ISOLATION OF A NEW DEOXYRIBOSIDIC COMPOUND, THYMIDINE DIPHOSPHATE RHAMNOSE

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Our previous studies (Okazaki and Okazaki, 1958, in press; Okazaki, Okazaki and Kuriki, 1959; Kuriki and Okazaki, in press) have shown the presence of a derivative of thymidine diphosphate in the acid-soluble extract from two strains of bacteria, Lactobacillus acidophilus R-26 and Escherichia coli 15T⁻, and its possible participation in DNA synthesis. The present paper reports the isolation and further characterization of this compound, which has now been identified as thymidine diphosphate rhamnose.

The acid-soluble extract was prepared with cold perchloric acid from the cells of L. acidophilus R-26 (deoxyriboside requiring bacteria) subjected to a treatment causing the accumulation of deoxyribosidic compounds, i.e. depletion of uracil and deoxyriboside followed by replenishment with thymidine (Okazaki and Okazaki, in press). The neutralized extract was added onto a column of Dowex-1 (formate) and fractionated by the gradient-elution chromatography with the "formic acid system" of Hurlbert et al. (1954). The compound under consideration was eluted at the position between those of thymidine monophosphate and thymidine diphosphate. The crude fraction so obtained was lyophilized to remove formic acid and ammonium formate and then chromatographed on Whatman No.1 paper with isobutylic acid-1 N NH_4OH (10:6) system. The paper chromatography separated two major ultraviolet absorbing components, the one having Rf value of 0.35 and the other having that

of 0.20. The former component was eluted from the paper and further purified by adsorption to Norit A and elution from the charcoal with ammoniacal ethanol-water, the obtained ammoniacal solution being evaporated in vacuo. In some cases, the solution of the compound was passed through a column of Dowex-50 to secure complete removal of ammonium ions.

The ultraviolet absorption spectra of the compound obtained as above were in excellent agreement with the spectra of thymidine both in acid and in alkali. This fact together with the observation reported previously (Okazaki, Okazaki and Kuriki, 1959) that the isotopic label was incorporated into this compound when the cells were incubated with labeled thymidine indicate the compound to be a thymidine derivative. Microbioassay after snake venom digestion (cf. Okazaki and Okazaki, 1958) proved the presence of deoxyriboside in the amount expected from ultraviolet absorption. The compound contained two moles of organic phosphate per mole of thymidine, and upon treatment with 1 N HCl at 100° for 10 min. half the phosphate was liberated in the inorganic form. Acid hydrolysis at the same time caused appearance of the reducing power measurable by the method of Park and Johnson (1949).

Thus the compound appeared to be a derivative of thymidine diphosphate conjugated with some reducing substance. Various color reactions for sugars were hence carried out on the purified samples. Only positive result was obtained in the cysteine-sulfuric acid test for methylpentose (Dische and Shettles, 1948). In this reaction, the purified compound formed a reaction product with a sharp absorption maximum at 400 mμ, just as did rhamnose and fucose which were used as standards. The whole absorption curve of the reaction product formed by the compound was in complete agreement with that formed by standard methylpentoses. The test for the stability of the reaction product to dilution with

Table I

Analytical data of thymidine diphosphate rhamnose

	μmoles
Thymidine	1.00
Total phosphate	1.90
Labile phosphate	1.07
Methylpentose	0.95

water (Dische and Shettles, 1951) also provided evidence that the reacting substance in the purified deoxyribosidic compound is methylpentose. If calculated as rhamnose or fucose, the molar ratio of methylpentose to thymidine in the compound was approximately 1 : 1.

Paper chromatography of the acid hydrolysate was performed with six different solvent systems. Rf values of the sugar component in the hydrolysate agreed well with Rf values of rhamnose (Table II), but differed from those of fucose. Thus it appears that the methylpentose contained in this deoxyribosidic compound is rhamnose.

Table II

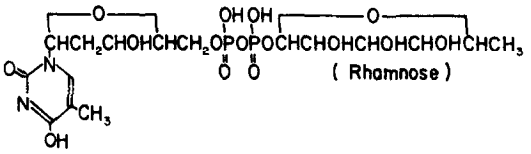
Rf values of the sugar component in the hydrolysate from the deoxyribosidic compound and of authentic rhamnose

Solvents	Sugar component in hydrolysate	L-rhamnose
Butanol-acetic acid-water(4:1:5)	0.43	0.43
Butanol saturated with water	0.23	0.24
Butanol-ethanol-water(3:1:3)	0.51	0.50
Phenol saturated with water	0.58	0.58
Ethylacetate-acetic acid-water(3:1:3)	0.18	0.19
Ethylacetate-pyridine-water(8:2:1)	0.26	0.27

In order to obtain further information on the structure of the compound, the purified sample was digested with snake venom phosphodiesterase which possessed also a strong nucleotide pyrophosphatase activity, and the split products were separated by Dowex-1

chromatography. The enzymatic treatment resulted in the release of two phosphate containing substances. The one component possessed equimolar amounts of thymidine and phosphate and was eluted at the position corresponding to that of thymidine monophosphate, while the other component, eluted just before the former, contained equimolar amounts of methylpentose and phosphate. Since the latter substance showed reducing activity only after liberation of phosphate by acid hydrolysis, the phosphate seemed to be attached to the anomeric carbon of methylpentose. From these observations it is evident that hydrolysis of the new deoxyribosidic compound with snake venom phosphodiesterase gave equimolar amounts of thymidine monophosphate and rhamnose-1-phosphate. The result may be explained on the basis of the nucleotide pyrophosphatase activity of the enzyme preparation, and provides evidence that in the compound thymidine monophosphate is linked to rhamnose-1-phosphate through a pyrophosphate bond. Treatment with crude snake venom resulted in the release of thymidine from the compound, indicating that one of the phosphate is esterified at the 5' position of deoxyribose of thymidine.

Summarizing the observations, the following structure is proposed for the new deoxyribosidic compound:



The possibility that more components are contained in the compound is not necessarily excluded, but we have evidence that there is no more nitrogen than that accounted for by thymidine moiety.

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