COMPARATIVE INVESTIGATIONS ON THE METABOLISM OF FORMALDEHYDE IN THE PRESENCE OF RIBOSE-5-PHOSPHATE IN CELL-FREE EXTRACTS OF YEASTS GROWN ON METHANOL

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1. Introduction

In microorganisms which are able to utilize methane or methanol as the sole carbon source, formaldehyde may be metabolized via allulose-6-phosphate as described by Kemp and Quayle [1] for *Pseudomonas methanica*. The enzyme system (hexosephosphate synthetase = HPS) responsible for the acyloinaddition of formaldehyde to ribose-5-phosphate was also detected in other methanol-utilizing bacteria [2, 3]. Furthermore Fuji and Tonomura [4] could establish, that [^{14}C]methanol fixed by a *Candida* sp. appeared predominantly in hexosephosphates within an incubation time of several seconds. We therefore tested four methanol-assimilating yeasts from our collection for their ability to incorporate [^{14}C]formaldehyde into hexosephosphates.

2. Materials and methods

The strains used were: *Kloeckera* sp. No. 2201*, *Candida boidinii* No. 0302**, *Pichia pastoris* M3**, and *Hansenula minuta* No. 0303**. The cells were grown in a minimal medium as described by Ogata et al. [5] containing either 2% methanol or 5% glucose as the sole carbon source. Glucose cultured cells were harvested when concentration of glucose had decreased to 3%. Cell-free extracts were prepared by mechanical disruption and centrifugation at 50 000 *g*, 2°C for 1 hr.

* Kindly supplied by Dr. K. Ogata.
** Isolations from the microbial laboratories of the IfGB.

HPS-activity was assayed according to Lawrence et al. [2] with the exception that the precipitated protein was finally removed by centrifugation. For scintillation measurements a 'corumatic system 200' (ICN/Tracerlab, USA) was used, with the scintillation cocktail for aqueous solutions as described by Bruno and Christian [6]. One unit of HPS-activity is defined equivalent to the incorporation of 1 μmole formaldehyde per minute and mg protein. Protein was determined essentially by the method of Lowry et al. [7].

The method of Stafford et al. [8] was used to measure the activity of a NAD(P) dependent enzyme reducing hydroxypyruvate. Carbohydrates were separated by one-dimensional TLC on silica gel (Merck, Darmstadt) after treating the reaction mixture with acid phosphatase from potato (Boehringer, Mannheim). Solvent systems contained acetone–water (90:10 v/v) or ethyl acetate–acetic acid–methanol–water (60:20:10:10 v/v). Reducing sugars were stained with aniline–diphenylamine–phosphoric acid [9] as well as with urea–HCl [11]; ketoses were stained with anthron reagent [10]. Radioautography was done by exposing 'Orsay-T4' X-ray films (Agfa, Belgium) for 100 hr to a total radioactivity of 5–50 nCi.

3. Results

As is shown in table 1 there exist significant differences in the HPS-activity in cell-free extracts from four methanol assimilating yeasts. Highest enzyme activity was measured in the *Kloeckera sp.* of Ogata [5] and next to this in the *Candida boidinii* isolated in our laboratories. A comparatively low activity was

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Table 1
HPS-activity in cell-free extracts of methanol-assimilating yeasts.

	HPS-activity ($IU \cdot 10^{-3}$)*
Kloeckera sp.	50
Candida boidinii	20
Pichia pastoris	5
Hansenula minuta	1
(Saccharomyces cerevisiae	<1)

* IU = International units after 3 min of reaction time.

found in cell-free extracts of *Pichia pastoris*, whereas enzyme activity of *Hansenula minuta* is in the range of the detection limit of the HPS-assay and is comparable to that of *Saccharomyces cerevisiae* which is not able to utilize methanol.

The standard error of the HPS-Test was calculated to be ± 19%. Because of the rather small reaction rates the tests were repeated as a function of reaction time (fig. 1). The results show increasing maximal values in the same order as mentioned above and a relatively high initial reaction velocity in all organisms (fig. 1a). Without adding ribose-5-phosphate we found a rather high basic level of radioactivity incorporated as is shown for the *Kloeckera sp.* in fig. 1b.

In thin-layer chromatographic separations of the dephosphorylated sugar phosphates the main radioactivity was found in spots indentified as hexoses. In addition to the radioactivity in the origin there appeared a rather weak labeling in the hexoses after 30 sec incubation time (fig. 2A). After incubating for 300 sec however the main radioactivity was found in fructose (fig. 2B). In both cases some highly active spots at high R_f values appeared, which could not as yet be identified. In the absence of ribose-5-phosphate the bulk radioactivity remains at the origin of the TLC (fig. 2C).

Adding [^{14}C]methanol to the assay mixture instead of [^{14}C]formaldehyde an essential part of the radioactivity appeared also in the fructose after 10 min incubation time.

In further experiments we could show that the HPS-enzyme system has a broad pH-optimum at pH 7 in phosphate buffer as well as imidazole–HCl buffered media. In contrast to the results of Lawrence et al. [2] and of Stieglitz and Mateles [3] with methanol bacteria we could not detect any Mg^{2+}-dependency of the enzyme system in yeasts.

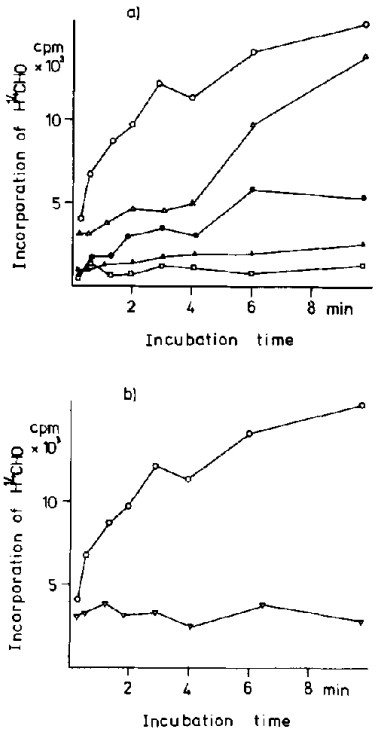

Fig. 1. Incorporation of $H^{14}CHO$ (50 nCi total radioactivity 0.5 ml. For further details see [2]) into hexosephosphates in cell-free extracts of methanol-assimilating yeasts as a function of time. a) Incubations with ribose-5-phosphate: (○—○—○) *Kloeckera sp.* No. 2201 and b) (△—△—△) *Candida boidinii* No. 0302, (●—●—●) *Pichia pastoris* M3, (▲—▲—▲) *Hansenula minuta* No. 0303, (□—□—□) *Saccharomyces cerevisiae*, b) ▽—▽—▽ *Kloeckera sp.* No. 2201 without ribose-5-phosphate.

After cultivating the *Kloeckera sp.* on glucose we found a much lower specific activity of HPS-enzyme which was in the range of one fifth of that of methanol grown organisms.

Hydroxypyruvate reductase by converting 3-hydroxy-2-ketopropionic acid to glycerate is known to be a key enzyme in bacteria incorporating C_1 via the glycine–serine pathway [2, 3]. By using the test system of Stafford et al. [8] we were not able to detect any NADH or NADPH dependent enzyme reaction after addition of hydroxypyruvate to extracts of our methanol assimilating yeasts.

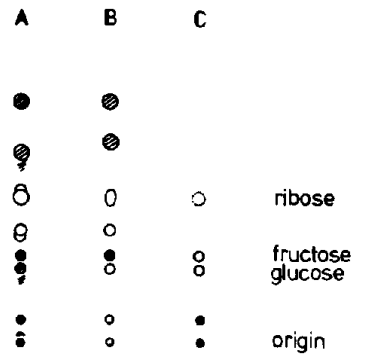

Fig. 2. TLC separation and autoratiography of dephosphorylated products of HPS-reaction: In the presence of ribose-5-phosphate after 30 sec (A), after 300 sec (B); and in the absence of ribose-5-phosphate after 300 sec (C) reaction time. (○) Stained carbohydrates; (●) main radioactivity; (•) minor radioactivity. Elution system was acetone–water. For further details see [1] and Materials and methods.

4. Discussion

The hexosephosphate synthetase activity of the investigated methanol grown yeasts is in rather good agreement with their ability to assimilate this carbon source*. Thus the *Kloeckera sp.*, which grows best on methanol, shows the highest specific HPS-activity. *Candida* and *Pichia* show a slower growth, whereas *Hansenula* uses methanol only very poorly. Compared to the results of Lawrence et al. [2] we obtained only rather low specific enzyme activities. It seems to be a peculiarity of methanol yeasts that their HPS-activities are lower by a factor of about 25 than the HPS-activities in methanol bacteria. Presumably by this reason we only were able to obtain reproducible results after precipitating protein from the test solution by adding ethanol. In spite of the fact that allulose could be separated very well from glucose and fructose by TLC we were not able to detect any labeled allulose ‡ in our chromatograms [12]. Thus allulose-6-phosphate, if synthesized at all, should be metabolized very rapidly to other hexoses in yeasts.

Nevertheless we suppose that *Kloeckera*, *Candida*, and *Pichia* yeasts contain a ribose-5-phosphate dependent formaldehyde incorporating enzyme system, which is induced during growth on methanol as carbon source but which is much less active in the presence of glucose as shown for the *Kloeckera sp.*

At the moment it seems to us that several reactions are superimposed and responsible for the incorporation of formaldehyde into yeast metabolism. In addition to the HPS-reaction there is evidence for a NAD dependent formaldehyde dehydrogenase [13, 14]. It is further known that a competition reaction exists as a consequence of the non-enzymatic [15] or enzymatic [16] reaction of formaldehyde with tetrahydrofolic acid. As the HPS-activities in *Hansenula minuta* as well as in *Saccharomyces cerevisiae* are very low we suggest that one of the competition reactions should be used in these organisms.

* Growth kinetics have been determined by Bronn et al. (publication will be prepared).

‡ Allulose as a reference compound was a generous gift from Dr. M.B. Kemp.

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