic digests of DNA from herring sperms, followed by removal of the purine bases from solution by absorption on resin columns.

Purified herring roe DNA (4.5 g) in solution in water (450 ml) was digested in the usual manner with deoxyribonuclease and alkaline phosphatase (see digestion of cod roe DNA). The precipitated solid ($MgNH_4PO_4$) was filtered off and found to correspond to 85 % dephosphorylation of the nucleic acid. Barium hydroxide (13 g) in hot water (100 ml) was added and the resultant precipitate removed by centrifuging. The solid residue was washed with hot water (3 × 100 ml) and the combined washings and supernatent liquid were evaporated under diminished pressure to smaller volume (100 ml). Solid carbon dioxide was added to precipitate excess barium ions and the solid formed was removed. After addition of acetic acid to bring the pH value to 6 the solution was evaporated under diminished pressure to small volume (25 ml) and then dried in the frozen state (residue 2.7 g). Chromatographic analysis of the mixture revealed that the following bases were present: Guanine, hypoxanthine (trace), adenine, cytosine, thymine, and uracil (formed by deamination of cytosine). The detection of uracil deoxyriboside in enzymic hydrolysates of DNA from various sources has been reported by other workers also [11,13,27]. The nucleoside mixture (2.7 g) dissolved in ammonium acetate solution [8] (0.1 *M*, 20 ml) was treated with acetic acid to give a solution of pH value 4.9. The solution was filtered onto a column (20 cm × 4 cm diameter) of zeocarb 225 resin, which had been prepared by washing the resin with 4 *N* hydrochloric acid (500 ml) and then distilled water (2.5 litres): thereafter it was treated with 5 % ammonium hydroxide (500 ml) and again with water (2.5 litres) and finally the column was treated with ammonium acetate buffer (0.1 *M*,

pH 3.9) until the eluate showed a pH value of 3.9. The column was developed with ammonium acetate buffer and the eluate (350 ml) contained thymidine and uracil deoxyriboside. The column was then eluted with 5 % ammonium hydroxide (500 ml) which gave a nucleoside mixture of the deoxyribosides of guanine, adenine and cytosine. This eluate was evaporated to dryness under reduced pressure and any ammonium acetate present was removed by heating at 40°/0.01 mm Hg. Finally, a white powdered residue (0.915 g) was obtained. This material was dissolved in water (15 ml) and 0.1 *N* hydrochloric acid (15 ml) was added. The solution was heated at 100° for 10 minutes, then cooled and filtered onto a mixed bed of Amberlite resins IR—120 (H) (100 g) and IR—4B (OH) (100 g). The resin column was eluted with water (500 ml) until the eluate gave a negative diphenylamine test. The eluate was concentrated to dryness by evaporation under reduced pressure followed by drying in the frozen state. The colourless residue (0.2 g) was induced to crystallise and was identified by chromatography as 2-deoxy-D-ribose. On treatment with freshly distilled aniline (31 mg) in absolute ethanol, by heating under reflux for 3.5 hours, the solid residue (40 mg) afforded shining crystals (15 mg) of aniline 2-deoxy-D-riboside, m.p. 163—164° (after recrystallisation twice from ethanol), alone or in admixture with an authentic specimen. (Found: C 63.3; H 7.3 N 6.8. Calc. for $C_{11}H_{15}O_3N$: C 63.1; H 7.2; N 6.7).

RESULTS AND DISCUSSION

In recent years one of us (W.G.O.) with other colleagues have described numerous attempts to synthesise 2-deoxy-D-ribose in good yield, by chemical methods from easily accessible initial materials [28,29]. Although worthwhile improvements have been achieved, highly pure 2-deoxy-D-ribose is still difficult to prepare in large quantities. Our original intention for the work described in this paper, was to try and improve the yield of the deoxy-sugar obtainable from the acidic hydrolysis of purine deoxy-ribonucleosides. We proposed to use ion-exchange resins to separate successively the enzymic digest of DNA into purine and pyrimidine deoxyribonucleosides, and then to free the sugar from the purine bases, in the acidic hydrolysate.

Initial experiments using this two-stage procedure with nucleoside mixtures obtained by treating DNA from soft herring roes with deoxyribonuclease [15,16] and alkaline phosphatase [17] were not encouraging and from hydrolysates of purine nucleosides with dilute hydrochloric acid only relatively poor yields of the crystalline 2-deoxy-D-ribose were obtained. We soon discovered that passage of a mixture of deoxyribonucleosides through a column consisting of a strongly acidic resin and a weakly basic resin, resulted in hydrolysis of the purine nucleosides by the strongly acidic resin, and simultaneously the free purine-bases so formed were retained on the resin column, as also were cytosine deoxyriboside and inorganic impurities. The eluate contained 2-deoxy-D-ribose and thymidine, since the latter is not sufficiently acidic to be retained by the resin. Acetone fractionation of this mixture afforded pure crystalline samples of both substances.

Due to the similarity in density of the resins used in the column of mixed resins it was not possible to separate them by the usual flotation method and so the column could not be regenerated. It was shown, however, that by successively shaking a solution of nucleosides with the acidic resin and then the weakly basic resin, a solution containing only 2-deoxy-D-ribose and thymidine resulted. The amounts of these substances isolated in crystalline form were approximately the same as obtained when a column of mixed resins

was used. The method should be applicable to the preparation of 2-deoxy-D-ribose in relatively large amounts. Although the overall yield of the sugar (0.27 g) from DNA (1.88 g) might not appear too good, it should be borne in mind that at the same time thymidine is obtained in excellent yield, and also that it is not necessary to use highly polymerised samples of DNA, since samples of commercial origin are quite adequate.

The authors are grateful to Professor M, Stacey, F. R. S., for his close interest in the work, the expenses of which were met by a grant from the *British Empire Cancer Campaign, Birmingham Branch* and *Norges Almenvitenskapelige Forskningsråd.*

SUMMARY

A method is described for the isolation in crystalline form from enzymic digests of DNA, of both 2-deoxy-D-ribose and thymidine. The method has been applied to DNA prepared from soft herring roes and cod roes.

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Received November 9, 1953.