Proteolytic changes in lactose hydrolysed UHT milks during storage

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Proteolytic changes in lactose unhydrolysed and hydrolysed direct UHT-treated milks were studied during a storage period of 12 weeks. Enzymatic hydrolysis was performed either before (=prehydrolysed) or after (=posthydrolysed) UHT-treatment. The enzymatic hydrolysis of lactose resulted in an increase in proteolysis compared to unhydrolysed milk, during the storage regardless whether hydrolysis was performed before or after the UHT-treatment. The highest degree of proteolysis was found at the highest storage temperature tested (45°C), while proteolysis was almost non existent at the lowest storage temperature of 5°C as measured by α–amino nitrogen/total nitrogen or as changes in SDS-PAGE analyses. Proteolysis was also noticed in unhydrolysed milk where it was caused by the plasmin enzyme system and possibly by the microbial contaminants in milk. The pH in milks decreased during the storage at 22, 30 and 45°C respectively and there was high correlation (r^2=0.865) between pH drop and α–amino nitrogen/total nitrogen change.

Proteolytische Veränderungen bei laktosehydrolysierten UHT-Milchen während der Lagerung

Proteolytische Veränderungen bei nicht-laktosehydrolysierter und hydrolysierter direkt UHT-behandelter Milch wurden während einer Lagerungszeit von 12 Wochen untersucht. Die enzymatische Hydrolyse wurde entweder vor (= prehydrolysed) oder nach (= posthydrolysed) der UHT-Behandlung durchgeführt. Die enzymatische Hydrolyse der Laktose führte im Vergleich zu nichthydrolysierter Milch zu einer erhöhten Proteolyse während der Lagerung unabhängig davon, ob die Hydrolyse vor oder nach der UHT-Behandlung stattfand. Der höchste Hydrolysegrad wurde bei der höchsten untersuchten Lagerungstemperatur (45°C) festgestellt, während die Proteolyse bei der niedrigsten Lagerungstemperatur von 5°C nahezu nicht stattfand – gemessen am Verhältnis α–Aminostickstoff/Gesamtstickstoff oder als Veränderunge in den SDS-PAGE-Analysen. Auch in nichthydrolysierter Milch wurde eine Proteolyse verursacht; sie wurde durch das Plasminenzymsystem und möglicherweise durch die mikrobiellen Kontaminanten in Milch festgestellt. Die pH-Werte in Milch sanken während der Lagerung bei 22 bzw. 30 und 45°C ab, und es bestand eine hohe Korrelation (r^2=0,865) zwischen dem Abfall des pH-Wertes und der Veränderung im Verhältnis von α–Aminostickstoff/Gesamtstickstoff.

34 UHT milk (lactose hydrolysis and proteolysis)   **34 UHT-Milch** (Laktosehydrolyse und Proteolyse)

1. Introduction

The changes during storage of UHT-milk have been followed in several studies (1, 2, 3, 4, 5). A major factor causing quality decline and reducing the shelf life of milk is proteolysis usually based on indigenous or bacterial proteases (1, 6-13). Proteolytic enzymes of psychrotrophic bacteria can be very heat stable (6). During storage at 20°C or higher, furosine usually increases and undenatured β-lactoglobulin content lowers (1, 5). Direct UHT treatment is usually found to be more gentle than indirect UHT-treatment (5, 14, 15, 16).

The effects of UHT-treatments on lactose hydrolysed milk have been studied in very few studies (2, 3, 17, 36). During recent years lactose hydrolysed and lactose free milks have gained a significant market share in several market areas (18). Enzymatic hydrolysis of lactose during manufacturing of lactose reduced or lactose free milks releases glucose and galactose from lactose. This doubles the molar concentration of

reducing sugars, which make lactose hydrolysed milk more vulnerable to the Maillard reaction than normal milk (3, 19, 20, 21). In the Maillard reaction lysine damage takes place through reactions between reducing sugars and free amino groups in lysine side chains. Lysine is not the limiting essential amino acid in milk, but when consumed together with cereals the milk lysine becomes a limiting amino acid of this combination. Therefore lysine should stay as available as possible (22). Evangelisti *et al*. (2) concluded that for UHT-treated lactose hydrolysed milk storage < 4°C should be compulsory in order to limit protein damage.

In this study the effect of lactose hydrolysis on proteolysis in skim milk during storage at 5, 22, 30 and 45°C respectively for 3 months was followed after direct UHT treatment. Also the effect of lactase addition stage was studied.

2. Materials and methods

Milk was normal skim milk received at the Valio UHT-plant, Turenki, Finland. The same milk batch was used both for the unhydrolysed and posthydrolysed milk tests. Very similar milk was used for the prehydrolysed milk test (Table 1).

Table 1: Analysis results of raw material milk

Test run	Un-hydrolysed	Pre-hydrolysed	Post-hydrolysed
pH	6.8	6.8	6.8
Fat	0.1	0.09	0.1
Dry matter (%)	9.23	9.19	9.23
Sensory evaluation (max.5)	5	5	5
Standard plate count cfu/ml	3500	3500	3500
Coliforms (cfu/ml)	0	0	0
B. cereus (cfu/ml)	0	0	0
Temperature (°C)	4.4	3.8	4.4

Three test-runs were performed: the first was treated with the UHT-process and packed into 1 L TetraBrik carton packages with no lactase addition (= unhydrolysed), the second was prehydrolysed with 0.09 % GODO YNL2 (Godo Shusei, Japan) (received 12/05) in milk in a tank at 10°C for 20 h before UHT-treatment and packed in similar 1 L carton packages (= prehydrolysed), and the third was hydrolysed after the UHT-treatment in packages with GODO YNL2. The enzyme was added aseptically into the package (0.003 %) (= posthydrolysed). The cartons were divided into four groups to be stored at temperatures of 5, 22, 30 and 45°C, respectively. The cartons were stored for 12 weeks and samples were taken for analyses at least every 4 weeks. The samples were frozen at −70°C and then transferred to −21°C for storage. The direct UHT treatment for all three types of milk was identical.

2.1 The description of the UHT-process

The UHT-treatment of milks was carried out by a direct UHT process based on infusion technology (APV, Denmark). Milk was first preheated to 75°C for about 20 s by plate heat exchangers and then heated very rapidly to 141°C by steam infusion. After a holding time of 1 s the milk was first cooled in a flash vessel to 70°C, homogenised with 180/50 bar and cooled by plate heat exchanger to 20°C.

2.2 Analyses

Samples were analysed every 4 weeks for pH and α-amino-N. Samples for SDS-PAGE analyses were taken directly after packing of milk, after 4 and 12 weeks of storage. pH was measured with a Mettler Delta 320 pH meter (Mettler-Toledo Ltd, Halstead, UK). α-amino-N was analysed according to LIESKE and KONRAD (23). Analysis was performed in four replicates, standard deviations and mean values were calculated. SDS-PAGE analyses were done according to (24) using 10 µg protein per sample well. Ready-made 18% Tris-HCl polyacrylamide gels (Bio-Rad, Hercules, CA, USA) were used. Protein bands were visualized by staining with Coomassie G-250 (GelCode® Blue Stain Reagent,

Pierce, USA) and compared with molecular weight markers (Prestained SDS-PAGE standards, broad range, Bio-Rad, USA). Milk samples were analysed for fat (25), total nitrogen (26), dry matter (27), sensory evaluation (35), standard plate count (28), coliforms (29), *Bacillus cereus* (30). Temperature was measured with a calibrated thermometer.

3. Results

Table 2: Composition of UHT-treated milk directly after packaging.

	Unhydrolysed	Prehydrolysed	Posthydrolysed
Protein (%)	3.42	3.54	3.49
Fat (%)	0.08	0.09	0.07
Lactose (%)	4.5	0.1	2.5
Glucose (%)	-	2.3	1.2
Galactose (%)	-	2.2	1.0

Results from the chemical and the microbiological analyses from the raw milks are presented in Table 1. Microbial counts were very low and sensorically milks were rated the highest class. The composition of the milks was very similar. The same raw milk lot was used for unhydrolysed and posthydrolysed milk tests.

The composition of the freshly packed test milks are shown in Table 2. Differences can be seen in the sugar contents of the lactose hydrolysed milks. In posthydrolysed milk the lactase remained active and hydrolysis continued, reducing the difference against prehydrolysed milk during the storage.

3.1 Changes in α-amino nitrogen/total nitrogen in non hydrolysed and hydrolysed milk during storage

The development of α-amino nitrogen/total nitrogen reflects the total proteolytic activity in milk during storage. This activity may originate from milk itself (indigenous proteases), from microbial contaminations of milk or from the lactase enzyme preparation used. The natural milk protease plasmin may be activated in the UHT-process (31), which means that in lactose hydrolysed UHT-milks it may have a synergic effect with other proteases. All commercial enzyme preparations have several side activities and among these proteolytic ones are common. Figure 1a-c shows the development of α-amino nitrogen/total nitrogen in unhydrolysed, prehydrolysed and posthydrolysed milk during the storage at different temperatures. In unhydrolysed milk only small changes in α-amino nitrogen/total nitrogen were seen. However, α-amino nitrogen/total nitrogen was the highest in samples stored at 45°C. Addition of lactase enzyme either before or after UHT-treatment caused a significant increase in proteolysis. No significant difference between prehydrolysed and posthydrolysed milk could be noticed. In prehydrolysed milk the enzyme dosage was 30 times higher as the time available for hydrolysis was much shorter than for the posthydrolysed milk. Although heat treatment had inactivated part of the proteolytic activity, much had survived into the packed milk causing proteolysis comparable to that of posthydrolysed milk.
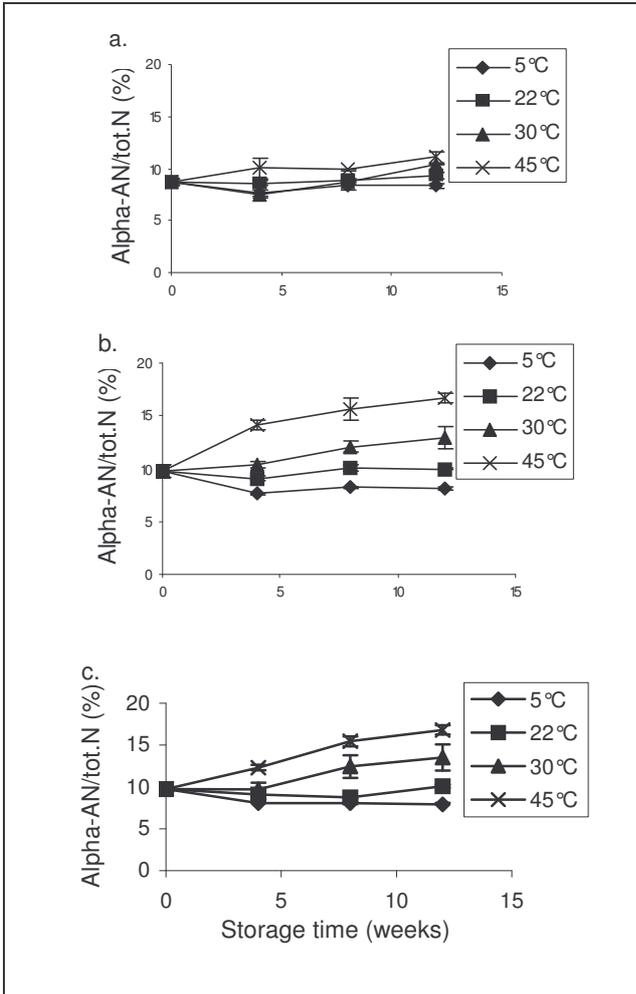
Fig. 1: Effect of storage temperature, time and lactase addition on α-amino nitrogen/total nitrogen in UHT-treated skim milk at different storage temperatures: a) unhydrolysed, b) prehydrolysed, c) posthydrolysed.

3.2 SDS-PAGE analyses of UHT milks during storage

Proteolysis was studied more closely by analysing milks with SDS-PAGE. Figure 2 a-c shows SDS-PAGE analyses with 18 % gels with milks stored for 0 or 4 weeks at temperatures 5, 22, 30 and 45 °C. Significant proteolysis took place during the storage at 22, 30 and 45 °C already during 4 weeks of storage. The casein bands were the weaker the higher the storage temperature. In lactose hydrolysed milks stored at 45 °C for 4 weeks casein bands were significantly weakened compared to those in unhydrolysed milk.

Mainly α_s- and β-caseins were hydrolysed during the storage, but ß-lactoglobulin and α-lactalbumin have survived partly in prehydrolysed and posthydrolysed samples. No significant changes were seen at 5 °C.

Commercial β-galactosidase contained proteolytic side activities, which enhanced proteolysis compared
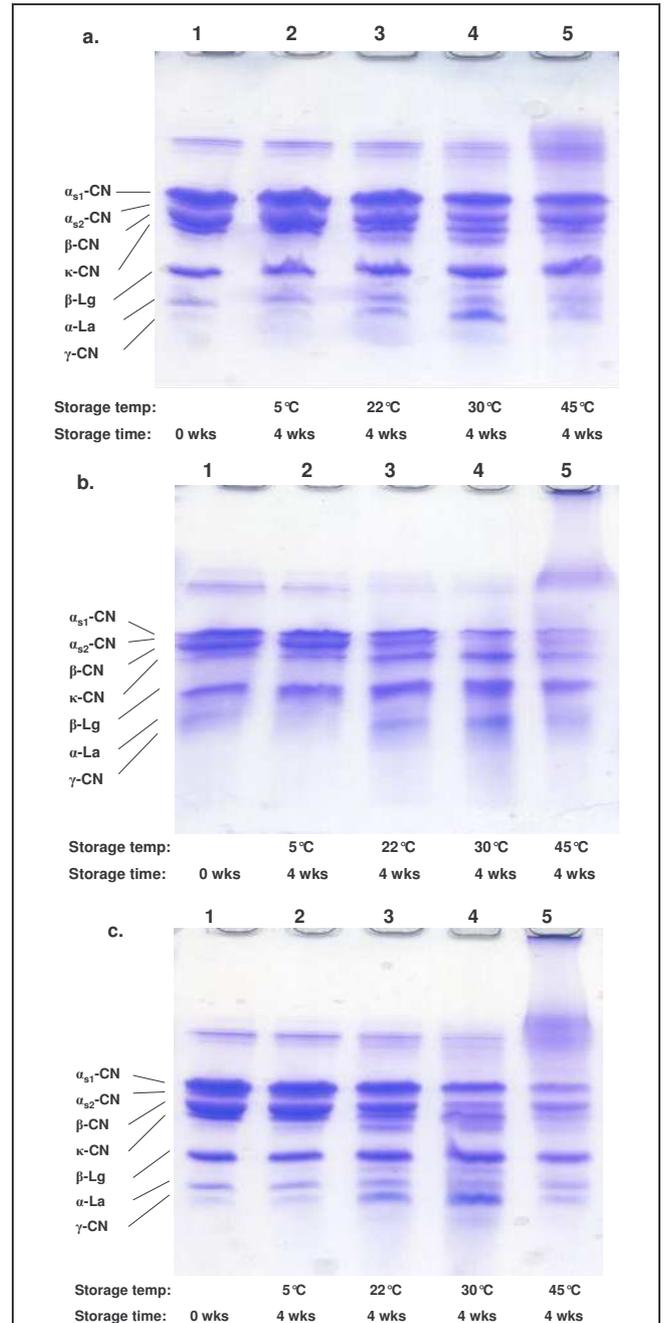

Fig. 2: Gels a-c show the unhydrolysed, prehydrolysed and posthydrolysed milk samples respectively stored at 5, 22, 30 and 45 °C after 0 and 4 weeks of storage. Lanes: 1. 0 weeks, 2. 4 weeks at 5 °C, 3. 4 weeks at 22 °C, 4. 4 weeks at 30 °C, 5. 4 weeks at 45 °C.

to unhydrolysed milk. Also milk, where lactase was not added, was affected by milk's natural proteolytic enzyme, plasmin or by the proteolytic enzymes from contaminating microbes. Formation of γ-casein refers to the action of milk plasmin enzyme (31, 13, 9). In test milks γ-casein was found both in lactose unhydrolysed and hydrolysed milks (2 a-c, 3 a-b).
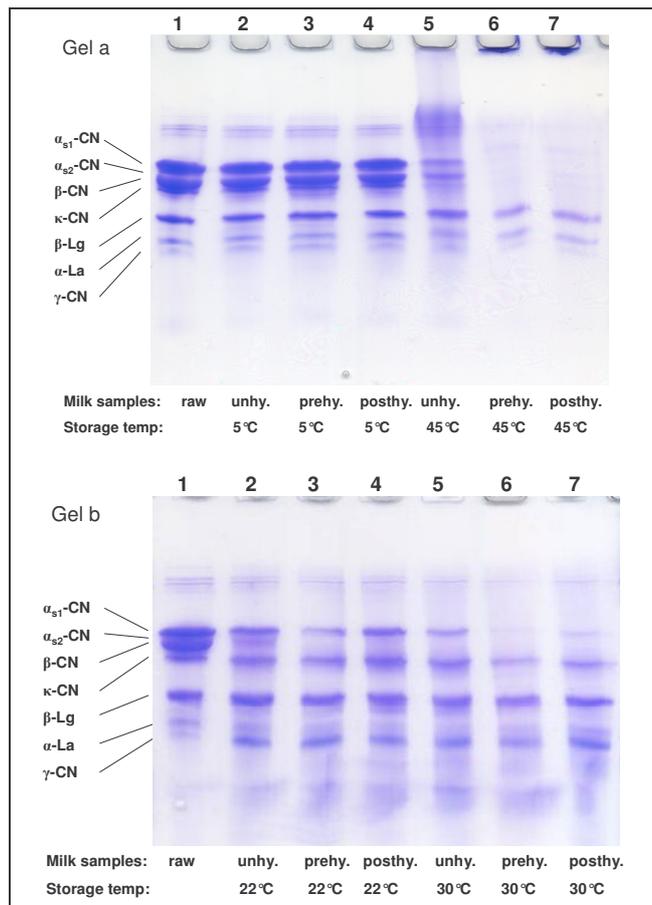
Fig. 3: SDS-PAGE analyses (18% gel) of UHT milks stored for 12 weeks at different temperatures. Gel a: Lanes: 1. raw skimmed milk, 2. unhydrolysed 5 °C, 3. prehydrolysed 5 °C, 4. posthydrolysed 5 °C, 5. unhydrolysed 45 °C, 6. prehydrolysed 45 °C, 7. posthydrolysed 45 °C. Gel b: Lanes: 1. raw skimmed milk, 2. unhydrolysed 22 °C, 3. prehydrolysed 22 °C, 4. posthydrolysed 22 °C, 5. unhydrolysed 30 °C, 6. prehydrolysed 30 °C, 7. posthydrolysed 30 °C.

Figure 3 a-b shows SDS-PAGE gels of milk samples after 12 weeks of storage at different temperatures. Again the casein bands were the weaker the higher the storage temperature. Figure 3a shows that, compared to skimmed raw milk, no significant changes happened in milks stored at 5 °C. At 45 °C in lactose hydrolysed milks practically all caseins have disappeared. Only the whey proteins, ß-lactoglobulin and α-lactalbumin, were found in low concentrations. In unhydrolysed milk part of caseins was survived.

Figure 3b shows samples stored at 22 or 30 °C and here proteolysis was clearly milder than at 45 °C. However, the same order can be found as at 45 °C, unhydrolysed milk had the smallest proteolytic changes, milks containing added lactase before or after UHT had clearly stronger changes. Prehydrolysed milk seemed to have had slightly stronger proteolysis than posthydrolysed milk. Proteolysis affected especially α_{s1}- and α_{s2}-caseins but also β-casein. K-casein was the one least affected. The whey proteins ß-lactoglobulin and α-lactalbumin persisted against proteolysis better than did caseins.

A number of new hydrolysis products can be seen in the gels (Figures 2a and 2c) below κ-casein, α-lactalbumin and γ-casein. In Fig. 3b new hydrolysis products are visible especially below α-lactalbumin and γ-casein. In the gels of lactose hydrolysed milks stored at 45 °C (Fig. 2b, lane 5, Fig. 2c, lane 5, Fig. 3a, lanes 6 and 7) very large molecular proteinous material was found close to the start well. These large aggregates were formed in milks where lactase was added either before or after heat treatment. However, these aggregates were not found in milks stored at 5, 22 or 30 °C. They may be due to advanced Maillard reaction products formed at 45 °C.

3.3 Changes in pH

Changes in pH during the storage were significant. pH in fresh lactose hydrolysed milks was slightly lower than in unhydrolysed milk. The higher the storage temperature, the higher the drop in pH during storage. The highest decrease was found in lactose hydrolysed milks at 45 °C. The pH changes were mainly due to the hydrolysis of the peptide bonds and release of H^+ molecules in ratio to the hydrolysed peptide bonds (32). Change in pH may partly also be due to the formation of organic acids (acetic and formic acids) from monosaccharides during heating at high temperatures (33, 34). However, their formation would require long holding times at above 120 °C. Fig. 4 shows the high correlation ($r^2 = 0.865$) between pH change and α-amino nitrogen/total nitrogen change in UHT-milk samples during the storage of 12 weeks.
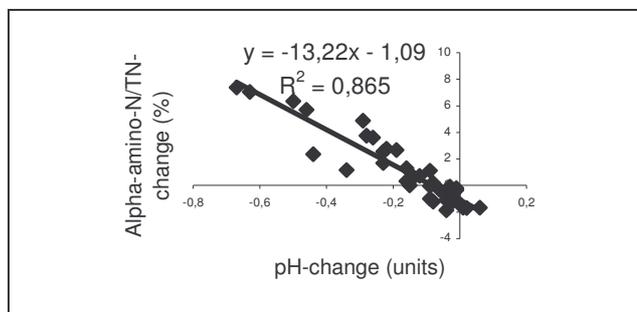

Fig. 4: Correlation between pH change and α–amino nitrogen/total nitrogen change in UHT-milks stored for 12 weeks at 5, 22, 30 and 45 °C.

It can be concluded that the drop in pH during storage of UHT-treated lactose hydrolysed milk can be used as a rough indicator of the extent of proteolysis.

4. Discussion

Although it is well known that commercial lactases contain proteolytic side activities, it was surprising that significant proteolysis occurred in lactose hydrolysed milks even when lactase treatment took place before UHT-treatment. This was clearly seen in the changes in α-amino nitrogen/total nitrogen and in the SDS-PAGE analyses. This remarkable proteolysis was not caused by poor raw milk quality as can be seen from the difference between unhydrolysed milk, where no lactase was added, and posthydrolysed milk. Both milks were

prepared from the same milk batch. In all three test milks the raw milk was of very similar quality (Table 1).

A rather long hydrolysis time is needed if lactose hydrolysis takes place before the UHT-treatment and a low hydrolysis temperature is used. Thus a higher lactase dosage is needed than in posthydrolysed milk, where the lactase has a much longer time to hydrolyse the lactose. Hydrolysis of lactose after the UHT-treatment with lactase aseptically filtered into the carton did not help to avoid the proteolysis. Although the dosage was very low, all the proteolytic side activities remained active at storage temperatures of 22, 30 and 45 °C.

The lactase dosage in prehydrolysed milk was 30 times higher than in posthydrolysed milk but proteolysis was only slightly stronger than in posthydrolysed milk. This means that most of the proteolytic side activity of the lactase was destroyed during the UHT-treatment of prehydrolysed milk. Thus increasing the heat treatment during the UHT process could destroy more of the harmful proteolytic activities in milk. However, this may lead also to increased protein damage due to an enhanced Maillard reaction and browning of the product. NEWSTEAD *et al.* (31) concluded that preheating ranging from 90 °C, 30 s to 90 °C, 60 s is needed to successfully limit the plasmin type activity in reconstituted milk in the direct steam injection UHT-process. Corzo *et al.* (1) studied proteolysis in indirect and direct UHT-treated milks during storage. They found that proteolysis was greatest in milks having the most severe heat treatments. The authors suggest that this may have been due to the differences in microbiological quality of milks. The higher heat treatments also may have activated plasmin enzyme system in milk. All stages of heat treatments were not described.

NEWSTEAD *et al.* (31) followed proteolytic changes during storage of UHT-milk. They found that pH decrease during storage of 8 months at 20 or 30 °C correlated well with the hydrolysis rate of β–casein. These results support the observation that the changes in pH observed were related to the extent of proteolysis.

5. Conclusions

Commercial lactase products contain proteolytic side activities which can cause product quality deterioration in UHT-treated milk when stored at ambient temperature. This leads to a limited shelf life of the product. Also a number of other factors such as the quality of raw milk, hydrolysis conditions, heat treatments of UHT-process as well as the storage temperature each have an impact on the shelf life of lactose hydrolysed milk. The problem may be avoided by optimising these different factors and keeping the storage temperature close to 5 °C.

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