AN ALTERNATIVE ROUTE FOR D-GALACTOSE CATABOLISM SHARED WITH THAT FOR DULCITOL DEGRADATION ROUTE IN MYCOBACTERIA

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1. Introduction

During the growth of saprophytic mycobacteria on galactose, a ketohexose accumulated in the media. A similar compound appeared after incubation of glucose-grown cells with dulcitol. This finding suggested that dulcitol and galactose may be metabolised by a common route. According to the literature, dulcitol is dehydrogenated to tagatose [1]; dulcitol-6-phosphate can either be dehydrogenated to tagatose-6-phosphate [2,3] or to galactose-6-phosphate [4]. It appears, however, that the complete pathway for catabolism of dulcitol has not yet been described.

However, the metabolism of galactose can start in three different ways: by phosphorylation at either C_1 [5] or C_6 [6]; oxidation to either galactohexodialdose [7] or galactonate [9]; and by isomerisation to tagatose [9].

Our previous report [10] indicated that the main route of galactose catabolism in Mycobacteria occurs via phosphorylation at C_1; it has also been established that the catabolism of the polyols is initiated by their dehydrogenation to ketoses [11]. This study provides evidence for a pathway involving tagatose as a common intermediate for both galactose and dulcitol.

2. Materials and methods

The bacteria used were: *Mycobacterium* sp. 279, *M. jucho, M. butyricum, M. muris, M. friburgensis* and *M. phlei.* The strains were obtained from the Department of Plant Physiology, University of Lublin.

The organisms were grown statically at 37°C on the surface of glutamate–citrate–salts medium (pH 6.8) in Roux flasks [12]. The source of carbon was galactose (3%) or dulcitol (2.5%) supplemented with galactose (0.5%) for stimulation of growth. After 4–7 days of growth, the cells were collected and washed with water.

For the preparation of cell-free extracts, 5 g packed cells were suspended in 20 ml 50 mM phosphate buffer (pH 7.2) containing 5 mM 2-mercaptoethanol and 1 mM EDTA and disrupted in a MSE 100 W ultrasonic vibrator (20 kc/s, 2 × 5 min). The homogenate was centrifuged at 16 000 rev./min for 45 min at 4°C to remove cell debris. Cell extracts were preincubated with DNase (2 mg/100 ml) at 20°C for 30 min and, after cooling, treated with solid ammonium sulfate. The proteins precipitating between 35–70% saturation were taken up and chromatographed first on Sephadex G-100 then on DEAE-cellulose columns as reported [11].

Polyol dehydrogenases were assayed spectrophotometrically at 340 nm as in [11]. Dulcitol dehydrogenation was measured in a mixture that contained in 1.7 ml total vol.: carbonate–bicarbonate buffer, pH 10.5 (100 μmol); dulcitol (125 μmol); NAD^+ (0.5 μmol); and enzyme. The assay for galactose isomerase was performed at 37°C using: phosphate buffer, pH 7.5 (100 μmol); galactose (20 μmol); and enzyme; in 2 ml total vol. Tagatokinase was monitored spectrophotometrically [13] at 340 nm in a mixture contained in 1.7 ml: Tris–HCl buffer, pH 7.5 (100 μmol); $MgCl_2$ (2 μmol); ATP (2 μmol; phosphoenolpyruvate (2 μmol); NADH (0.2 μmol); tagatose (3 μmol); and an excess of enzyme, pyruvate kinase and lactate dehydrogenase.

Paper chromatography of sugars was performed with Whatman no. 1 filter paper, water-saturated phenol and *N*-butanol–pyridine–water (3:2:1.5, by vol.) as solvents. Sugars were detected by spraying the chromatogram with alkaline silver nitrate, resorcinol hydrochloride or orcinol trichloracetate reagents.

Alditol acetates and trimethylsilyl derivatives of sugars were prepared and subjected to gas chromatography by standard procedures [14,15]. Total sugars were determined with cysteine–H_2SO_4 method [16], ketoses with cysteine-carbazol [17] and Roe [18] methods and glyceraldehyde-3-phosphate with its dehydrogenase.

3. Results and discussion

All the organisms used were capable of growing on medium containing galactose. A chromatographic analysis of the culture medium revealed accumulation of a ketohexose, especially high in *M. phlei* (fig.1). To identify the metabolite the culture fluid was concentrated with use of rotary evaporator, the bulk of galactose converted to galactonate by bromine oxidation with subsequent removal of the acid by Dowex-1/$HCOO^-$/ treatment, then purified by preparative paper chromatography.

The purified metabolite, when applied to the paper chromatogram, showed a single red spot with resorcinol and yellow with orcinol sprays. In water-saturated phenol it migrated like authentic tagatose and was separated from fructose and sorbose. Applied to gas chromatography in the form of the alditol acetate or trimethylsilyl derivative, it behaved in the same way as did the authentic tagatose derivatives. The absorption spectrum in the cysteine–H_2SO_4 reaction [16] and the rate of colour development in this test ($t_{1/2}$ = 7 min) of the metabolite corresponded to the tagatose standard. Thus, it is concluded that the metabolite may be the tagatose.

The formation of tagatose during growth of mycobacteria on galactose medium suggested isomerisation of the substrate used. Indeed, assay of galactose isomerase as performed in cell-free extract showed a small but measurable activity (~12 munits/mg protein). It should be added that galactose isomerase was not observed earlier in mycobacteria.

In contrast to galactose, dulcitol was utilised for growth less readily. Of 14 *Mycobacterium* strains tested, only 4 grew in dulcitol media, i. e., *M. friburgensis, M. butyricum, M. muris* and *Mycobacterium* sp. 279.

Attempts to phosphorylate dulcitol with ATP were unsuccessful; also negative were tests for conversion of dulcitol to galactose, phosphorylation of galactose to galactose-6-phosphate and isomerisation of the latter to tagatose-6-phosphate. Therefore, on the analogy with other polyols which undergo dehydrogenation to their respective ketoses [11], we expected the same reaction with dulcitol. Indeed, in extracts concentrated with ammonium sulfate, a slow NAD-dependent dehydrogenation of dulcitol in glycine–NaOH buffer was observed. However, introduction of carbonate–bicarbonate buffer accelerated the reaction almost 3-times and the highest activity in this buffer was found to be near pH 11 (fig.2). Like the sorbitol and ribitol dehydrogenases, dulcitol dehydrogenase was competetively inhibited by Tris.

We have reported the occurence in mycobacteria of 3 types of NAD-dependent polyol dehydrogenases [11]. To find out which of the enzyme is responsible for dulcitol dehydrogenation, the enzyme preparations were analysed by gel- and exchange-chroma-

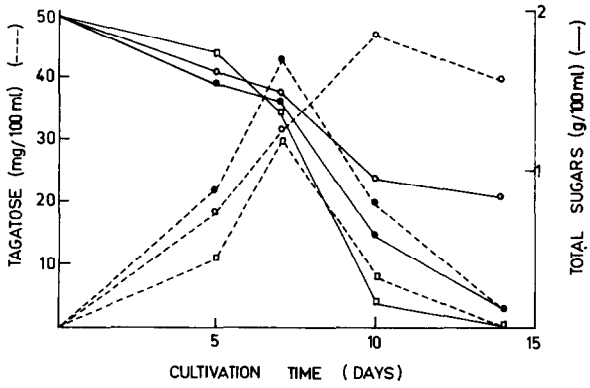


Fig.1. Galactose disappearance and tagatose formation in growth media of mycobacteria: (□) *Mycobacterium* sp. 279; (○) *M. phlei* (tagatose content reduced by 50%); (●) *M. jucho.*

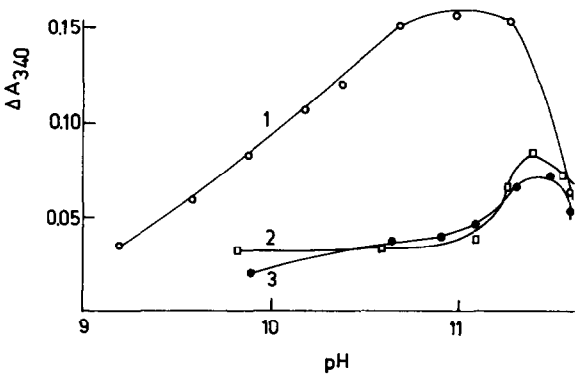


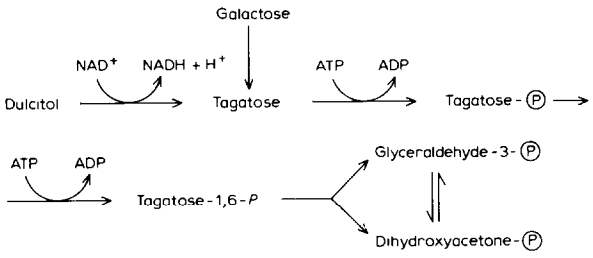
Fig.2. Dehydrogenation of dulcitol at various buffers: (1) carbonate–bicarbonate buffer; (2) glycylglycine–NaOH buffer; (3) glycine–NaOH buffer.

tography and gel electrophoresis. We found that sorbitol and dulcitol dehydrogenation activities were non-separable at these conditions, at least in relation to *Mycobacterium* sp. 279.

The experiments showed that tagatose was a metabolite common to both galactose and dulcitol catabolism. We have recently found that tagatose is phosphorylated with ATP to tagatose-6-phosphate by the enzyme preparations from dulcitol–galactose-grown Mycobacteria. The reaction is catalysed by a new kinase of high specificity towards tagatose. The results of purification and properties of the enzyme will be published elsewhere.

To recognize the further reactions of the pathway, tagatose-6-phosphate was prepared from the tagatokinase incubation mixture by a procedure modified from that for galactose-1-phosphate [19]. When incubated with ATP, Mg^{2+} and crude enzyme preparations, glyceraldehyde-3-phosphate was formed; this was trapped with hydrazine [8]. No aldehyde was found if ATP or hydrazine was omitted.

The formation of glyceraldehyde-3-phosphate from tagatose-6-phosphate suggested a possible phosphorylation of the latter to tagatose-1,6-phosphate with its subsequent cleavage to triose-phosphates. Thus, the entire pathway suggested might be:



The above pathway is, however, a side process for galactose degradation in mycobacteria. As reported in [10], the main route proceeds via galactose-1-phosphate as originally proposed by Leloir [5].

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