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The metabolic fate of doubly labelled lactose- ^{13}C , ^{15}N]ureide after pre-dosing with different ureides

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Running title: **Lactose- ^{13}C , ^{15}N]ureide and pre-dosing**

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Background: To date, the measurement of the oro-caecal transit time (OCTT) with lactose- ^{13}C ureide usually requires a pre-dosing with the analogous substrate in its unlabelled form.

Objective: In the present study, the enzyme induction provoked by different unlabelled sugar ureides in OCTT measurements when using doubly labelled lactose- ^{13}C , ^{15}N ureide (DLLU) was evaluated.

Methods: Thirteen healthy adults (age: 22-58 years) received 500 mg DLLU together with a standardised breakfast. Expired air, urine, and faeces were collected over a period of 14, 48, and 72 h, respectively. After 1 and 2 weeks, the test was repeated after pre-dosing of 3x120 mg glucose ureide (GU) and 3x200 mg cellobiose ureide (CU), respectively, on the day prior to study begin. The ^{13}C - and ^{15}N -enrichments were measured by isotope ratio mass spectrometry. The OCTT was calculated by the detection of a significant $^{13}\text{CO}_2$ -rise.

Results: In comparison to the period without pre-dosing (7.8 ± 2.2 hours) the measured OCTT was significantly lowered either after GU pre-dosing (5.8 ± 1.9 hours, $P = 0.033$) or CU pre-dosing (6.0 ± 2.2 hours, $P = 0.039$). The respective renal ^{13}C - and ^{15}N -excretions amounted to 24.5 and 45.6, 24.7 and 54.0, and 22.5 and 50.1 %, respectively, whereas the faecal ^{13}C - and ^{15}N -excretions amounted to 12.1 and 45.8, 4.8 and 21.5, and 9.6 and 39.8 %, respectively.

Conclusions: Pre-dosing with unlabelled GU and CU prior to the administration of DLLU led to an unequivocal induction of the enzyme activity and resulted in a definitive estimation of the OCTT, clearly demonstrating that glucose- ^{13}C ureide is the matrix of the bacterial degradation in the caecum.

Keywords:

Cellobiose ureide, glucose ureide, lactose- ^{13}C , ^{15}N]ureide, oro-caecal transit time, pre-dosing

Introduction

Within the last decade the non invasive lactose- ^{13}C]ureide (^{13}C -LU) breath test for measuring the oro-caecal transit time (OCTT) has been re-established and is meanwhile generally accepted and applied by a broad number of scientific teams (Berthold *et al.*, 2009; Christian *et al.*, 2002; Geypens *et al.*, 1999; Leitzmann *et al.*, 1998; Morrison *et al.*, 1999; Priebe *et al.*, 2004; Van den Driessche *et al.*, 2000; Wutzke and Sattinger, 2006). The molecular bond between the sugar moieties and ^{13}C]urea of ^{13}C -labelled glycosyl ureides, synthesised under acidic conditions from reducing sugars and ^{13}C]urea, has been shown to approach the caecum undigested, but then to be split by *Clostridium innocuum* (Mohr *et al.*, 1999). In our previous studies ^{13}C -LU, glucose- ^{13}C]ureide (^{13}C -GU), and cellobiose- ^{13}C]ureide (^{13}C -CU) were confirmed to yield reliable results when pre-dosing with the analogous unlabelled ureides, lactose ureide (LU), glucose ureide (GU) and cellobiose ureide (CU), respectively (Wutzke *et al.*, 1997; Wutzke and Glasenapp, 2004). Furthermore, it could be demonstrated that the induced enzyme activity is short-lived and no longer exists after 1 week. (Wutzke and Schütt, 2007). Therefore, a pre-treatment with LU on the day prior to ^{13}C -LU ingestion is essential for OCTT measurements. However, Morrison and co-workers described the pre-dosing with GU prior to ^{13}C -LU ingestion but without measuring the faecal ^{13}C -excretion (Morrison *et al.*, 2003). In recently published papers, assessing the effects of pro- and prebiotics on the intestinal ammonia metabolism in humans when using lactose- $^{15}\text{N}_2$]ureide (^{15}N -LU) as biomarker, the faecal ^{15}N -excretion has been investigated (Jackson *et al.*, 2004; de Preter *et al.*, 2004; Geboes *et al.*, 2005). However, the faecal ^{13}C -excretion and the corresponding total ^{13}C -partitioning, in breath + urine + faeces = remainder, of doubly labelled lactose- $^{13}\text{C},^{15}\text{N}$]ureide (DLLU) is still unknown. In continuation of these

investigations, the metabolic balance of both isotopes and particularly the enzyme induction of DLLU with other unlabelled glycosyl ureides, that are chemically not identical with the administered DLLU, was of great interest with respect to proof clearly that ^{13}C -GU and the corresponding enzyme *glucose ureide hydrolase* are the stakeholders of the degradation process of ^{13}C -labelled sugar ureides in the caecum. Therefore, the aim of the present study was to validate a possible enzyme induction by GU and CU when using DLLU for evaluating the OCTT and the additional measurement of the metabolic fate of both isotopes in breath, urine and faeces.

Methods

Tracer substance

Lactose- ^{13}C , ^{15}N]ureide and unlabelled GU and CU, were synthesised according to our method as described in detail (Wutzke *et al.*, 1997). Lactose and urea were obtained from Merck Darmstadt Germany, whereas ^{13}C - and $^{15}\text{N}_2$]urea (each 99 atom %) were obtained from Campro Scientific Berlin. Based on the 9:1 mixture of ^{13}C - and $^{15}\text{N}_2$]urea used for the DLLU synthesis, the ^{13}C - and ^{15}N -enrichments amounted to 89.2 and 10.2 atom % respectively, in the urea moiety of DLLU.

Subjects

Thirteen healthy adults (9 female, 4 male, age: 22-58 years, body mass index: 20.1-26.3 kg/m²) volunteered for this study. They were in good health throughout the study. None of the subjects were receiving any medication or had a history of gastrointestinal diseases. Furthermore, none of the subjects regurgitated after having received the sugar ureides. The testing protocol was approved by the Committee on Ethics of the Faculty of Medicine of the University of Rostock (registration number: II HV 41/2004). Written consent was obtained from all subjects.

Study protocols

Subjects were studied after an overnight fast. All volunteers received a continental breakfast made up of 1 wheat flour roll, 30 g butter, 1 slice of cheese, 25 g jam and 300 ml of black tea at 07 45. Six hours after breakfast, all volunteers received an individually standardised regular lunch at 14 00. Every single subject was allowed to choose his personal lunch menu but it was similar during the 3 tests (2 fried eggs, sunny side up, and 2 slices of wholemeal bread). Furthermore, the breakfast was identical in all 13 subjects on the days of DLLU administration. No other food or drink was allowed until 6 hours after breakfast.

Baseline breath, urine, and faeces samples were collected 15, 30 and 60 minutes, respectively, prior to the ingestion of DLLU to determine the baseline abundance of ^{13}C and ^{15}N . During breakfast, 500 mg DLLU, corresponding to a ^{13}C - and ^{15}N -excess dose of 14.42 and 3.67 mg, respectively, was administered as a single oral pulse strewn on a small area of the butter at 08 00 to ingest the label with one bite. Two expired breath samples were taken in 30-minute intervals over a period of 14 hours by direct exhalation into ExetainersTM (IVA Analysentechnik, Meerbusch, Germany). The urine was collected quantitatively in 2-, 4-, and 6-hour intervals over 48 hours, whereas faeces were collected quantitatively over 72 hours.

After a 1-week wash out period the subjects received a pre-dosage of 3x120 mg GU in 4-hour intervals (at 12 00, 16 00, and 20 00) 1 day prior to the repeated DLLU administration. After a further 1-week wash out period the subjects repeated the test with pre-dosing of 3x200 mg CU (equimolar to the GU dosage). The procedure of food intake, pre-dosing, DLLU administration, breath, urine, and faeces collection was identical in all 3 tests.

Analytical techniques

Isotope ratio mass spectrometry

The ^{13}C -enrichment in breath was measured by isotope ratio mass spectrometry (Tracer mass 20-20, SerCon, Crewe, U.K.) as detailed previously (Barrie *et al.*, 1989; Wutzke *et al.*, 1997). The data were expressed either as $\delta^{13}\text{C}$ -enrichment over baseline (DOB), as atom percent excess (atom % exc), as cumulative percentage dose recovery (CPDR) in breath, or as cumulative percentage urinary and faecal ^{13}C - and ^{15}N -excretion (CPE).

^{13}C -and ^{15}N -excess dose

The ^{13}C -and ^{15}N -excess dose (D) is the product of the DLLU intake (I), the ^{13}C -and ^{15}N -enrichment of the urea moiety (E), and the number of labelled ^{13}C -and ^{15}N -atoms, respectively, divided by the molecular weight (MW) of 403.4 g/mol times 100 (Wutzke and Glasenapp, 2004).

$$D = I \cdot E \cdot n/100 \cdot \text{MW}$$

Cumulative percentage ^{13}C -dose recovery (CPDR)

The CPDR is the product of an assumed CO_2 -production rate (V_{CO_2}) of 300 mmol/m²/hour (Schoeller *et al.*, 1980, Wutzke *et al.*, 1997), the calculated cumulative ^{13}C -data ($\Delta\delta^{13}\text{C}_{\text{cum}}$), the isotope ratio of $^{13}\text{CO}_2$ standard gas (0.01123), and the body surface area (BSA) in m² divided by the ^{13}C -excess dose (^{13}CD) in mmol times 10.

$$\text{CPDR} = V_{\text{CO}_2} \cdot \Delta\delta^{13}\text{C}_{\text{cum}} \cdot (0.01123) \cdot \text{BSA} / 10 \text{ }^{13}\text{CD}.$$

$$^{13}\text{CD} = 1.109 \text{ mmol}.$$

Cumulative percentage urinary and faecal ^{13}C -and ^{15}N -excretion (CPE)

The CPE is the product of the urinary or faecal ^{13}C - or ^{15}N -excretion (E) in mmol, respectively, times 100 divided by the ^{13}C - or ^{15}N -excess dose (D) in mmol, respectively.

$$\text{PCE} = E \cdot 100 / D.$$

$$^{15}\text{ND} = 0.245 \text{ mmol.}$$

Orocaecal transit time

The OCTT was calculated from the interval between DLLU intake and the detection of a significant, sustained and non-reversing rise of $^{13}\text{CO}_2$ in breath of 2 DOB or more (Wutzke *et al.*, 1997).

Statistical analysis

The Kruskal-Wallis test and the Mann-Whitney U-test, on program SPSS 15.0 for Windows were used for statistical analysis. All results are quoted as means \pm s.d.

Results

$^{13}\text{CO}_2$ -signals were observed in the expired air of all subjects beginning 6.0 hours after DLLU administration without pre-dosing. The mean maximum $^{13}\text{CO}_2$ -enrichment of 6.2 ± 0.0 DOB was reached after 14.0 hours (Figure 1). The pre-dosing of GU and CU 1 day prior to the DLLU ingestion caused a shifting of the $^{13}\text{CO}_2$ -rise beginning after 4.5 hours (maximum enrichment 7.0 ± 0.0 DOB at 6.0 hours; and 8.8 ± 0.0 DOB at 7.5 hours, respectively, Figure 1). In contrast to the delayed OCTT of 7.8 ± 2.2 hours measured without pre-dosing, the rise of $^{13}\text{CO}_2$ -enrichment after pre-dosing with both GU and CU was faster (approximately 2 DOB/hour) and led to a definitive estimation of the mean OCTT of 5.8 ± 1.9 and 6.0 ± 2.2 hours, respectively, when using a threshold of 2 DOB (Table 1). The observed OCTT differed significantly from the data without pre-dosing ($P = 0.033$ and 0.039 , respectively) whereas the data of the pre-dosing tests were not significantly different (Table 1). The respective CPDR amounted to 17.7 ± 8.4 , 28.7 ± 9.2 , and 26.1 ± 10.8 %, respectively, and differed significantly from the data without pre-dosing ($P = 0.014$ and 0.035 , respectively) whereas the values of the pre-dosing tests were not significantly different (Table 1,

Figure 2). The respective renal ^{13}C - and ^{15}N -CPE amounted to 24.5 ± 7.9 and 45.6 ± 9.6 , 24.7 ± 6.7 and 54.0 ± 10.5 , and 22.5 ± 4.6 and 50.1 ± 14.6 %, respectively, whereas the faecal ^{13}C - and ^{15}N -CPE amounted to 12.1 ± 11.7 and 45.8 ± 14.3 , 4.8 ± 3.4 and 21.5 ± 15.9 , and 9.6 ± 6.4 and 39.8 ± 24.5 %, respectively (Table 2, Figure 2). The calculated data of the urinary ^{13}C - and ^{15}N -CPE were not significantly different, whereas significantly different numbers of the faecal ^{13}C - and ^{15}N -CPE were observed without- vs GU- ($P = 0.026$), GU- vs CU- ($P = 0.006$), and without- vs GU-pre-dosing ($P = 0.002$), respectively. After administration of DLLU without and pre-dosing with GU and CU, the total ^{13}C -elimination in breath, urine, and faeces after the time-span of 14, 48, and 72 hours, respectively, amounted to 56.9 % on average in all 3 tests (54.3, 58.2 and 58.2 %, respectively), whereas the corresponding ^{15}N -excretion in all 3 tests was found to be 85.6 % on average (91.4, 75.5 and 89.9 %, respectively, Figure 2).

Discussion

Since the primary publication in 1995, $^{13}\text{CO}_2$ -breath tests for measuring the OCTT preferentially by using ^{13}C -LU have become generally accepted worldwide (Berthold *et al.*, 2009; Heine *et al.*, 1995; Riemmele *et al.*, 1997; Geypens *et al.*, 1999; Jackson *et al.*, 1999; Van den Driessche *et al.*, 2000; Christian *et al.*, 2002; Morrison *et al.*, 1998, 1999, 2003; Schneider *et al.*, 2007; Wutzke and Sattinger, 2006). It is commonly assumed that ^{13}C -GU and the corresponding *glucose ureide hydrolase* are the key substance and the key enzyme, respectively, of the enzymatic sugar ureide degradation process that is exclusively initiated by *Clostridium innocuum* in the human caecum (Mohr *et al.*, 1999; Wutzke and Glasenapp, 2004). Our previous studies revealed that the application of ^{13}C -LU, ^{13}C -GU, and ^{13}C -CU lead to significantly shorter OCTT data of 419 vs 311 ($P = 0.028$), 415 vs 287 ($P = 0.017$), and

401 vs 266 ($P= 0.012$) min, respectively, when pre-dosing with the respective unlabelled ureides LU, GU, and CU (Wutzke and Glasenapp, 2004; Wutzke and Schütt, 2007). Therefore, we concluded that pre-dosing with the analogues substrate in its unlabelled form is essential for OCTT measurements to obtain reliable results. Furthermore, ^{15}N -LU has been additionally applied as a biomarker for studying colonic ammonia metabolism to assess the effects of pro- and prebiotics on the intestinal flora in humans, and for measuring the transfer of ^{15}N (Jackson *et al.*, 2004; de Preter *et al.*, 2004; Geboes *et al.*, 2005).

In the present study, pre-dosing with GU and CU, both chemically different from the subsequent ingested DLLU, resulted in a faster increase of the initial $^{13}\text{CO}_2$ output and correspondingly in a higher ^{13}C -signal, thus, in comparison to the measured OCTT without pre-dosing (7.8 hours), leading to reliable OCTT of 5.8 and 6.0 hours, respectively. The rise of the $^{13}\text{CO}_2$ -enrichment in the expired air reflected the microbial degradation of DLLU. These observations of an apparently shorter OCTT after pre-dosing with chemically different sugar ureides strongly suggest a preceding induction of the enzyme activity as already claimed earlier when pre-dosing with the same ureide in its unlabelled form (Wutzke *et al.*, 1997; Wutzke and Glasenapp, 2004; Wutzke and Schütt, 2007). Furthermore, it could be demonstrated that pre-dosing with merely 3x120 mg GU as well as the equimolar dosage of 3x200 mg CU is high enough to induce the enzyme activity and to give reliable results. In previously published papers, higher dosages of up to 5x1 g LU have been used in adults (Morrison *et al.*, 2003; Wutzke and Glasenapp, 2004). The relatively low dosage of GU and CU, respectively, applied in the present study minimise a potential influence of pre-dosing on OCTT. For this reason, a longer pre-dosing procedure would be counterproductive.

Our measured data are in agreement with the OCTTs of 320, 292, 287, and 246 min published by Leitzmann *et al.*, 1998; Geypens *et al.*, 1999; Wutzke and Glasenapp, 2004; and Wutzke and Sattinger, 2006, respectively, in healthy adults. The observed CPDR- and CPE-data were also in the same order of magnitude with the values published earlier (Wutzke and Glasenapp, 2004; Wutzke and Schütt, 2007). In contrast to the measurement of the OCTT along with the mandatory pre-treatment procedure, our findings demonstrate that (except for the GU pre-dosing with a high s.d., presumably caused by an incomplete homogenisation of the faeces) neither the urinary nor the faecal total CPE are affected by pre-dosing with both unlabelled ureides (Table 2). Similar observations have been made by De Preter and Verbeke, 2006, when using ^{15}N -LU and the respective unlabelled LU. The observed total urinary ^{13}C - and ^{15}N -enrichments immediately after DLLU ingestion indicate that the undigested tracer substance and/or glucose[^{13}C , ^{15}N]ureide (^{13}C , ^{15}N -GU) are partially absorbed from the small bowel (Wutzke *et al.*, 1997; Heine *et al.*, 1995, Morrison *et al.*, 2003). After the bacterial degradation of the DLLU in the colon, the originated end products $^{13}\text{CO}_2$ and $^{15}\text{NH}_3$ follow separate metabolic pathways. The majority of the carbon isotope is exhaled as $^{13}\text{CO}_2$ in breath and is urinary excreted as [^{13}C]urea and partly as undigested DLLU and/or ^{13}C , ^{15}N -GU (Wutzke *et al.*, 1997; Morrison *et al.*, 2003). Assuming a nearly complete ^{13}C -elimination in breath, urine, and faeces, the total recovery in all 3 tests of 57 % on average indicates that the remaining 43 % may be incorporated into products of bacterial metabolism, may be retained in the body bicarbonate pool or may be excreted as methane. In contrast, the nitrogen isotope is mainly urinary excreted as [$^{15}\text{N}_2$]urea and $^{15}\text{NH}_3$, and partly as undigested DLLU and/or ^{13}C , ^{15}N -GU as well (Wutzke *et al.*, 1997). The total recovery in all 3 tests of 86 % on average indicates that the remaining 14 % of the $^{15}\text{NH}_3$ are incorporated into bacterial protein or are absorbed across the colon wall re-entering the alpha-amino-

nitrogen pool. It is assumed that after hydrolysis of the [$^{13}\text{C},^{15}\text{N}_2$]urea moiety of the DLLU, the originated $^{15}\text{NH}_3$ is absorbed, mixed with unlabelled NH_3 and finally re-synthesised to [$^{15}\text{N}^{14}\text{N}$]urea in the liver (Geboes *et al.*, 2004). In a previously published paper we observed that after DLLU administration the ^{15}N -labellings of urinary urea and urinary ammonia were simultaneously detectable with the $^{13}\text{CO}_2$ -enrichment. In the present study, the retry of analysing this effect by a corresponding ^{13}C -measurement in urinary urea was technically not feasible.

Based on the information available in the scientific literature, only 1 paper describes in merely 4 subjects the use of GU for pre-dosing prior to ^{13}C -LU ingestion but without measuring the faecal ^{13}C -excretion (Morrison *et al.*, 2003). The measured OCTT amounted to 5.8 hours and was similar with the data of the present study. Nevertheless, the authors claimed that little or no ^{13}C -tracer is excreted in the faeces of subjects having consumed either ^{13}C -LU or ^{13}C -GU. In contrast to their speculation, the findings of the present study after DLLU administration clearly prove a significant faecal ^{13}C -excretion in all 3 tests of 8.8 % on average (Table 2, Figure 2).

In summary, our data clearly demonstrate that it is regardless which unlabelled ureide is used for the pre-dosing procedure prior to the ingestion of the ^{13}C -labelled ureide for measuring the OCTT, as long as ^{13}C -GU is the matrix. Seen in this light, our findings make an important additional contribution to prove the fact that glucose- ^{13}C ureide and the associated *glucose ureide hydrolase* are definitely the key figures of the degradation process of ^{13}C -labelled sugar ureides in the human caecum.

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Conflict of interest

The authors declare no conflict of interest.

Table 1

Orocaecal transit times estimated after ingestion of lactose- ^{13}C , ^{15}N]ureide without and with pre-dosing with unlabelled glucose- and cellobiose ureide

subject no.	without pre-dosing	with glucose ureide	with cellobiose ureide
1	9.0	5.0	6.5
2	4.5	3.5	4.5
3	-	5.5	12.0
4	9.5	5.5	7.0
5	9.5	4.5	5.0
6	6.5	6.5	6.0
7	12.0	6.0	5.0
8	9.5	10.0	7.5
9	9.0	5.0	3.5
10	5.0	3.5	4.0
11	6.0	-	-
12	8.0	8.5	5.5
13	6.0	6.0	5.0
mean	7.83¹	5.79²	5.96³
SD	2.2	1.9	2.2

-: no data

Kruskal-Wallis test: P= 0.048

Mann Whitney U-test: ¹ vs ² P= 0.033; ¹ vs ³ P= 0.039; ² vs ³ P= 0.977

Table 2

Faecal ^{13}C - and ^{15}N -excretions after ingestion of lactose- ^{13}C , ^{15}N]ureide without and with pre-dosing with unlabelled glucose- and cellobiose ureide

subject no	^{13}C -excretion			^{15}N -excretion		
	without	glucose ureide	cellobiose ureide	without	glucose ureide	cellobiose ureide
1	2.62	2.93	3.05	22.95	2.26	16.5
2	14.14	14.76	11.54	71.51	46.16	16.14
3	10.42	4.32	11.6	49.1	32.47	64.57
4	47.16	5.8	6.42	51.35	17.89	70.0
5	3.14	6.2	7.84	16.7	30.08	50.3
6	0	3.43	6.4	44.45	18.66	28.88
7	10.5	1.54	25.76	53.18	3.13	86.35
8	7.68	6.28	19.12	33.89	9.1	0.9
9	8.55	3.71	5.34	41.46	28.0	39.03
10	7.03	4.61	6.54	54.37	17.73	44.5
11	16.67	-	-	41.66	-	-
12	7.17	2.86	5.03	60.65	51.4	45.15
13	22.71	1.62	5.91	54.54	1.26	14.69
mean	12.14¹	4.84²	9.55³	45.83⁴	21.51⁵	39.75⁶
SD	11.7	3.4	6.4	14.4	15.9	24.5

-: no data

Kruskal-Wallis test: P= 0.017, 0.018

Mann Whitney U-test: ¹ vs ² P= 0.026; ¹ vs ³ P= 0.574; ² vs ³ P= 0.006;

⁴ vs ⁵ P= 0.002; ⁴ vs ⁶ P= 0.376; ⁵ vs ⁶ P= 0.160

Figure legends

Figure 1: Mean $^{13}\text{CO}_2$ -enrichment after ingestion of lactose- $^{13}\text{C},^{15}\text{N}$ ureide without and with pre-dosing with unlabelled glucose ureide and cellobiose ureide

Figure 2: Mean percentage ^{13}C - and ^{15}N -excretion in breath, urine, and faeces, respectively, after ingestion of lactose- $^{13}\text{C},^{15}\text{N}$ ureide without and with pre-dosing with unlabelled glucose ureide and cellobiose ureide

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Fig. 1

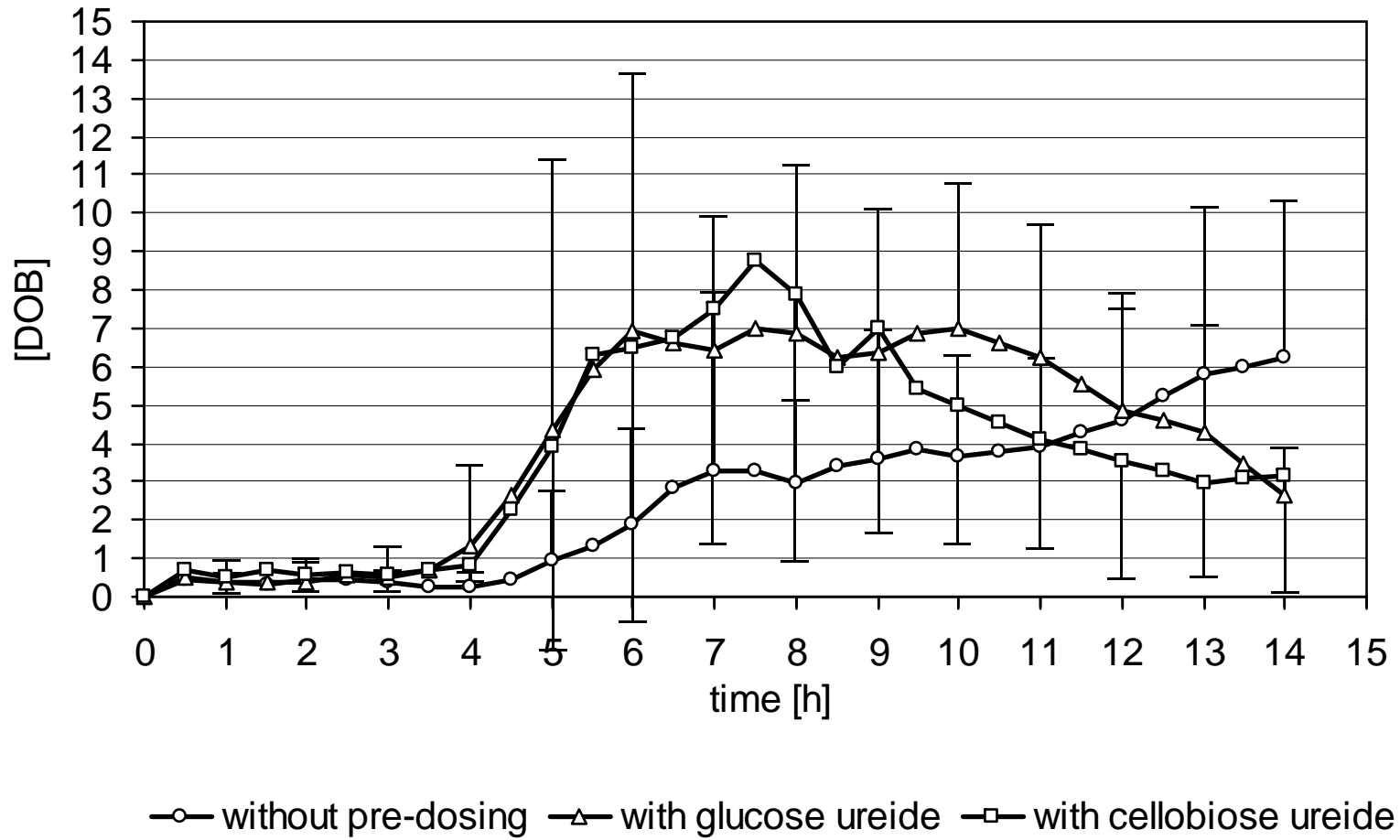


Fig. 2

