ACTA CHEMICA SCANDINAVICA 8 (1954) 603—610

The Sugar Component of Deoxypentosenucleic Acid from *Mycobacterium phlei*

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In a previous paper [1] one of us with another colleague described a convenient method for the isolation of the sugar component of the purine nucleosides of deoxypentosenucleic acids (DNA). In the case of DNA from soft herring roe and cod roe the sugar component was shown to be 2-deoxy-D-ribose. Very little is known about the sugar of DNA from microorganisms. Only in the case of DNA from *Mycobacterium tuberculosis* has chromatographic data provided by Chargaff *et al.*[2], suggested the presence of 2-deoxy-D-ribose in the purine nucleosides. Consequently it was considered of great interest to identify conclusively the sugar in a microbial DNA. The present work is concerned with the qualitative and quantitative determination of the sugar component of purine nucleosides of DNA from *Mycobacterium phlei*. This organism was chosen because it provided a fairly convenient source for the preparation of sufficient quantities of DNA.

The possibility of the existence of linkages of the type: sugar(1)-phosphate in the DNA under investigation, as suggested by Overend, Stacey and Webb [3] for herring roe DNA has also been examined.

EXPERIMENTAL

Analytical methods

Nitrogen was estimated by means of a micro-Kjeldahl method.

For the phosphorus estimations the colorimetric method of Allen modified by Jones, Lee and Peacocke [5] was used.

The pentosenucleic acid (RNA) content of the nucleic acid was determined by the method of Euler and Hahn [6] using yeast RNA as standard.

Enzymes

The deoxyribonuclease (DRNase) was prepared from oxpancreas according to the procedure of McCarthy as modified by Overend and Webb [7] (For the freeze dried preparation, Found: P 2.0 %). The alkaline phosphatase was isolated from calf small intestine by the method of Klein [8]. The enzymes were stored in the freeze dried state.

The isolation and purification of DNA from *Mycobacterium phlei*

Nucleic acid was isolated from *M. phlei* by the method of Jones [9]. The bacterial cells were disintegrated in the presence of sodium arsenate (0.01 *M*) by shaking with ballotini glass beads. The suspension was diluted and allowed to stand at 0° C for 24 hours. The nucleoproteins were precipitated from solution by the addition of cetyltrimethylammonium bromide and purified by four reprecipitations from 1 *M* sodium chloride solution by dilution with water (3 vols.). The protein, cell debris and cetyltrimethylammonium bromide were removed by treatment with chloroform and octanol, the nucleic acid precipitated with ethanol, dissolved in distilled water, dialyzed and dried *in vacuo* in the frozen state. The white fibrous product contained after extensive drying *in vacuo* at 110° C: N 12.2, P 7.6 and RNA 12 %.

To remove the RNA it was decided in this case to use the charcoal method (Chargaff and Zamenhof [10], Dutta, Jones and Stacey [11]) as this is convenient when the RNA content of the nucleic acid is small.

Impure DNA (0.55 g) afforded 0.26 g of a highly fibrous product. It contained after extensive drying *in vacuo* at 110° C: N 13.2, P 7.6 and RNA <1 %.

The purine and pyrimidine composition of DNA from *M. phlei* has been reported in a previous paper [13].

Enzymic degradation of DNA

Purified DNA (moist product, 0.26 g) was dissolved in water (15 ml) and 0.1 *N* NaOH (3.5 ml) added. The solution immediately became less viscous and was kept at room temperature for 30 minutes. It was subsequently neutralized by the addition of 2 *N* sulphuric acid, and magnesium sulphate (0.196 g with water of crystallisation) in water (4.5 ml) and DRNase (0.0025 g) in water (0.5 ml) added. The mixture was kept in a stoppered flask at 37° C for 24 hours. At intervals the pH of the solution was adjusted to 6 by addition of minute amounts of conc. ammonia. Aliquots (0.06 ml) were withdrawn with a micropipette and the amount of phosphorus present determined. From the volume of the digest the total amount of phosphorus was calculated to be 0.0184 g. (The amount of phosphorus present in the DRNase could be ignored.)

To the DRNase digest were added ammonium sulphate (0.196 g), phosphatase (0.01 g), and the pH adjusted to 9 by addition of conc. ammonia. To prevent growth toluene (2 ml) was added. The mixture was kept for 10 hours at 37° C and the pH again adjusted to 9. The digest was kept for further 38 hours at 37° C, the precipitated magnesium ammonium phosphate filtered off quantitatively on a sintered-glass filter after the mixture had been kept at 0° C for 12 hours, washed with icecold water (10 ml), redissolved on the filter in 2 *N* sulphuric acid and the solution made up to 50 ml with water. The phosphorus content of the solution was determined. By applying a correction for the solubility of magnesium ammonium phosphate at 0° C in the digestion mixture, the percentage of dephosphorylation of the DNA was calculated to be 98.3 %.

The filtrate containing the nucleosides was dried in vacuum in the frozen state. The mixture of salts and nucleosides was then dissolved in water (40 ml) (the pH of the solution was 4.8), insoluble phosphatase removed at the centrifuge, and the precipitated enzyme washed with acetate buffer, pH 4.8 (2 × 2.5 ml). The protein precipitate contained negligible amounts of DNA nucleosides as shown by the negative Dische test. The solution containing the nucleosides and the buffer used for washing the precipitate were transferred quantitatively to a volumetric flask and the total volume made up 50 ml.

Determination of the amounts of thymidine and 2-deoxy-D-ribose of the purine nucleosides in an aliquot of the nucleoside mixture

The mixed bed ion exchange resin technique described in a previous paper [1] for the isolation of a mixture of thymidine and 2-deoxy-D-ribose was applied. A suitable mixed bed ion exchange resin was prepared by mixing with stirring in a glass column wet Amber-

lite IR-120 (H) with an equal volume of Amberlite IR-4B (OH) (the particle size of the resins was 40—60 mesh). An aliquot (5 ml, equal to 1/10 of original digest) of the solution (total volume 50 ml) containing the nucleosides was transferred to the top of the column (diameter 2 cm, length 4 cm) and washed through slowly with water until the eluate gave a negative Dische reaction and contained no ultra-violet absorbing material. (100 ml of water was necessary and the eluate passed through the column at a rate of 0.5 ml/min.). The eluate was concentrated by evaporation in the frozen state in vacuum and then quantitatively transferred to a volumetric flask and the volume made up to 50 ml. The amount of thymidine and 2-deoxy-D-ribose present in the solution was determined in the following way.

(I) Quantitative determination of thymidine. An aliquot of the above solution was diluted suitably with water (2 ½ times) and the ultra-violet absorption was determined in the Beckman spectrophotometer in 1 cm cells using water as blank.

(II) Quantitative determination of 2-deoxy-D-ribose. The colour produced with an aliquot of the solution to be tested (2 ml) and the Dische reagent (5 ml) was measured at 590 mμ in a Colman spectrophotometer. The Dische reagent employed was that described by Deriaz *et al.*[14].

The solution being examined contained as the only components thymidine and 2-deoxy-D-ribose (see later chromatographic evidence) and hence a standard solution consisting of known amounts of thymidine and 2-deoxy-D-ribose has been used. The standard solution was prepared in the following way.

Thymidine (prepared from cod roe DNA by the method of Laland and Overend [1]) was recrystallized twice from acetone and had m.p. 183—184° C. 2-Deoxy-D-ribose was a synthetic product recrystallized twice from ethyl acetate and had m.p. 78—80° C. Approximately 0.005 g of each was accurately weighed and dissolved in water (25 ml). Three solutions were prepared: (1) 2-deoxy-D-ribose solution (5 ml) + water (5 ml), (2) thymidine solution (5 ml) + water (5 ml), (3) 2-deoxy-D-ribose solution (5 ml) + thymidine solution (5 ml).

The optical densities of the colours developed with the Dische reagent and equal volumes of solution (1) and (3) were 0.33 and 0.37 respectively, thus showing that the presence of thymidine had only a small effect on the Dische colour developed by 2-deoxy-D-ribose.

By examining the ultra violet absorption spectrum of solution (2) and (3) it was shown that the presence of 2-deoxy-D-ribose approximately equal to that of thymidine had no effects upon its spectrum.

Solution (3) was employed as the standard solution for determining the amounts of 2-deoxy-D-ribose and thymidine in the eluate from the mixed bed resin. The quantities of these two components in 1/10 of the DNA digest (nucleoside mixture) was thymidine, 1.87 mg and 2-deoxy-D-ribose, 3.25 mg.

Paper chromatography of the eluate containing 2-deoxy-D-ribose and thymidine

An aliquot of the concentrated eluate containing 37 μg of thymidine and 64 μg of 2-deoxy-D-ribose was put on a paper strip and the chromatogram developed with the top layer of a *n*-butanol-ethanol-water-ammonia (conc. aqueous) mixture (40 : 10 : 49 : 1, v/v/v/v). The strip was photographed in the ultra-violet light and only one spot with R_F value identical to a reference spot of thymidine was visible. On subsequent spraying with a phenylene diamine reagent [15] one spot only was visible when examined under the ultra-violet lamp. It had R_F value identical to a reference spot of 2-deoxy-D-ribose.

Method for detection of "free" 2-deoxy-D-ribose in the mixture of salts and nucleosides (from the DNA digest)

The remainder of the solution containing the nucleoside mixture (45 ml, representing 9/10 of the total DNA digest) was dried in the frozen state in vacuum. The residue was dissolved in water, (10 ml), two aliquots (0.5 ml, representing 9/200 of the original mixture of nucleosides) were withdrawn and transferred to small flasks (25 ml). To one of the

flasks was added an aqueous solution (10 μl) containing 2-deoxy-D-ribose (50 μg). The solutions in the two flasks were evaporated to dryness in the frozen state in vacuum. To each of the residues were added absolute acetone (5 ml) (2-deoxy-D-ribose is easily soluble in acetone) and left at room temperature for 48 hours with occasional shaking. The insoluble material was filtered off and washed with acetone (3 ml). The filtrates were concentrated to dryness in vacuum and the two residues were each dissolved in water (300 μl; pH of these solutions was 5.5). On a strip of paper was put the following four spots: 10 μg and 20 μg of synthetic 2-deoxy-D-ribose and 120 μl of each of the above solutions. The solution which contains 2-deoxy-D-ribose added initially, is designated 1) and the other designated 2). The paper strip was developed with the *n*-butanol-ethanol-water-ammonia mixture described previously. The dried strip was photographed in the ultra-violet light. The chromatographic runs of solutions 1) and 2) exhibited on the photographic print three well defined spots with R_F values respectively 0.19, 0.29 and 0.50 (the last spot being thymidine). The paper strip was then sprayed with the phenylene diamine (as hydrochloride) reagent and examined under the ultra-violet lamp. Solution 1) exhibited three spots with the following R_F values: 0.19, 0.29 and 0.40. Solution 2) gave only two spots with R_F values: 0.19 and 0.29. The reference runs of respectively 10 μg and 20 μg of 2-deoxy-D-ribose gave only one spot each with R_F value 0.40. Consequently 2-deoxy-D-ribose was only present in solution 1) to which synthetic sugar had been added originally. An inspection of the chromatogram also revealed that the aliquot (120 μl) of solution 2) on the chromatogram contained less that 10 μg of 2-deoxy-D-ribose (since the 10 μg reference spot could be detected). The appearance of the two slowest moving spots in solution 1) and 2) showing up with the sugar spraying reagent (R_F values 0.19 and 0.29) and having R_F values identical with the two slowest moving nucleosides, is caused by the hydrolysis of the nucleosides with the acidic spraying reagent.

Isolation and characterization of thymidine and 2-deoxy-D-ribose

The remainder of the solution containing the mixture of the nucleosides (9 ml, representing 8/10 of the original nucleoside mixture) was passed down a mixed bed resin as previously described using proportional quantities of resins and eluate, combined with what was left of the 2-deoxy-D-ribose and thymidine obtained from 1/10 of the original digest. The combined solutions were evaporated to dryness in the frozen state in vacuum and the syrupy residue treated with dry acetone (1 ml) for a week at 0° C. The precipitated crystalline material (12 mg) was filtered off, washed with dry, cold acetone (2 ml) and recrystallized from acetone. The highly crystalline material had m.p. 183—185° C, alone and in admixture with thymidine isolated from cod roe DNA. (Found. C 49.6, H 5.7; Calc. for $C_{10}H_{14}O_5N_2$ (242): C 49.6, H 5.8.)

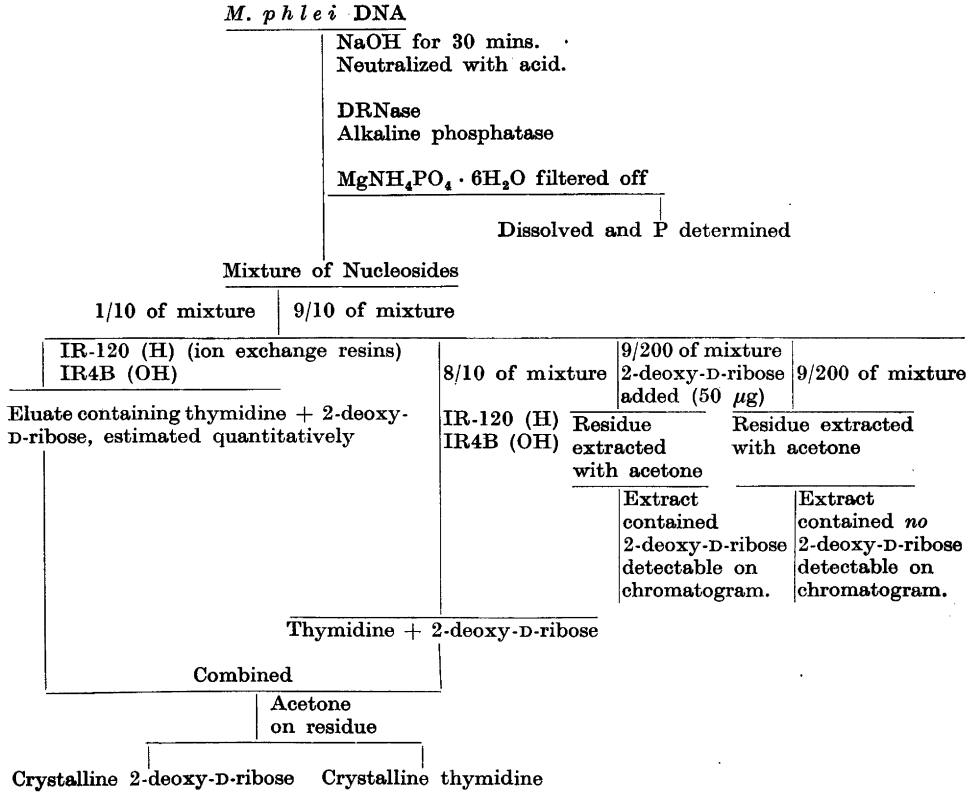
The combined acetone filtrate was concentrated to dryness *in vacuo* and the residue dissolved in water (1.5 ml). Estimation of the amounts of 2-deoxy-D-ribose and thymidine present, was carried out as already described and amounted to respectively 21 mg and 2.1 mg. The optical rotation of the solution (at equilibrium) was determined in a micro-rotation tube (0.5 dm). Taking into account the rotation of the small amount of thymidine ($[\alpha]_D^{20} = +62°$) present, the observed rotation for the 2-deoxy-D-ribose was: $[\alpha]_D^{20} = -64°$. Deriaz *et al.*[16] quote for synthetic 2-deoxy-D-ribose, $[\alpha]_D^{20} = -58°$.

The solution of the 2-deoxy-D-ribose used for the determination of the optical rotation was evaporated to dryness in the frozen state in vacuum. The resulting syrup was seeded with 2-deoxy-D-ribose and when crystallization had terminated ethyl acetate (1—2 ml) was added and the solid filtered off after 24 hours. The faintly yellow product (9 mg) had m.p. 70—80° C (micro melting point) alone or in admixture with authentic 2-deoxy-D-ribose (synthetic 2-deoxy-D-ribose had m. p. 78—80° C). Because of the small amount of sugar isolated no attempt was made to recrystallize it in order to obtain a higher m.p. Instead the anilide was prepared by the procedure given by Deriaz *et al.*[16]. The shiny crystals of the anilide so obtained had m.p. 163—164° C (micro melting point) alone or in admixture with authentic 2-deoxy-D-ribose anilide.

RESULTS AND DISCUSSION

The purified DNA from *M. phlei* employed in the present experiments gave negative protein tests (Millons and Sakaguchi) and contained less than 1 % RNA. Its fibrous nature indicated that extensive degradation has not occurred during the isolation procedure. The fairly low nitrogen and phosphorus values suggest the presence of impurity in the nucleic acid preparation. This "impurity" is not removed by prolonged dialysis or by exhaustive drying *in vacuo* at 110° C and therefore does not appear to be inorganic material or water. The presence of acidic polysaccharide is a possibility (see Dutta, Jones and Stacey [11] and Zamenhof *et al.*[17]), but so far no definite evidence of the presence of polysaccharide in the DNA from *M. phlei* has been obtained. Attempts to raise the nitrogen and phosphorus values by fractionation with cetyltrimethylammonium bromide, calcium and ethanol, copper precipitation and by digestion with sodium hydroxide at 37° C followed by precipitation with dilute acid have been unsuccesful. The presence of impurity in no way invalidates the values of the present investigation, although the presence of phosphorus in the "impurity" may explain why less than 4 moles of base per 4 atoms of phosphorus were found [13].

Table 1. Fractionation scheme of M. phlei DNA digest.



A general outline of the enzymic breakdown of the DNA and the various manipulations of the obtained mixture of nucleosides is seen in Table 1. The method and conditions employed for the degradation of DNA and the isolation of the mixture of 2-deoxy-D-ribose (the recovery of the sugar from a mixed bed resin as employed in this investigation is 100 %) and thymidine permitted a quantitative determination of these two compounds to be made in relation to the DNA phosphorus. Thus it was possible to calculate the moles of thymidine and 2-deoxy-D-ribose per 4 atom of DNA phosphorus. The figures for thymidine and 2-dexoy-D-ribose should agree respectively with that for thymine and total purines obtained by the quantitative chromatographic analysis of *M. phlei* DNA (reported in an earlier paper [13]). All these figures are presented in Table 2.

Table 2. The quantities of various constituents of M. phlei DNA. The figures denote moles per 4 atom of phosphorus.

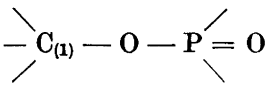
From chromatographic analysis		Present investigation	
Thymine	0.49	Thymidine	0.53
Total purines	1.57	2-Deoxy-D-ribose	1.66

It will be seen that the agreement between the figures in the horizontal rows is fairly good. The figure obtained for the total purines by a method [18] developed by one of us (S.G.L.) is 1.64 and hence the agreement with the present investigation is better.

When attempting to explain the discrepancies between the results of the two methods represented in Table 2, it should be borne in mind, that in the chromatographic analysis of the purines and pyrimidines the total nitrogen of these accounted for only 92.5 % of the total nitrogen of the DNA. The figures quoted from the chromatographic method in Table 2 could therefore possibly be too low.

It is important to note that the method described in this paper for the determination of the purine bound 2-deoxy-D-ribose and thymidine per 4 atom of phosphorus is general applicable to DNA's.

The initial treatment of DNA with alkali (preceding the enzymic degradation) was intended to break linkages of the type (Overend, Stacey and Webb [3]):



In the formulae $C_{(1)}$ is the carbon No. 1 of the 2-deoxy-D-ribose moiety. It would be expected that 2-deoxy-D-ribose molecules involved in the original DNA in linkages of the above type should appear in the final enzymic digest as "free" 2-deoxy-D-ribose. "Free" 2-deoxy-D-ribose would also be present if the enzyme preparation contained some nucleoside-cleaving enzyme. The interpretation of the presence of the "free" sugar could therefore be uncertain

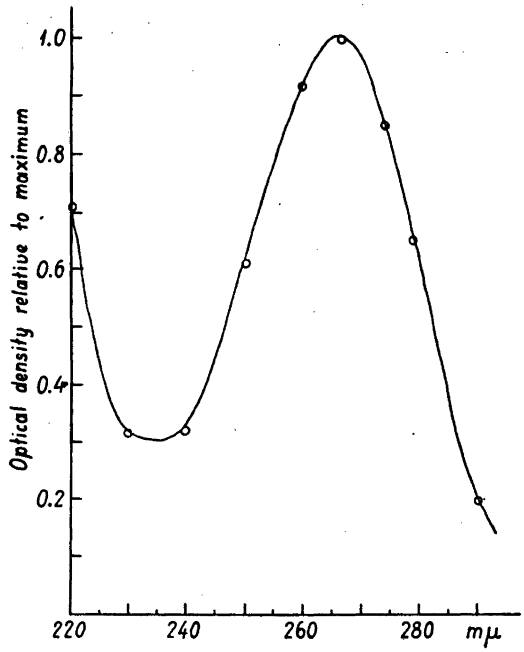


Fig. 1. Ultra-violet absorption spectra in distilled water. The drawn curve is the spectrum of thymidine isolated from DNA cod roe and the open circles represent the spectrum of thymidine from DNA isolated from Mycobacterium phlei.

unless the presence of such an enzyme could be excluded. However, in the absence of the "free" sugar the results could be interpreted with absolute certainty, and this was the case in the present investigation. A chromatographic method was adopted for the detection of any "free" sugar. This method was shown to be capable of detecting the presence of as little as 10 μg of 2-deoxy-D-ribose in an aliquot of the digestion mixture. As no 2-deoxy-D-ribose could be detected, the aliquot of the mixture of nucleosides contained less than 10 μg of 2-deoxy-D-ribose. Thus it could be calculated that the presence of "free" 2-deoxy-D-ribose in the mixture of nucleosides occurred to an extent of less than 1 % of the total sugar content. Consequently the presence of linkages of the $C_{(1)}$—phosphate type in the original DNA would occur in less than 1 % of all the sugar molecules.

The presence of thymidine and 2-deoxy-D-ribose in the nucleic acid from *M. phlei* was finally proved by the isolation and characterization of these substances. On the basis of mixed melting point of the isolated sugar and its anilide with the corresponding synthetic products, analysis, the optical rotation and its chromatographic behaviour it is concluded that the sugar component of the purine nucleosides in *M. phlei* DNA is 2-deoxy-D-ribose. The crystalline thymine nucleoside which was isolated was shown to be identical with thymidine isolated from cod roe DNA on the basis of analysis,

mixed melting point, chromatographic behaviour and ultra-violet absorption properties. A comparison between the two thymine nucleosides is seen in Fig. 1.

SUMMARY

1. A quantitative estimation of the amounts of thymidine and 2-deoxy-D-ribose (originating from the purine nucleosides) per 4 atom of phosphorus in DNA from *Mycobacterium phlei* has been carried out. These figures agreed fairly well with what could be expected from the figures of the chromatographic purine and pyrimidine analysis of the DNA under examination.

2. The presence in *Mycobacterium phlei* DNA of 2-deoxy-D-ribose-(1)-phosphate linkages occurs in less than 1 % of the total sugar molecules.

3. The sugar component of the purine nucleosides in DNA from *Mycobacterium phlei* has been isolated in the crystalline state. It has been shown to be identical with synthetic 2-deoxy-D-ribose. The nucleoside containing thymine was isolated and shown to be identical with the corresponding nucleoside from cod roe DNA.

The authors wish to thank Professor M. Stacey F.R.S. for interest in this work and *Medical Research Council* for financial assistance for part of this investigation. One of us (S.G.L.) wishes to thank *Norges Almenvitenskapelige Forskningsråd* for financial support.

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Received November 11, 1953.