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Preparation of Desoxyribonucleosides from Thymonucleic Acid

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The preparation of desoxyribonucleosides from tymonucleic acid (DNA) has been tried by different authors after enzymatic hydrolysis of the polynucleotide. The used enzymes were usually crude mixtures of phosphatases. Levene [1] and coworkers and Klein [2] have especially worked on this problem, and the latter has worked out a detailed method which allows the preparation of each nucleoside. This method is rather laborious and it seems, at least in one case [3], to have been impossible to reproduce the yields obtained by Klein. Especially the preparation of pyrimidine desoxyribosides seems to be difficult. For these in particular Schindler [4] recently has worked out a new method in which, after a preliminary separation according to Klein's principles, he separates the pyrimidine desoxyribosides on an adsorption chromatogram of alumina. But even in this case the yield of desoxycytidine, in particular, is rather low.

Our interest in these problems arose when we wanted to study the utilisation of isotopically marked desoxyribonucleosides in the rat. It was thought that the simplest method for the preparation of the marked nucleosides would be first the preparation of biologically marked DNA and then, from that, the isolation of the constituent nucleosides. For this reason it was first necessary to work out a method which allows the preparation of the desoxyribosides in a reasonably good yield from rather small amounts of DNA. Such a method is described in the present paper. The application of the method to the metabolic problem will be described in a following paper.

It was thought that, after a preliminary preparation of the nucleosides according to Klein's principles, the use of ion exchange chromatography with Dowex and starch chromatography might make it possible to obtain better yields. Chromatography on Dowex has been successfully used for the first

time by Cohn [5,6] for the separation of purine and pyrimidine bases and ribo mononucleotides. Cohn used for these separations both the anion exchanger Dowex 1 and the cation exchanger Dowex 50.

The nucleosides formed by the enzymatic hydrolysis of DNA are the desoxyribosides of hypoxanthine, guanine, thymine and cytosine. From a theoretical point of view it was first considered better to use an anion exchanger for their separation. At this time, however, it was impossible to obtain the Dowex anion exchanger and so the separation was tried with the cation exchanger (Dowex 50). The pyrimidine desoxyribosides were easily separated from each other by the use of 0.5 N HCl as eluting agent. A complete separation of all nucleosides could be achieved by running the chromatogram first with 0.1 M ammonium acetate buffer of pH 3.9 and then with 5 % ammonia. The buffer could be completely removed from the different fractions of the effluent by evaporation *in vacuo* at 40°, and the nucleosides further purified by crystallisation. In the case of desoxycytidine before crystallisation one more chromatogram was run on Dowex 50 with 0.5 N HCl as eluting agent. This method allowed the preparation of all four nucleosides in a pure crystalline state in gram amounts from about 20 g of DNA.

When the amount of DNA available was much less, a chromatogram on starch developed with butanol-water could be used after the Dowex chromatogram as a further step of purification instead of crystallisation. Chromatography on starch has been successfully used by Reichard [7] for the separation of the ribonucleosides. In the case of the desoxyribonucleosides the separation was not quite as good, since desoxyguanosine and desoxyhypoxanthosine did not separate completely. However, in the present case, this was of minor importance as the starch chromatogram was only used after a preliminary complete separation of the desoxyribosides on Dowex 50. This type of purification was used in the isotope experiments. Freedom of the starch chromatogram-prepared desoxyribonucleosides from foreign nitrogen was established by controlling the ratio between light absorption and amount of nitrogen for each component. Standard values for these ratios had been previously obtained on pure crystallized substances.

EXPERIMENTAL

DNA. The method of isolation of the DNA chosen for the enzymatic preparation of desoxyribosides proved to be of great importance. The DNA's prepared by the two different methods of Hammarsten [8,9] did not give any appreciable amount of free phosphate during the digestion. When a 10 % solution of this type of sodium nucleate in $N/10$ NaOH was boiled for one

hour still only between 10—20 % of its phosphorus was split off by a later enzymatic digestion. Much better results were, however, obtained when a sample of Feulgen's b-nucleic acid [10] was tried. This "DNA" was obtained by the action of unpure pancreas desoxyribonuclease on a higher polymer form of DNA. It thus consisted of oligonucleotides, probably of about the size of trinucleotides [11]. This mixture of oligonucleotides was readily attacked by the intestinal phophatases and as in the case of Klein more than 90 % of its phosphorus was set free during the course of digestion.

Preparation of crude desoxyguanosine and hypoxanthosine. For obtaining the crude desoxyribosides the methods of Klein [2] and Schindler [4] were used with some slight modifications.

Twenty g of b-nucleic acid according to Feulgen [10] (wet weight % P = 6.60) were suspended in 200 ml water and dissolved with the aid of conc. ammonia. The pH of the solution was adjusted to 8.8. Two hundred and fifty ml of ammonia-ammonium sulfate buffer (1 molar with respect to NH_4) of pH 8.8 and 100 ml 0.3 *M* magnesium sulfate solution were added. To this mixture was added the enzyme dissolved in 75 ml water with the aid of ammonia (final pH 8.8). The enzyme was prepared and once purified by precipitation at pH 4.7 as described by Klein [12], the only difference being that pig intestine was used instead of beef, since the former material was easy to obtain in a frozen state from the slaughter house. The amount of enzyme that was used in the present experiment corresponded to 50 g of mucosa. The mixture was allowed to stand for two days at 37°.

Purification of the nucleosides from most salts and the separation of the crude desoxyguanosine and desoxyhypoxanthosine from the pyrimidine desoxyribosides were carried out as described by Schindler [4]. One fifth of all the volumes used by him was taken.

Preparation of desoxyguanosine. The first two desoxyriboside precipitates, obtained by the method of Schindler, were combined, suspended and almost completely dissolved in 20 ml of warm water. The solution was extracted with 100 ml of a chloroform : ethanol (2 : 1) mixture as described by Schindler. Three layers were formed on standing in a separatory funnel and the lowest removed. This procedure was repeated 20 times. Once or twice during the procedure, when the separation of the layers became incomplete a few ml of water were added to the mixture. The combined chloroform extracts were evaporated to dryness *in vacuo*, giving a white dry residue weighing 1.36 g. A chromatogram on Dowex 50 (see below) revealed that this fraction contained besides smaller amounts of pyrimidine desoxyribosides almost as much desoxyhypoxanthosine as desoxyguanosine. This seems not to be in accordance with Schindler's statement that chloroform : ethanol extracts hardly any

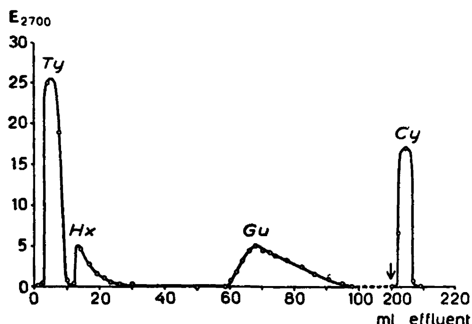

Fig. 1. Chromatogram on Dowex 50 of approximatively 3 mg of each desoxyriboside of thymine, hypoxanthine, guanine and cytosine. Length of column 140 mm, diameter 9 mm. Solvent: first, 0.1 M ammonium acetate buffer pH 3.9; after the arrow, 5 % ammonia.

desoxyhypoxanthosine. Even after three recrystallisations this fraction contained about 20 % desoxyhypoxanthosine. Therefore, the whole amount was dissolved in 100 ml warm ammonium acetate buffer and fractionated on Dowex 50 as described below. The desoxyguanosine fraction was combined with the corresponding fractions from the desoxyhypoxanthosine and pyrimidine chromatograms. Water and ammonium acetate were practically completely removed from the combined fractions by evaporation in an oil pump vacuum at 40° C. The dry white residue weighed 0.91 g. It was easily crystallizable from water and gave after two crystallisations 0.71 g of a product with the following analytical values: C = 42.1 %, H = 5.21 %, N = 24.8 %. Desoxyguanosine. H_2O gives theoretical: C = 42.1 %, H = 5.30 %, N = 24.6 %.

Preparation of desoxyhypoxanthosine. The crude desoxyhypoxanthosine was washed twice with 10 ml of 90 % ethanol and dried with ethanol and ether. It weighed 1.15 g. It was dissolved in 50 ml warm ammonium acetate buffer and after running a chromatogram on Dowex 50 as described below the main fraction was combined with the desoxyhypoxanthosine fractions of the other Dowex chromatograms. The water and buffer were removed *in vacuo.* The dry residue weighed 2.51 g. After three crystallisations from water 1.63 g of desoxyhypoxanthosine were obtained with the following analytical values: C = 47.9 %, H = 4.64 %, N = 22.4 %. Theoretical: C = 47.6 %, H = 4.79 %, N = 22.2 %.

Preparation of pyrimidine desoxyribosides. The mother liquor after the first crystallisation of desoxyhypoxanthosine according to Schindler was evaporated *in vacuo* until an oily residue was left. This contained the pyrimidine desoxyribosides together with a considerable amount of desoxyhypoxanthosine. Fractionation experiments with chloroform : ethanol as suggested by Schindler did not meet with success as all desoxyribosides showed considerable solubility

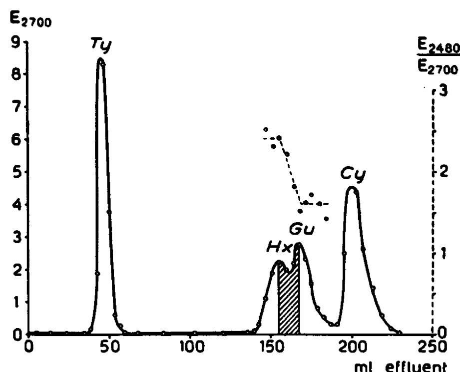

Fig. 2. Chromatogram on starch of ca. 2 mg of each desoxyriboside of thymine, hypoxanthine, guanine and cytosine. Length of column 290 mm, diameter 19 mm. The dash line gives the ratio E_{2480}/E_{2700} and indicates where desoxyhypoxanthosine starts to become contaminated with desoxyguanosine. The area under the diagonal lines indicates the mixed fractions.

in this medium. It was thought that extraction with propanol as used by this author would not be of any greater advantage. Instead, after dissolving the residue in 20 ml of ammonium acetate buffer a chromatogram on Dowex as described below was run directly. The purinedesoxyribosides obtained from the chromatogram were combined with the corresponding main fractions. From the fractions containing thymidine water and buffer were removed and the dry residue was three times crystallized from methanol: ethanol. The crude product which weighed 2.12 g gave 1.31 g crystalline thymidine with the following analytical values: C = 49.9 %, H = 5.70 %, N = 11.5 %. Theoreical: C = 49.6 %, H = 5.83 %, N = 11.6 %.

The desoxycytidine fractions, which were obtained from the column by elution with 5 % ammonia were the most impure. After removal of water and ammonia by evaporation *in vacuo* it proved to be of great value to rerun the crude product on another Dowex 50 column (diameter 50 mm, length 100 mm) with 0.5 *N* HCl as solvent. The fractions containing desoxycytidine were located by their characteristic ultra violet light absorption which was measured in the Beckman quartz spectrophotometer. From the combined fractions the excess HCl was carefully removed by repeated evaporation *in vacuo*. The white residue proved to be the hydrochloride of desoxycytidine. It weighed 1.37 g. It was easily crystallized from methanol-ethanol. After two crystallisations 0.91 g desoxycytidine. HCl were obtained with the following analytical values: C = 40.8 %, H = 5.24 %, N = 15.6 %, Cl = 13.7 %. Theoretically: C = 41.0 %, H = 5.35 %, N = 15.9 %, Cl = 13.5 %.

Chromatography of desoxyribosides on Dowex 50. For each chromatogram of the three crude desoxyriboside fractions a column of Dowex 50 (mesh size ca 300) of 50 mm diameter and 140 mm length was used. The commercial ion exchanger was first washed with 4 *N* HCl, then water, 5 % ammonia and at last 0.1 *M* ammonium acetate buffer. The buffer was prepared by titrating

1 M ammonia with glacial acetic acid to pH 3.9, the pH of the solution being determined with the aid of a glass electrode. The buffer was diluted ten times before use.

When the effluent after the last washing reached a pH of 3.9 the crude desoxyriboside fraction was dissolved in 20—100 ml of the buffer, placed on the top of the column and run in by air pressure. The chromatogram was then run with the buffer. Ten ml fractions were taken from the beginning. After the appearance of thymidine the fraction size was approximately 50 ml. The fractions were analyzed by measuring their light absorption in the ultra violet with the Beckman quartz spectrophotometer. Fractions containing the same type of light absorption were combined. When the desoxyguanosine had come out from the column the solvent was shifted to 5 % ammonia. This caused desoxycytidine to come out immediately.

Fig. 1 shows a model chromatogram of this type run on a smaller scale.

Chromatography of desoxyribosides on starch. Chromatography on starch was used as a method of purification when only very small amounts of desoxyribosides were available. It was used instead of crystallisation and was always preceeded by a chromatogram on Dowex 50. Thus the desoxyribosides had already been separated and freed from most salts.

Fig. 2 shows a model experiment in which a mixture of all four desoxyribosides was subjected to chromatography. The starch column was prepared in the way described by Stein and Moore [13]. The chromatogram was run with butanol-water (86.5 parts butanol + 13.5 parts water). Two ml fractions were taken and analyzed by their ultra violet absorption in the usual way. The separation of desoxyhypoxanthosine and desoxyguanosine was incomplete. The mixed fractions containing both desoxyribosides could be indentified by determining the ratio of the light absorption at 2480 Å and 2 700 Å for each fraction. This ratio differs for the two desoxyribosides. The procedure was thus principally the same as that used by Reichard [14] for guanine and xanthine.

Criteria of purity of components. When large amounts of crystallized substances were available elementary analyses were carried out. After starch chromatograms, however, the desired degree of purity was only a relative one. This type of purification was used when the material contained the stable isotope N^{15}. The only desired purity was thus the purity from foreign nitrogen. This was determined in each case by reference to a ratio used previously [15], a comparison between the maximum light absorption and the amount of nitrogen present in the sample $\frac{E_{max}}{\gamma N/ml}$. These ratios were first determined on crystalline standard samples and found to be: thymidine (E_{max} at 2670 Å)

= 0.341; desoxyhypoxanthosine (E_{max} at 2490 Å) = 0.201; desoxyguanosine (E_{max} at 2550 Å) = 0.176; desoxycytidine (E_{max} at 2790 Å) = 0.303. All values were determined in $N/10$ HCl.

SUMMARY

A method is described for the preparation of desoxyribosides, after enzymatic hydrolysis of thymonucleic acid, by chromatography on Dowex 50 and on starch.

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