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Accumulation of D-tagatose by D-galactose-grown Mycobacteria

Kumulacja D-tagatozy przez mykobakterie hodowane na galaktozie

Аккумулирование D-тагатозы микробактериями, возрастающими в среде с галактозой

Saprophytic strains of mycobacteria are known to utilize variety of sugars and sugar alcohols as a sole source of carbon and energy. Being strict aerobes they can assimilate nutriments very efficiently and hence, they do not use to accumulate partially oxidized metabolites in culture media, unless special approaches are employed. The use of "resting cells" technique (2) and "underlayering" procedure (5, 8) as well as specific inhibitors (2) and nutritional mutants with metabolic blocks (5) have permitted to observe some of the metabolites extracellularly released.

Among sugars with clear metabolic characteristics in mycobacteria are: glucose (6), fructose (7), mannose (4) and xylose (5). However, the data on the metabolism of galactose and its derivatives are still lacking. To resolve the problem detailed studies have been undertaken in this Laboratory, which indicated that Leloir route and modified Doudoroff pathway might function in mycobacteria. In the course of the work extracellular accumulation of unknown metabolite of galactose was observed.

The present report deals with the isolation and identification of tagatose as a metabolite of galactose in mycobacteria.

MATERIALS AND METHODS

Microorganisms and cultivation. The following strains were used: *M. phlei, M. friburgensis, M. jucho, Mycobacterium* sp. 279 and *Mycobacterium* sp. 607. The strains were obtained from the Department of Plant Physiology, University in Lublin. The cells were grown aerobically on the surface of liquid glutamate--citrate-salts medium as described earlier (5). The source of carbon was glucose or galactose at final concentration of 2 to 4 per cent. The medium (100 ml per Erlenmeyer flask of 250 ml) was inoculated with cell suspension from Löwenstein slant and incubated at 37°C for 4—7 days or longer, if needed. In the course of growth samples of medium were sterically withdrawn with Pasteur pipette.

Paper chromatography. N-butanol-pyridine-water (3 :2 : 1.5), n-propanol--ethyl acetate-water (7 : 1 : 2) and water saturated phenol were employed as solvents for separation of sugars. Detection of sugars was performed with alkaline silver nitrate, orcinol trichloracetate and resorcinol hydrochloride sprays. Whatman No 1 paper was used in all the experiments.

Gas chromatography. Gas chromatographic apparatus type GCHF 18.3.4. Veb Chromotronic Berlin (GDR), equipped with FID detector was used. Glass column (0.3×200 cm) was filled with Gas Chrom Q (100—120 mesh) covered with ECNSSM (3%). Column temperature — 180°C. Flow rate of gases: argon — 80 ml/min, hydrogen — 40 ml/min, oxygen — 100 ml/min. Alditol acetates were prepared according to the standard procedure.

Analytical methods. Ketohexoses were identified and estimated by the colour reactions given by resorcinol hydrochloride, and cysteine-carbazol-H_2SO_4. Total sugars were determined with cysteine-H_2SO_4 (1) reagent. Dowex-1 ($HCOO^-$) was prepared according to the standard procedure.

RESULTS

Paper chromatographic analysis of galactose medium after growth of *M. phlei* showed repeatedly the appearance of unknown metabolite (Fig. 1) which has been tentatively identified as a ketohexose due to its red colour given with resorcinol hydrochloride reagent. Next, it was established that other strains of saprophytic mycobacteria were also able to accumulate the metabolite extracellularly, though they varied in time--course profile of the metabolite formation. *M. phlei* was distinguished by its relative high and persistent accumulation of the metabolite (Table 1).

In another approach, 7-day old glucose-grown cultures of *M. phlei* were carefully deprived of the medium and underlayered with buffered (*pH* 7) galactose solution. In this case, the accumulation of the metabolite was higher than earlier and dependent on galactose concentration. If the complete galactose medium was applied in the underlayering procedure, the accumulation of the metabolite was lower and transient (Fig. 2). Further, the metabolite was also produced when the cultures

Fig. 1. Paper chromatography of the galactose medium of *M. phlei*. Chromatography was performed in n-butanol pyridine-water (3 : 2 : 1.5). Detection was obtained with alkaline silver nitrate spray

Table 1. Accumulation of tagatose in the galactose medium of various mycobacterial strains

Strain	Days				
	5	7	10	14	21
M. smegmatis	31.5	44.8	5.0	0	0
M. friburgensis	13.3	18.3	11.6	0	0
M. sp. 607	18.3	38.2	18.2	3.3	0
M. butyricum	16.6	14.1	3.3	0	0
M. jucho	23.2	43.2	19.9	2.5	0
M. sp. 279	11.6	29.9	8.3	0	0
M. phlei	38.2	63.0	94.6	79.6	54.8

Strains were grown in the gluatamate-citrate-salts medium containing 2% galactose. Tagatose was expressed in mg per 100 ml of the medium.

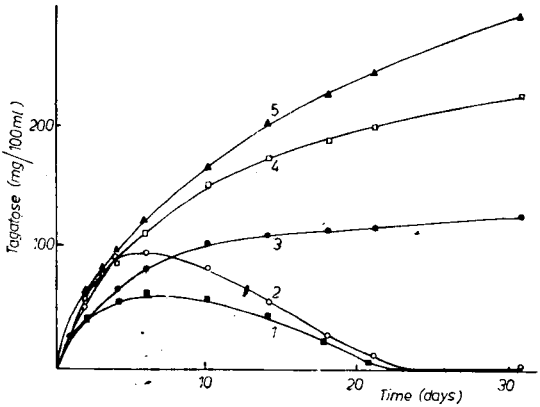

Fig. 2. Extracellular accumulation of tagatose by *M. phlei* under different conditions. Glucose-grown cultures were underlayered with respective solutions; 1 — 2% galactose in complete growth medium, at 37°C, 2 — 2% galactose in complete growth medium, at 45°C, 3 — 1% galactose in 10 mM phosphate buffer, *pH* 7.0, 4 — 2% galactose in 10 mM phosphate buffer, *pH* 7.0, 5 — 4% galactose in 10 mM phosphate buffer, *pH* 7.0

were underlayered with buffered dulcitol solution. In case of the cultures which had been previously starved on sugar-free medium, an increase of the metabolite accumulation was observed. To the contrary, chloramphenicol (100 μg/ml) significantly diminished the accumulation.

To obtain the metabolite which was next submitted to identification, *M. phlei* was grown in the basal production medium as specified in the Methods with the use of galactose as a source of carbon. One liter of the medium collected after 10 days of growth containing 720 mg of ketohexose (Roe method, fructose standard) was concentrated in a rotary evaporator to 80 ml at 60°C and, after cooling, deproteinized with 60% perchloric acid solution (20 ml). The precipitate was centrifuged, supernatant neutralized with 10 N KOH solution and left overnight in cold for $KClO_4$ precipitation. The solution was decolorized with Norit (5 g), diluted to 300 ml and subjected to galactose oxidation. $BaCO_3$ (25 g) was added to the solution, followed by bromine (6 ml) and kept in the dark for 3 days, with stirring. After that time the mixture was centrifuged, barium was removed with 4 N H_2SO_4 solution (52 ml) and the excess of bromine blown away with N_2. The remaining solution was treated with Dowex-50 (H^+) resin (5 g) and passed through Dowex-1 ($HCOO^-$) resin (200—400 mesh, 50 g) column to remove galactonate. The eluate was evaporated to dryness, dissolved in ethanol and analyzed. It appeared that the preparation contained 400 mg of ketohexose and was still contaminated with galactose. Therefore, further purification was performed by preparative paper chromatography with the use of pyridine-butanol-

-water solvent by descending technique. The eluate was rechromatographed, if needed, to receive galactose-free metabolite samples.

The purified metabolite was applied to paper chromatography. A single spot was found by spraying with reagents as described in the Methods. With resorcinol spray the spot was red, with orcinol spray the spot was yellow. The latter was fluorescent under Uv irradiation. The results are in favour of ketohexose and against ketopentose.

Suitable solvents for distinct separation of ketohexoses are lacking. In butanol-pyridine-water and propanol-ethyl acetate-water solutions, for instance, the migrations of the metabolite, authentic tagatose and fructose were the same, having R_{fI}-0.41 and R_{fII}-0.39. Hansen and Anderson (3) reported that they were able to separate ketohexoses with the use of water saturated phenol. Their results expressed in $R_{fructose}$ values were: sorbose — 0.83, tagatose — 0.88 and psicose — 1.08. Our results are: metabolite — 0.88, tagatose — 0.89 and sorbose — 0.84, which indicated that the metabolite was neither fructose nor psicose and it behaved like tagatose.

The metabolite was also tested by gas chromatography in the form of alditol acetate and compared with authentic tagatose. As a result of borohydride reduction of carbonyl group of authentic tagatose, two epimeric polyoles — talitol and dulcitol — were formed. Their respective t_R values in relation to sorbitol were 0.83 and 0.90; for metabolite the obtained values were: 0.80 and 0.90.

The absorption spectrum of cysteine—H_2SO_4 reaction (Fig. 3) and the rate of colour development in this test (Fig. 4) in relation to various sugars have also been reported. According to Dische and Devi (1) $t_{1/2}$ color development are: fructose — 40 min, psicose — 15 min and

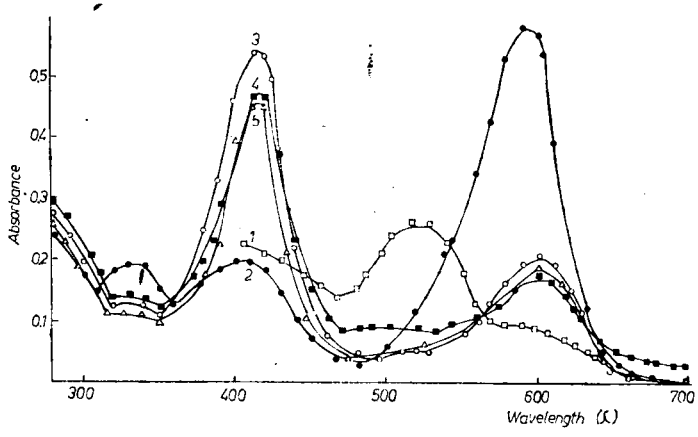

Fig. 3. Absorption of various ketoses in Dische and Devi's (1960) test; 1 — xylulose, 2 — sorbose, 3 — fructose, 4 — purified tagatose, 5 — authentic tagatose

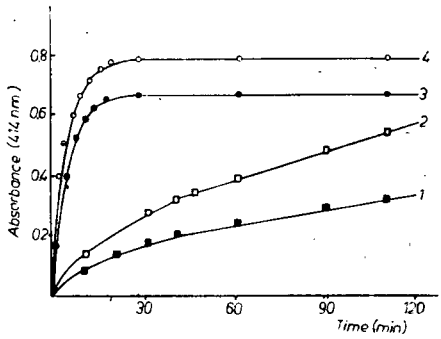

Fig. 4. Effect of time on absorption of ketoses in Dische and Devi's (1960) test, at 414 nm; 1 — sorbose, 2 — fructose, 3 — purified tagatose, 4 — authentic tagatose

tagatose — 7 min. Our results showed the same values for metabolite and tagatose. On the other hand, the absorption spectrum or yellow solution typical for ketohexoses distinguished metabolite from sorbose.

We have recently found in mycobacteria tagatokinase a very specific new enzyme which, apart from tagatose, has no activity towards fructose and sorbose and several other common sugars. It appeared that tagatokinase was able to phosphorylate the metabolite.

The obtained results indicate that the metabolite accumulated in the culture media of mycobacteria is tagatose.

DISCUSSION

The data obtained in the present work have indicated that the saprophytic strains of mycobacteria possess mechanisms which permit to form tagatose from dulcitol and galactose *in vivo*. These mechanism are expected to be isomerisation of galactose and dehydrogenation of dulcitol. In agreement with the experiments *in vivo* we have been able to observe low (about 0.01 μmol/min/mg protein) but reliable isomerase activity towards galactose in the cell-free extracts of the galactose-grown mycobacteria. Dehydrogenation of dulcitol has also been observed and the reaction was NAD-dependent.

The question arises concerning the catabolism of tagatose. As it has been mentioned before, a very specific tagatokinase occurs in mycobacteria, which phosphorylates tagatose to tagatose-6-phosphate. Further steps of this pathway might be phosphorylation of the latter to tagatose-1,6-phosphate with its subsequent cleavage to glyceraldehyde-3-phosphate and phosphodihydroxyacetone, as known metabolites of glycolytic cycle. The conclusion is based on the finding that glyceraldehyde-3-phosphate is formed from tagatose-6-phosphate during its incubation with ATP, magnesium ions and crude enzyme preparations.

The reason why the extracellular release of tagatose in mycobacteria occurs is unknown. Taking into account that the accumulation of tagatose was especially intense in underlayering procedure in which the glucose-grown cells were used, the conclusion might be drawn of strict inducible character of tagatokinase; it is assumed that the enzyme is absent in the original cultures. Moreover, the nitrogen source (and presumably also the other constituents of the medium) are considered to be needed for inducible tagatokinase and for further tagatose catabolism as well.

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STRESZCZENIE

Saprofityczne szczepy mykobakterii kumulują w podłożu galaktozowym nieznany metabolit — ketoheksozę. Kumulacja była największa w przypadku szczepu *Mycobacterium phlei* i zależała od stężenia galaktozy w podłożu. Chloramfenikol hamował gromadzenie ketoheksozy. Z podłoża wzrostowego *M. phlei* wyizolowano ketoheksozę i zidentyfikowano ją metodami chromatograficznymi i spektralnymi jako D-tagatozę. Dyskutowano mechanizm tworzenia się i rozpadu tagatozy u mykobakterii.

РЕЗЮМЕ

Сапрофитные штаммы микобактерий накапливали в среде с галактозой метаболит-кетогексозу. Самое большое количество метаболита, которое зависело от концентрации галактозы, найдено в штамме *Mycobacterium phlei*. Хлорамфеникол ингибировал накопление кетогексозы. Из исследованной среды изолировали метаболит, а применяя хроматографические и спектральные методы идентифицировали его как D-тагатозу.